ARTICLE

Deprotection of S-Acetamidomethyl Cysteine with Copper (II) and 1,2-Aminothiols under Aerobic Conditions

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Ring-opening by $CuSO_4$ of a 1,3-thiazolidine carbonyl structure (Thz) as an N-terminal cysteine (Cys) residue revealed that an intramolecular S–acetamidomethyl cysteine (Cys(Acm)) can also be deprotected with concomitant formation of a disulphide bond connecting the two Cys residues. A mechanistic study on the disulphide formation led to a general protocol for deprotection of the S-Acm group by $CuSO_4$ and a 1,2-aminothiol under aerobic conditions. Application of this new deprotection reaction allowed for the synthesis of Apamin, a peptide with two-disulphides in a one-pot/stepwise disulphide-bridging procedure.

Introduction

Acetamidomethyl (Acm) protection[1] of the sulphydryl group of cysteine has contributed significantly to the synthesis of cystine peptides including multiple disulphide-containing peptides.[2] The S-Acm group remains intact under acidic conditions employed for deprotection of conventional side chain protecting groups, and its removal can be achieved by metal salts such as $Hg(OCOCH_3)_2$[1] or AgOTf[3] to give cysteine peptides after treatment of the resulting S-metal peptides with thiols. Alternatively, oxidative conversion to a disulphide can be achieved by the action of I_2[4], $Tl(OCOCF_3)_3$[5], chlorosilane-sulphoxide[6] or sulphenyl chloride systems[7] (Figure 1).
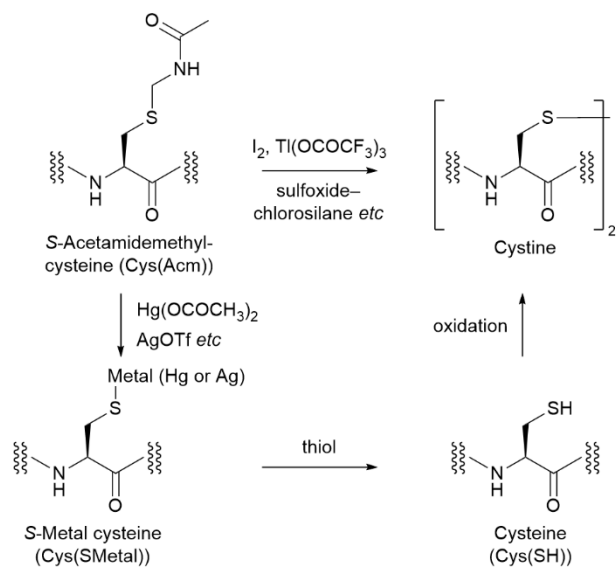

Figure 1. Deprotection of S-acetamidomethyl cysteine (Cys(Acm)).

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The unique chemical features of the S-Acm group support its widespread use in peptide/protein chemistry and the recent upward trend in native chemical ligation (NCL)[8] reactions for protein chemical synthesis has led to new methods for Acm removal.[9] In this context, we recently reported that a 1,3-thiazolidine-4-carbonyl (Thz)[10] residue as an N,S-protected cysteine derivative which is indispensable in NCL chemistry is deprotected by the action of $CuSO_4$ to regenerate a cysteine residue.[11] While evaluating the ring-opening of thiazolidine with $CuSO_4$, we found that chemical behaviour of the S-Acm residue with $CuSO_4$ is different when the Thz- and Cys(Acm)-containing peptide (Thz-**GALYR**-Cys(Acm)-**FG**-NH_2 (**1**)) was treated with $CuSO_4$ in the presence or absence of ascorbate. Under both conditions, ring-opening of the thiazolidine occurs but reaction with ascorbate followed by treatment with dithiothreithol (DTT) gave a thiazolidine-opened Cys(Acm) peptide (H-Cys(SH)-**GALYR**-Cys(Acm)-**FG**-NH_2 (**2**)), whereas reaction in the absence of ascorbate afforded an Acm-deprotected peptide (H-Cys(SH)-**GALYR**-Cys(SH)-**FG**-NH_2 (**3**))[12,13]. Additionally, replacement of DTT with EDTA affords intermolecular and intramolecular disulphide peptides **4** (Figure 2 and Figure S1 (Fig S1) in the SI).
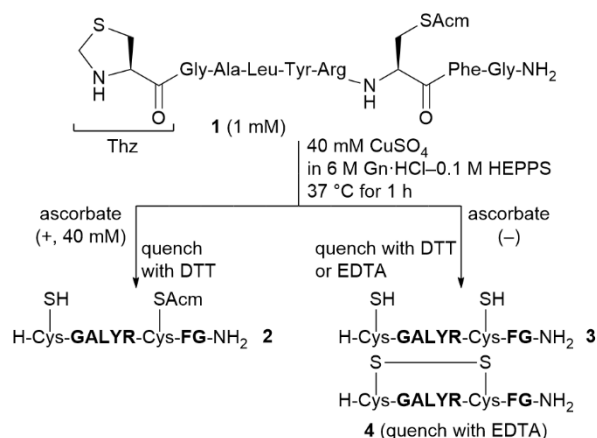

**Figure 2.** Ring-opening reactions of Thz with and without ascorbate.

Table 1. Evaluation of contribution of N-terminal amino acids to deprotection of Acm group.

Peptide	N-terminal residue (Xaa)	Removal of Acm	Product
1	Thz	+	**3**[a] or **4**[b]
5	Ala	−	**5**[a]
2	Cys(SH)	+	**3**[a,c] or **4**[b,c]
6	Ac-Cys(SH)	−	**6**[a]

[a]Quench with DTT; [b]Quench with EDTA; [c]small amount of N-terminal cysteic acid peptide was detected (Fig S2).

