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COMMUNICATION

# Copper (II)-mediated C-H sulphenylation or selenylation of tryptophan enabling macrocyclization of peptides

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**Cu(II)-mediated C-H sulphenylation or selenylation of the Trp indole by a derivative of cysteine or selenocysteine enables access to the tryptathionine unit or its selenium congener. The mechanism of these protocols which allow macrocyclization of Trp- containing peptides has been studied.**

Regioselective oxidation of the N-terminal cysteine (Cys) residue in peptides with copper sulphate ($CuSO_4$) under aerobic conditions leads to disulphide bond formation between N-terminal and internal S-acetamidomethyl (Acm) Cys residues (Fig. 1(a)).[1] The number in parentheses indicates the oxidation state of the sulphur atom. In this reaction, an electrophilic Cys sulphenic acid, (Cys(OH), S(0)), or congeners at the same oxidation state, resulting from the oxidation of the cysteine, reacts with the nucleophilic sulphide of Cys(Acm) (S(-2)) to generate a disulphide (S(-1) x 2) with the simultaneous release of the Acm cation. Recently, we reported that S-*p*-methoxybenzyl (MBzl) cysteine sulphoxide (Cys(MBzl)(O), S(0)) can serve as a crypto S-chlorocysteine (S(0)) under acidic conditions and engage in aromatic electrophilic substitution ($S_EAr$) with the indole of tryptophan (Trp, C2(-1)), affording tryptathionine (S(-2) and C2(1)) (Fig. 1(b)).[2] Such oxidative conversion of Cys(Acm) and Trp by a Cys-S-X species (X = heteroatom) led to speculation that under the influence of a Cu(II) salt, the N-terminal Cys could react with a Trp in peptides to form tryptathionine (Fig.1(b, This work)). The tryptathionine is an indispensable structure commonly found in bicyclic peptidic toxins.[3] It is useful for payloads of antibody-drug conjugates (ADC)[4] and functions as a potential linkage for peptide stapling.[2,5] Consequently, we attempted to develop the Cu(II)-mediated methodology to form the tryptathionine linkage. Application of the developed protocol to the C-H selenylation of tryptophan residue in peptides was also examined.

Our evaluation began with the Fmoc-based preparation of

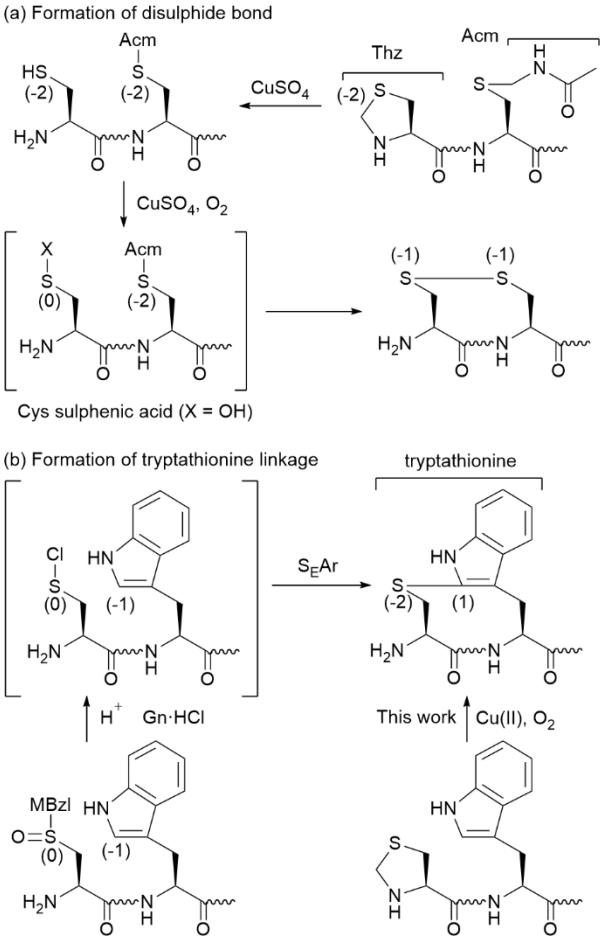


**Fig. 1.** Oxidation of S-acetamidomethyl cysteine (Cys(Acm)) and tryptophan with oxidized cysteine units. (a) Formation of disulphide bond. (b) Formation of tryptathionine linkage. Acm = acetamidomethyl; MBzl = *p*-methoxybenzyl; Thz = 1,3-thiazolidine-4-carbonyl. Number in the parenthesis: oxidation state of the sulphur or C2 of the indole.

the linear precursor peptide, (H-Thz-**NPI**-Trp-**GIG**-OH (**1**): Thz = 1,3-thiazolidine-4-carbonyl), leading to a toxic bicyclic octapeptide, the Amatoxin derivative (Pro2-Ile3-S-deoxo amaninamide (**2**),[3b] in which the Thz was incorporated as a protected N-terminal cysteine from which the cysteine can be regenerated by the action of Cu(II) salts.[6,7] Results obtained

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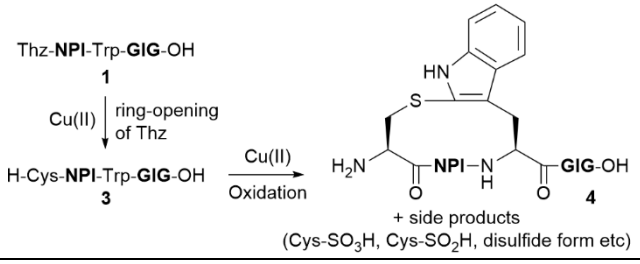
under different reaction conditions are summarized in Table 1, Fig. 3 and Fig. S1 in ESI†.