Results and Discussion

To shed light on these unexpected results, we evaluated the chemical behaviour of the peptides in Table 1. Initially, direct involvement of the Cu salt in the removal of Acm group was examined using peptide **5** which has an Ala-substituent on the N-terminal Thz. Treatment of **5** with CuSO$_4$ (40 eq.) in 6 M guanidine hydrochloride (Gn·HCl) in 3-[4-(2-hydroxyethyl)-piperazin-1-yl]propane-1-sulphonic acid (HEPPS) buffer at pH 7.0 did not affect the Acm group and led to recovery of the starting material, indicating that the Thz or regenerated Cys residue is involved in the Cu-induced removal of the Acm group. The N-terminal cysteine peptide **2** was subjected to the Cu-mediated reaction followed by DTT quenching to afford **3**. On the other hand, removal of Acm group did not occur in the reaction of *N*-acetyl Cys peptide **6** and reaction of **1** or **2** with CuSO$_4$ followed by quenching with EDTA gave disulphide peptides which could be intra- or intermolecularly paired.
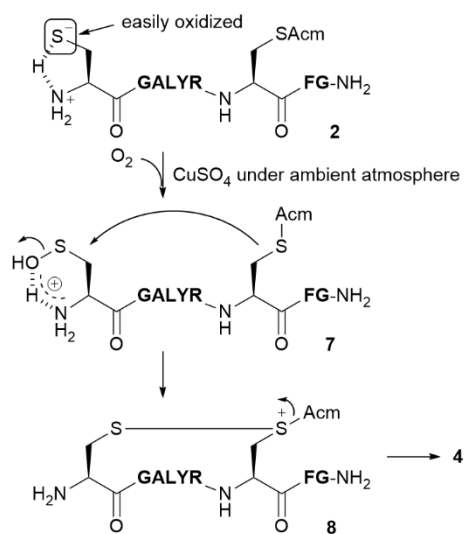
Figure 3. Plausible mechanism for the deprotection of Acm and the formation of disulphide peptide under Cu(II) conditions.

**Table 2**. Optimization of removal conditions of an Acm group[a].
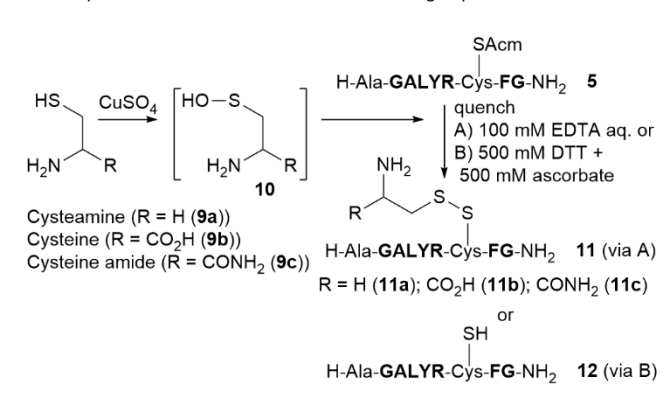
Entry	1,2-Aminothiol (mM)	CuSO$_4$ (mM)	Quench[b]	Product (Conversion (%)), **5** (remaining (%))[c]
1	**9a** (25)	100	A	**11a** (41)[d], **5** (59)
2	**9a** (50)	100	A	**11a** (73)[d], **5** (27)
3	**9a** (100)	100	A	**11a** (> 95), **5** (ND)
4	**9a** (100)	100	B	**12** (78)[e]
5	**9a** (200)	100	A	**11a** (18), **5** (82)
6	**9a** (300)	100	A	**11a** (5), **5** (95)
7	**9a** (25)	25	A	**11a** (71), **5** (29)
8	**9a** (50)	50	A	**11a** (98)[d], **5** (2)
9[f]	**9a** (100)	100	A	**11a** (59), **5** (41)
10	**9b** (100)	100	A	**11b** (40), **5** (60)
11	**9c** (100)	100	A	**11c** (66), **5** (34)

[a]Reactions were conducted at 37 °C, pH 5 under ambient atmospheric conditions for 3 h; [b]Quench method: (A) Addition of 100 mM EDTA aq. (B) Addition of 500 mM DTT and 500 mM ascorbate followed by stirring at 37 °C for 30 min; [c]Conversion (%) (or remaining (%)) proportions were determined by HPLC analysis with UV detection at 220 nm and calculated using the equation percent formation = 100 [(integ. **5** or **11** or **12**)/(integ. **5** + integ. **11** + integ. **12**)]: integ. = integration of peak area of the UV absorption; [d]Not separable, but small amount of the disulphide form of **12** was a contaminant in the eluted **11a** on HPLC analysis; [e]Isolated yield; [f]Under inert atmospheric conditions (Fig S3 in the SI).

From these results, we speculated that an N-terminal Cys residue as a 1,2-aminothiol moiety would be converted to an oxidizing species and function as an Acm deprotecting agent affording the disulphide. In contrast, reaction in the presence of ascorbate probably does not involve the formation of such an oxidizing species with the Acm group remaining intact. Generally, compared to an internal Cys residue, an N-terminal Cys is known to be easily oxidized[14] in the presence of metal salts producing the disulphide via formation of oxidizing species such as sulphenic acid. Although other possibilities cannot be eliminated, a possible explanation for the outcome of the reaction in the absence of ascorbate is that an N-terminal sulphenic acid moiety is formed by the action of Cu(II) and subsequent nucleophilic attack of the sulphur atom of *S*-Acm occurs and is followed by disulphide formation, as shown in Figure 3. This envisioned us that the reaction of a Cys(Acm)-containing peptide with CuSO$_4$ in the presence of 1,2-aminothiols such as cysteamine would allow for the oxidative removal of Acm group. We attempted to remove the Acm group by reagent mixtures with varying CuSO$_4$/cysteamine ratios as

shown in Table 2, where stoichiometry of the reagents was also changed. Use of excess cysteamine relative to copper ions prevented the deprotection from reaching completion (entries 3 vs 5 and 6). On the other hand, the reaction tolerated the excess amount of the copper ion relative to cysteamine. (entries 3 vs 1 and 2). A possible explanation for incomplete deprotection under conditions shown in entries 5 and 6 is that the use of excess cysteamine relative to $CuSO_4$ led to the formation of an insoluble white precipitate probably consisting of copper and cysteamine. A large excess amount of the mixture consisting of $CuSO_4$/cysteamine (1:1) achieved the quantitative conversion of **5** to the corresponding hetero disulphide dimer **11a** (entries 3 vs 7 and 8). Reaction of **5** (1 mM) with $CuSO_4$/cysteamine (1:1, 100 mM) in 6 M Gn·HCl–0.1 M HEPPS under an ambient atmosphere proceeded quantitatively in 3 h at 37 °C forming a pale yellow clear solution and giving **11a** in >95% conversion judged by HPLC analysis and the Cys-form **12** in 78% isolated yield after reduction with DTT in the presence of ascorbate (entry 4). Reaction under an inert atmosphere decreased the conversion rate to about 60%, which indicates that oxygen is involved in the oxidative conversion of *S*-Acm to disulphides (entry 9). From these results, a plausible mechanism can be envisioned and is shown in Figure 4. The thiol group of cysteamine could form an intramolecular hydrogen bond or zwitterion with the amino group and dissociating into a thiolate anion which is more easily oxidized than a thiol to an oxidizing species such as sulphenic acid with participation of the neighbouring amine. Electrophilic attack of the resulting sulphenic acid on the sulphide of Cys(Acm) followed by release of an acetoamidomethyl cation gives the hetero disulphide. This envisioned mechanism involving an electrophilic sulphenic acid moiety would be essentially identical to those of oxidative deprotection procedures including I_2-, sulphenyl chloride- and sulphoxide–chlorosilane-mediated systems.

Next, the capability of other thiols to deprotect of Acm group was evaluated. The cysteine–$CuSO_4$ system was not effective for the deprotection probably due to preferential formation of the zwitterionic carboxylate anion and ammonium cation in the cysteine molecule (entry 10).
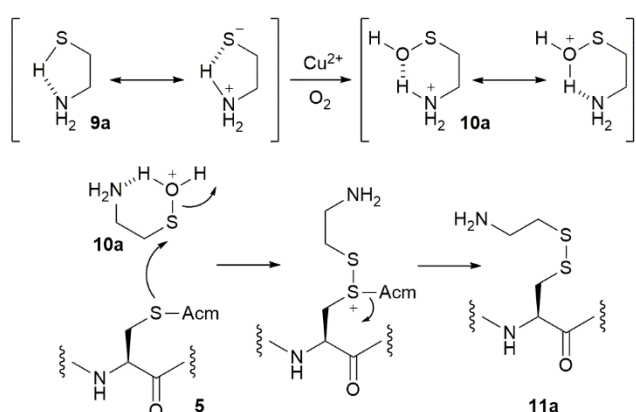
**Figure 4.** Plausible mechanism for the deprotection of Acm under Cu(II) and cysteamine conditions.

Table 3. Examination of deprotection of other *S*-protecting groups[a]

Entry	*S*-Protecting group	12 (Conversion (%)), 13 or 14 (remaining (%))
1	Trt	**12** (>95), **13** (ND)
2	MBzl	**12** (25)[b], **14** (75)

[a]Conversion (%) (or remaining (%)) were determined by HPLC analysis with UV detection at 220 nm and calculated using the equation, percent formation = 100[(integ. **12** or **13** or **14**)/(integ. **12** + **13** or **12** + **14**)]: integ. = integration of peak area of the UV absorption; [b]A small amount of sulphoxide of **14** was detected (Fig S4 in the SI).

A cysteine amide could be used as an alternative to cysteamine for the oxidative removal of Acm group, but in this case the removal was incomplete after 3 h reaction (entry 11).

Other *S*-protecting groups were subjected to the cysteamine–$CuSO_4$ system. N-Terminal alaninyl peptides containing *S*-triphenylmethyl cysteine (Cys(Trt)) or *S*-*p*-methoxybenzyl cysteine (Cys(MBzl)) (H-Ala-**GALYR**-Cys(Trt)-**FG**-NH$_2$ (**13**) or H-Ala-**GALYR**-Cys(MBzl)-**FG**-NH$_2$ (**14**)), respectively (Table 3) were examined. Quantitative removal of *S*-Trt group with $CuSO_4$–cysteamine was observed but deprotection of *S*-MBzl group remained incomplete. Such difference is probably attributable to the ease of formation of cations derived from *S*-Trt, *S*-MBzl or *S*-Acm. Reaction of Met-containing peptide with $CuSO_4$–cysteamine gave a small amount of sulphoxide and uncharacterized peptides (Fig S5 in the SI). In the suggested reaction mechanism, $CuSO_4$ participates in the oxidative removal of Acm through formation of a sulphenic acid species. Other metals can be involved in Acm removal in the presence of 1,2-aminothiols. The use for example of potassium ferricyanide, $K_3[Fe(CN)_6]$–cysteamine allows for the oxidative removal of the Acm group of peptide **2** but the reaction proceeded more slowly than reactions using $CuSO_4$ (Fig S6 in the SI). We next attempted to treat peptide **2** with H_2O_2 to disclose whether $CuSO_4$ functions as a generator of hydroxy radicals or as Lewis acid to accelerate the oxidation of the 1,2-aminothiol moiety. Attempted reaction afforded the disulphide dimer of **2** as a main product with accompanying negligible amount of the Acm-deprotected peptide **4** (Fig S7 in the SI).
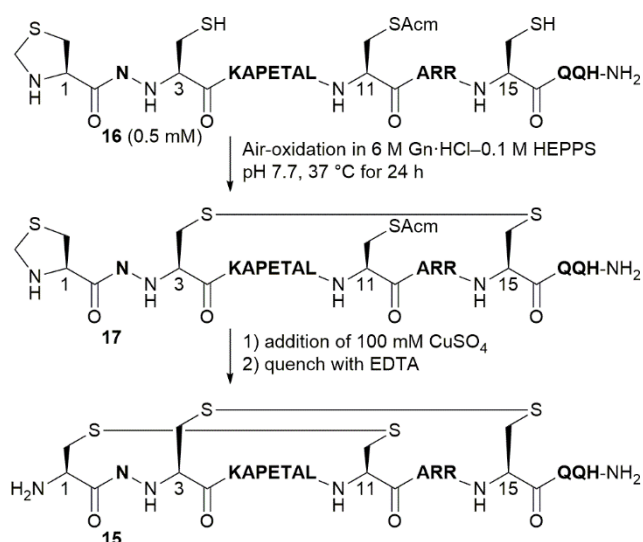
Figure 5. Examination of the CuSO₄–cysteamine system to establish the second disulphide bridge (Cys1–Cys11) of Apamin.
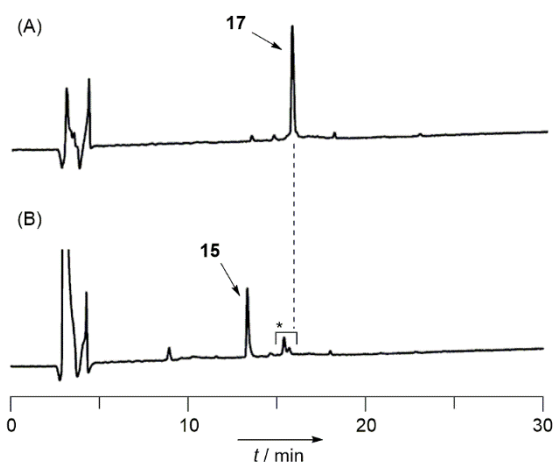
Figure 6. HPLC data from the one-pot/sequential disulphide-bridging in the synthesis of Apamin. (A) Air oxidation: t = 24 h; (B) Addition of CuSO₄: t = 5 h. Analytical HPLC conditions: linear gradient of 0.1% TFA/CH₃CN in 0.1% TFA/H₂O, 5% to 40% over 30 min. *N-Terminal cysteic acid peptide.

Subsequently, the CuSO₄–cysteamine system was applied to the synthesis of the bis-disulphide-bridging peptide, Apamin **15** as shown in Figures 5, 6, Figs S8 and S9 in the SI). Because Apamin has two disulphide bridges (Cys1–Cys11 and Cys3–Cys15), linear peptides are required for stepwise disulphide bond formation, and Thz1-**N**-Cys(SH)3-**KAPETAL**-Cys(Acm)11-**ARR**-Cys(SH)15-**QQH**-NH₂ (**16**), was initially prepared by an Fmoc-based solid-phase peptide protocol. 1,3-Thiazolidine carbonyl (Thz), triphenyl methyl-*S*-cysteine (Cys(Trt)) and Cys(Acm) were employed as protecting groups for cysteines at positions 1, 3 and 15, and 11. Protected peptide resin was treated with TFA to afford the linear peptide **16**, which was subjected to an air-oxidation step (0.5 mM peptide concentration) in 6 M Gn·HCl–0.1 M HEPPS buffer (pH 7.7) at 37 °C for 24 h to establish the first disulphide bond between Cys3 and Cys15. Then, a solution of CuSO₄ was added to the reaction mixture containing one-disulphide pairing peptide **17** to construct the second disulphide

bond, between Thz1 and Cys(Acm)11. The attempted CuSO₄-mediated reaction readily afforded the desired Cys1–Cys15 disulphide-linked peptide **15** in 61% isolated yield after EDTA quenching although small amounts of oxidative side products were also detected (Figure 6). Disulphide pairs of synthetic **15** was confirmed by comparative HPLC analyses of **15** and possible three isomers prepared by a regioselective disulphide-forming reaction[15](Fig S8 in the SI). Next, we conducted the disulphide-bridging in reverse order using Thz1-**N**-Cys(MBzl)3-**KAPETAL**-Cys(Acm)11-**ARR**-Cys(MBzl)15-**QQH**-NH₂ (**18**) (Fig S9 in the SI). However, attempts to construct the disulphide bond between Thz1 and Cys(Acm)11 with CuSO₄ under aerobic conditions resulted in the formation of significant amounts of Cys1-over-oxidized peptides. The reason for this difference in the CuSO₄-mediated oxidation steps might be attributable to the possibility that the initially formed disulphide bond of peptide **16** facilitates the second step disulphide-bridging with CuSO₄.

Conclusions

Studies on the ring-opening of the Thz residue in a Cys(Acm)-containing peptide with CuSO₄ under aerobic conditions led to the finding that formation of an intramolecular disulphide bond between the Thz and Cys(Acm) residues occurs. Experimental examination of the reason for the disulphide formation revealed the pivotal role of the Thz-derived thiol which is susceptible to Cu(II)-mediated oxidation under the influence of the neighbouring amino group. Consequently, such insights resulted in both the development of an *S*-Acm deprotection system mediated by CuSO₄–1,2-aminothiols under aerobic conditions and the synthesis of Apamin, a peptide containing two disulphides in a one-pot/sequential disulphide-bridging reaction. Finally, the development of a more sophisticated deprotection format for *S*-Acm groups from the CuSO₄–1,2-aminothiols system is in progress and will be reported in due course.

Experimental

General Information

Materials. All reagents and solvents were obtained from the Peptide Institute, Inc. (Osaka, Japan), FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), Tokyo Chemical Industry Co., Ltd (Tokyo, Japan), Nacalai Tesque, Inc. (Kyoto, Japan), Watanabe Chemical Industries, Ltd (Hiroshima, Japan), Merck KGaA (Darmstadt, Germany) and Sigma-Aldrich Co. LLC (St Louis, MO).

HPLC, MS. Each peptide was characterized by MS analysis as described below. Mass spectra were recorded on a LC-MS (Shimadzu, Japan, Prominence-I LC-2030, LCMS-2020) and a Cosmosil 5C₁₈-AR-II analytical column (Nacalai Tesque, Japan, 4.6 × 250 mm, flow rate 1 mL min⁻¹, were used, and eluting products were detected by UV at 220 nm and MS). For HPLC separation, HPLC was carried out on HITACHI L-7150 with an L-2400 detector using a Cosmosil 5C₁₈-AR-II analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate 1.0 mL min⁻¹), a Cosmosil 5C₁₈-AR-II semipreparative column (Nacalai

Tesque, 10×250 mm, flow rate 3.0 mL min^{-1}), or a Cosmosil 5C$_{18}$-AR-II preparative column (Nacalai Tesque, 20×250 mm, flow rate 10 mL min^{-1}) eluting with a linear gradient system (solvent A: 0.1% TFA in H$_2$O, solvent B: 0.1% TFA in CH$_3$CN)

SPPS. Unless otherwise stated, peptides used in this work were synthesized by Fmoc solid-phase peptide synthesis (Fmoc-SPPS) on Rink Amide AM resin (0.67 mmol g^{-1}) or Histidine 2-chlorotrityl resin (0.29 mmol g^{-1}). Fmoc SPPS was performed according to the following protocol. 1) Removal of Fmoc groups was carried out with 20% piperidine/DMF for 10 min at room temperature (RT). 2) The resin was washed with DMF (× 5). 3) A standard Fmoc-protected amino acid (4.0 equiv.) was coupled with the aid of *N,N*-diisopropylcarbodiimide (DIPCI) (4.0 equiv.) and 1-hydroxy-benzotriazole monohydrate (HOBt·H$_2$O) (4.0 equiv.) in DMF for 1.5 h at RT. Completion of the coupling reaction was confirmed by the Kaiser ninhydrin test. The coupling reaction was repeated until the Kaiser test became negative. 4) The resin was washed with DMF (× 3). A cycle of step 1 to 4 was repeated. Deprotection of acid-labile protecting groups with concomitant release of peptides from a resin was achieved using a cocktail of TFA/TES/H$_2$O (v/v, 95/2.5/2.5) (50 μL/1 mg resin) at RT for 1.5–2 h. After the resin was filtered off, cooled Et$_2$O was added to the filtrate and the precipitate was collected by centrifugation. The obtained precipitate was washed with cooled Et$_2$O and purified by preparative HPLC. The following side-chain protected amino acids were employed: Arg(Pbf), Asp(O*t*Bu), Cys(Acm), Cys(MBzl), Cys(*t*Bu), Cys(Trt), Glu(O*t*Bu), His(Trt), Lys(Boc), Ser(*t*Bu), Thr(*t*Bu), Tyr(*t*Bu).

Synthesis of peptides

*H-Thz-**GALYR**-Cys(Acm)-**FG**-NH$_2$* (**1**) (16.4 mg, 12.6 μmol, 38% isolated yield): Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 45% over 30 min, t_R = 19.5 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 14% to 24% over 30 min, t_R = 16.1 min. LRMS (M + H) calcd for C$_{47}$H$_{70}$N$_{14}$O$_{11}$S$_2$ 1071.5, found 1071.2.

*H-Cys-**GALYR**-Cys(Acm)-**FG**-NH$_2$* (**2**) (5.8 mg, 4.5 μmol, 27% isolated yield): Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 45% over 30 min, t_R = 19.2 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 15% to 25% over 30 min, t_R = 13.8 min. LRMS (M + H) calcd for C$_{46}$H$_{70}$N$_{14}$O$_{11}$S$_2$ 1059.5, found 1059.3.

*H-Ala-**GALYR**-Cys(Acm)-**FG**-NH$_2$* (**5**) (13.7 mg, 10.9 μmol, 33% isolated yield): Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 45% over 30 min, t_R = 18.6 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 14% to 24% over 30 min, t_R = 13.9 min. LRMS (M + H) calcd for C$_{46}$H$_{70}$N$_{14}$O$_{11}$S 1027.5, found 1027.2.

*Ac-Cys-**GALYR**-Cys(Acm)-**FG**-NH$_2$* (**6**) (11.4 mg, 9.4 μmol, 56% isolated yield): Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 45% over 30 min, t_R = 22.9 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 18% to 28% over 30 min, t_R = 17.1 min. LRMS (M + H) calcd for C$_{48}$H$_{72}$N$_{14}$O$_{12}$S$_2$ 1101.5, found 1101.6.

*H-Ala-**GALYR**-Cys-**FG**-NH$_2$* (**12**) (8.6 mg, 7.3 μmol, 26% isolated yield): Analytical HPLC conditions: linear gradient of solvent B in

solvent A, 5% to 45% over 30 min, t_R = 19.7 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 16% to 26% over 30 min, t_R = 14.7 min. LRMS (M + H) calcd for C$_{43}$H$_{65}$N$_{13}$O$_{10}$S 956.5, found 956.5.

*H-Ala-**GALYR**-Cys(MBzl)-**FG**-NH$_2$* (**14**) (1.7 mg, 1.3 μmol, 14% isolated yield): Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 45% over 30 min, t_R = 25.1 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 23% to 33% over 30 min, t_R = 15.6 min. LRMS (M + H) calcd for C$_{51}$H$_{73}$N$_{13}$O$_{11}$S 1076.5, found 1076.5.

*H-Ala-**GALYR**-Met-**FG**-NH$_2$* (**S1**) (8.7 mg, 7.1 μmol, 21% isolated yield): Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 45% over 30 min, t_R = 23.6 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 22% to 32% over 30 min, t_R = 10.2 min. LRMS (M + H) calcd for C$_{51}$H$_{73}$N$_{13}$O$_{11}$S 984.5, found 984.1.

Ratio of CuSO$_4$ and ascorbate

An aqueous solution of CuSO$_4$ (2.5 μL of 0.8 M; final concentration 40 mM) was added to a solution of **1** (1 mM, 50 μL) in 6 M Gn·HCl in 0.1 M HEPPS (pH 7.0) in the presence of Na ascorbate solution (2.5 μl of 0.8 M, 1.6 M or 2.4 M; final concentration 40 mM, 80 mM or 120 mM, respectively) or in the absence of ascorbate. The reaction was continued for 1 h at 37 °C and pH 5 under ambient atmosphere. To quench the reaction, 100 mM EDTA aq. or solid DTT was added to the reaction. After filtering off the precipitate, the resulting solution was analyzed by HPLC.

Analytical HPLC conditions of peptide **2**: linear gradient of solvent B in solvent A, 5% to 45% over 30 min, t_R = 18.9 min. LRMS (M + H) calcd for C$_{46}$H$_{70}$N$_{14}$O$_{11}$S$_2$ 1059.5, found 1059.7.

Analytical HPLC conditions of peptide **3**: linear gradient of solvent B in solvent A, 5% to 45% over 30 min, t_R = 20.4 min. LRMS (M + H) calcd for C$_{43}$H$_{65}$N$_{13}$O$_{11}$S$_2$ 988.5, found 988.6.

Analytical HPLC conditions of peptide **4**: linear gradient of solvent B in solvent A, 5% to 45% over 30 min, t_R = 18.8 min. LRMS (M + H) calcd for C$_{43}$H$_{63}$N$_{13}$O$_{10}$S$_2$ 986.4, found 986.6.

Evaluation of the contribution of the N-terminal amino acid to the deprotection of Acm group

CuSO$_4$ solution (2.5 μL of 0.8 M; final concentration 40 mM) was added to a solution of **1**, **2**, **5** or **6** (1 mM, 50 μL) in 6 M Gn·HCl in 0.1 M HEPPS (pH 7.0). The reaction was continued for 1 h at 37 °C and pH 5 under ambient atmosphere. To quench the reaction, 100 mM EDTA aq. was added to the reaction. After filtering off the precipitate, the resulting solution was analyzed by HPLC.

Analytical HPLC conditions of peptide **4**: linear gradient of solvent B in solvent A, 5% to 45% over 30 min, t_R = 18.7 min. LRMS (M + H) calcd for C$_{43}$H$_{63}$N$_{13}$O$_{10}$S$_2$ 986.4, found 986.5.

Optimization of conditions for removal of Acm group

Peptide **5** (1 mM, 50 μL) was treated with various concentrations of CuSO$_4$ (2.5 μL of 0.50 M, 1.0 M or 2.0 M; final concentrations 25 mM, 50 mM or 100 mM, respectively) in 6 M Gn·HCl–0.1 M HEPPS buffer (pH 7.0) in the presence of 1,2-aminothiols (**9a**, **9b** or **9c** at the concentrations shown in Table 2) and ethyl 4-hydroxybenzoate as internal standard. The reaction mixture was stirred for 3 h at 37 °C, pH 5 under ambient atmosphere and quenched with 100 mM EDTA aq. or 500 mM

ascorbate (solid form) and 500 mM DTT (solid form) followed by stirring for 30 min. Then, progress of reaction was monitored by HPLC analyses.

Analytical HPLC conditions of peptide **11a**: linear gradient of solvent B in solvent A, 5% to 45% over 30 min, t_R = 27.6 min. LRMS (M + H) calcd for $C_{45}H_{70}N_{14}O_{10}S_2$ 1031.5, found 1031.4.

Analytical HPLC conditions of peptide **11b**: linear gradient of solvent B in solvent A, 5% to 45% over 30 min, t_R = 27.6 min. LRMS (M + H) calcd for $C_{46}H_{70}N_{14}O_{12}S_2$ 1075.5, found 1075.4.

Analytical HPLC conditions of peptide **11c**: linear gradient of solvent B in solvent A, 5% to 45% over 30 min, t_R = 27.6 min. LRMS (M + H) calcd for $C_{46}H_{71}N_{15}O_{11}S_2$ 1074.5, found 1074.6.

Analytical HPLC conditions of peptide **12**: linear gradient of solvent B in solvent A, 5% to 45% over 30 min, t_R = 19.5 min. LRMS (M + H) calcd for $C_{43}H_{65}N_{13}O_{10}S$ 956.5, found 956.5.

Synthesis of Peptide Containing Cys(Trt)[16]

Trt-OH (1.0 mg, 4.0 μmol) was added to a solution of Peptide **12** (2.3 mg, 2.0 μmol) in HFIP (400 μL). The reaction mixture was stirred at RT for 6 h. Then, the solution was concentrated by N_2 gas, and the resulting crude material was purified by preparative HPLC.

H-Ala-*GALYR*-Cys(Trt)-*FG*-NH₂ (**13**) (1.0 mg, 0.70 μmol, 35% isolated yield): Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 95% over 30 min, t_R = 17.5 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 36% to 46% over 30 min, t_R = 12.4 min. LRMS (M + H) calcd for $C_{62}H_{79}N_{13}O_{10}S$ 1198.6, found 1198.5.

Evaluation of the stability of other *S*-protecting group in the presence of CuSO₄ and cysteamine

CuSO₄ solution (2.5 μL of 2.0 M; final concentration 100 mM) was added to a solution of **13** or **14** (1 mM, 50 μL) in 6 M Gn·HCl in 0.1 M HEPPS (pH 7.0) in the presence of cysteamine derivatives (100 mM). The reaction was continued for 3 h at 37 °C and pH 5 under ambient atmosphere. After quenched with 100 mM EDTA aq., the resulting solution was filtered and then analyzed by HPLC.

Treatment of Met-containing peptide with CuSO₄ in the presence or absence of cysteamine

CuSO₄ solution (2.5 μL of 2.0 M or 800 mM; final concentration 100 mM or 40 mM) was added to a solution of **S1** (1 mM, 50 μL) in 6 M Gn·HCl in 0.1 M HEPPS (pH 7.0) in the presence or absence of cysteamine (100 mM). The reaction was continued for 3 h or 45 min at 37 °C and pH 5 under ambient atmosphere. After quenched with 100 mM EDTA aq., the resulting solution was filtered and then analyzed by HPLC.

Analytical HPLC conditions of peptide **S2**: linear gradient of solvent B in solvent A, 5% to 45% over 30 min, t_R = 18.8 min. LRMS (M + H) calcd for $C_{45}H_{69}N_{13}O_{11}S$ 1000.5, found 1000.1.

Analytical HPLC conditions of peptide **S3**: linear gradient of solvent B in solvent A, 5% to 45% over 30 min, t_R = 24.0 min. LRMS (M + H) calcd for $C_{47}H_{74}N_{14}O_{11}S_2$ 1075.5, found 1075.0.

Examination of deprotection of Acm mediated by iron salts

A K_3FeCN_6 solution (2.5 μL of 2.0 M; final concentration 100 mM) was added to a solution of **2** (1 mM, 50 μL) in 6 M Gn·HCl in 0.1 M HEPPS (pH 7.0) in the presence of cysteamine (100 mM). The reaction was continued for 3 h at 37 °C and pH 5 under

ambient atmosphere. After quenched with 100 mM EDTA aq., the resulting solution was filtered and then analyzed by HPLC.

Examination of deprotection of *S*-Acm with H₂O₂

H_2O_2 solution (2.5 μL of 0.8 M; final concentration 40 mM) was added to a solution of **2** (1 mM, 50 μL) in 6 M Gn·HCl in 0.1 M HEPPS (pH 7.0). The reaction was continued for 45 min at 37 °C and pH 5 under ambient atmosphere. After filtering off the precipitate, the resulting solution was analyzed by HPLC.

Analytical HPLC conditions of peptide **4**: linear gradient of solvent B in solvent A, 5% to 45% over 30 min, t_R = 20.4 min. LRMS (M + H) calcd for $C_{43}H_{63}N_{13}O_{10}S_2$ 986.4, found 986.1.

Analytical HPLC conditions of peptide **S4**: linear gradient of solvent B in solvent A, 5% to 45% over 30 min, t_R = 20.4 min. LRMS (M + H) calcd for $C_{43}H_{63}N_{13}O_{10}S_2$ 1107.5, found 1107.0.

Analytical HPLC conditions of the dimer of peptide **2**: linear gradient of solvent B in solvent A, 5% to 45% over 30 min, t_R = 22.6 min. LRMS (M + 2H) calcd for $C_{92}H_{138}N_{28}O_{22}S_4$ 1058.5, found 1058.5.

Analytical HPLC conditions of peptide **S5**: linear gradient of solvent B in solvent A, 5% to 45% over 30 min, t_R = 21.9 min. LRMS (M + 2H) calcd for $C_{92}H_{138}N_{28}O_{23}S_4$ 1066.5, found 1066.0.

Application of the CuSO₄–cysteamine system to establish the second disulphide bridge (Cys1-Cys11) of Apamin

H-Thz-*N*-Cys-*KAPETAL*-Cys(Acm)-*ARR*-Cys-*QQH*-OH (**16**) The peptide was assembled on the Fmoc-His(Trt)-2-Cl-Trt resin (0.010 mmol) using the SPPS procedure as described above. The subsequent deprotection of the resin was carried out with TFA/TES/H₂O (v/v/v, 95/2.5/2.5) for 2 h to give a crude product, which was purified by preparative HPLC to yield the title compound (7.9 mg, 3.1 μmol, 31% isolated yield): Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 45% over 30 min, t_R = 13.9 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 16% to 26% over 30 min, t_R = 20.8 min. LRMS (M + 2H) calcd for $C_{83}H_{139}N_{31}O_{26}S_4$ 1058.0, found 1058.2.

To establish the first disulphide bridge, a solution of **16** (0.5 mM, 2 mL) in 6 M Gn·HCl in 0.1 M HEPPS (pH 7.7) was stirred for 24 h at 37 °C under the ambient atmosphere. After completion of formation of the first disulphide bridge, CuSO₄ solution (100 μl of 2.0 M; final concentration 100 mM) was added to the reaction mixture with additional stirring for 5 h at 37 °C. The reaction was quenched by the addition of 100 mM EDTA aq. to the mixture. After filtering off the precipitate, HPLC purification of the filtrate gave Apamin **15** in 61% (1.6 mg) isolated yield.

Analytical HPLC conditions of peptide **17**: linear gradient of solvent B in solvent A, 5% to 40% over 30 min, t_R = 15.6 min. LRMS (M + 2H) calcd for $C_{83}H_{137}N_{31}O_{26}S_4$ 1057.0, found 1057.1.

Analytical HPLC conditions of Apamin **15**: linear gradient of solvent B in solvent A, 5% to 40% over 30 min, t_R = 13.3 min. LRMS (M + 2H) calcd for $C_{79}H_{130}N_{30}O_{25}S_4$ 1014.4, found 1014.7.

Synthesis of disulphide isomers using regioselective disulphide-forming reaction[15]

Syntheses of isomers were conducted by the regioselective disulphide-forming reaction consisting of air-oxidation and subsequent I_2 treatment. Requisite peptides (Cys(SH)1, Cys(Acm)3, Cys(SH)11 and Cys(Acm)15-peptide, Cys(SH)1, Cys(Acm)3, Cys(Acm)11 and Cys(SH)15-peptide and Cys(SH)1, Cys(SH)3,

Cys(Acm)11 and Cys(Acm)15-peptide were synthesized by Fmoc-SPPS protocol using Cys(Trt) and Cys(Acm) as *S*-protected cysteine derivatives. Linear Cys(Acm) peptides were subjected to air-oxidation process to establish the first disulphide bond. After being purified by HPLC purification, mono-disulphide peptides were treated with I_2 in MeOH/0.5 M HCl aq. (4/1) to construct the second disulphide bond. The reactions were quenched by the addition of ascorbate until the reaction mixture becomes colorless. Then, the mixture was purified by preparative HPLC.

Cys1–Cys11 and Cys3–Cys15 isomer (Apamin): linear gradient of solvent B in solvent A, 5% to 40% over 30 min, t_R = 15.0 min.

Cys1–Cys15 and Cys3–Cys11 isomer: linear gradient of solvent B in solvent A, 5% to 40% over 30 min, t_R = 16.3 min.

Cys1–Cys3 and Cys11–Cys15 isomer: linear gradient of solvent B in solvent A, 5% to 40% over 30 min, t_R = 16.2 min.

Examination of the use of CuSO₄–cysteamine system to establish the first disulphide bridge (Cys1-Cys11) of Apamin

The peptide *H-Thz-**N**-Cys(MBzl)-**KAPETAL**-Cys(Acm)-**ARR**-Cys(MBzl)-**QQH**-OH* (**18**) was assembled on the Fmoc-His(Trt)-2-Cl-Trt resin (0.010 mmol) using the SPPS procedure as described above. Subsequent deprotection of the resin was carried out with TFA/TES/H_2O (v/v/v, 95/2.5/2.5) for 2 h to give a crude product, which was purified by preparative HPLC to yield the title compound (5.2 mg, 1.9 μmol, 18% isolated yield): Analytical HPLC conditions: linear gradient of solvent B in solvent A, 5% to 45% over 30 min, t_R = 19.9 min. Preparative HPLC conditions: linear gradient of solvent B in solvent A, 16% to 26% over 30 min, t_R = 15.1 min. LRMS (M + 2H) calcd for $C_{99}H_{155}N_{31}O_{28}S_4$ 1178.0, found 1178.4.

CuSO₄ solution (2.5 μL of 2.0 M; final concentration 100 mM) was added to a solution of **18** (1 mM, 50 μL) in 6 M Gn·HCl in 0.1 M HEPPS (pH 7.0). The reaction was continued for 1 h at 37 °C and pH 5 under ambient atmosphere. After quenched with 100 mM EDTA aq., the resulting solution was filtered and then analyzed by HPLC.

Analytical HPLC conditions of peptide **19**: linear gradient of solvent B in solvent A, 5% to 40% over 30 min, t_R = 21.0 min. LRMS (M + 3H) calcd for $C_{95}H_{148}N_{30}O_{27}S_4$ 757.3, found 757.4.

Examination of deprotection of *S*-Acm in de-aerated solvent.

CuSO₄ solution (2.5 μL of 2.0 M; final concentration 100 mM) was added to a de-aerated solution of **5** (1 mM, 50 μL) in 6 M Gn·HCl in 0.1 M HEPPS (pH 7.0) in the presence of cysteamine (100 mM). The reaction was continued for 3 h at 37 °C and pH 5 under an Ar atmosphere. After quenched with 100 mM EDTA aq., the resulting solution was filtered and then analyzed by HPLC.

Conflicts of interest

There are no conflicts to declare.

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