**Table 1.** Examination of reaction conditions enabling the C-H sulphenylation of Trp




| entry | Peptide (mM) | $Cu^{2+}$ (mM) | buffer | Formation of **4** (%)[a] |
|---|---|---|---|---|
| 1 | **1** (1) | $CuSO_4$ (40) | Gn·HCl (6 M)–HEPPS (100 mM) in $H_2O$ | ND[b] |
| 2 | **1** (1) | $CuCl_2$ (40) | Gn·HCl (6 M)– Tris·HCl (100 mM) in $H_2O$ | 8 |
| 3 | **1** (1) | $CuCl_2$ (40) | Gn·HCl (1.2 M)–Tris·HCl (20 mM) in HFIP–$H_2O$ (4:1) | 31 |
| 4 | **1** (1) | $CuCl_2$ (40) | Gn·HCl (1.2 M)–Tris·HCl (20 mM) in EtOH–$H_2O$ (4:1) | ND |
| 5[c] | **1** (1) | $CuCl_2$ (40) | Tris·HCl (100 mM) in HFIP–EG–$H_2O$ (8:1:1) | 75 |
| 6[c] | **1** (1) | $CuCl_2$ (40) | $FeSO_4$ (20 mM)–Tris·HCl (100 mM) in HFIP–EG–$H_2O$ (8:1:1) | >90 |
| 7[c] | **3** (1) | $CuCl_2$ (40) | $FeSO_4$ (20 mM)–Tris·HCl (100 mM) in HFIP–EG–$H_2O$ (8:1:1) | 16 |

Unless otherwise mentioned, reactions were carried out at 37 °C for 45 min under aerobic conditions (final pH = 4–5). The reaction was diluted fivefold with 100 mM EDTA and directly analyzed by HPLC. [a]Formation (%) was determined by HPLC analysis with UV detection at 220 nm and calculated using the equation: percent formation = 100 [(integ. **4**)/(integ. **1** + **4** + by-products)], where integ. = integrated peak area of the UV absorption. [b]ND = not detected. [c]37 °C for 2 h.

The reaction of the Thz peptide **1** (1 mM) under the same conditions used for the oxidation of Cys(Acm), (40 mM $CuSO_4$ in 6 M guanidine hydrochloride (Gn·HCl)–0.1 M 3-[4-(2-hydroxyethyl)piperazin-1-yl]propane-1-sulphonic acid (HEPPS) (pH 7.0) at 37 °C for 45 min), led to no desired material **4** being detected. A complex mixture in which oxidized peptides, Cys-$SO_3H$, Cys-$SO_2H$ and disulphide peptides were the main components was produced (Table 1, entry 1 (Fig. S1(a))).‡ Research on the oxidation of thiols has shown that various oxidation routes are responsible for producing the side products, but we simplified the reactions as shown in Fig. 2 to explore the optimum reaction conditions. The thiol **3** formed in the $CuSO_4$-mediated ring-opening of the Thz dissociates into a thiolate anion with participation of the adjacent amino group. This thiolate anion is oxidized to the corresponding thiyl radical with the aid of the Cu(II) ion.[8] The reaction of the thiyl radical with oxygen affords the peroxyl radical **5**.[9] Reduction of **5** with thiolate anion or Cu(I), followed by protonation, gives the peroxy species **6** and a Fenton-like reaction of **6** affords a hydroxy radical and a Cys(OH) intermediate **7** that is a key intermediate in the reactions. The resulting Cys(OH) intermediate **7** could participate in the desired product-forming path to the product through the nucleophilic attack of a chloride anion,

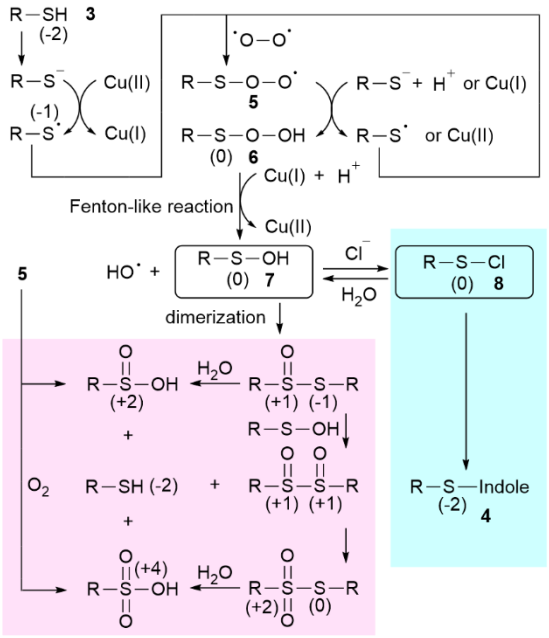



**Fig. 2.** Plausible reaction mechanism responsible for formation of products in the reaction. blue: desired product-forming path; red: by-product-forming path. R-SH = N-terminal Cys peptide **3**.

leading to S-chlorocysteine **8** which can achieve the electrophilic substitution of indole (Fig. 2, blue).[2,10] Alternatively, dimerization[11] of **7** or further oxidation of **5**[12] leads to the formation of several by-products (Fig. 2, red: by-product-forming path). Based on this speculative mechanism, we attempted to prioritize the preferential formation of **8** using suppression of dimerization of **7** by increasing the concentration of chloride anion in the reaction. Replacement of HEPPS and $CuSO_4$ with Tris·HCl and $CuCl_2$, respectively, showed no significant improvement (entry 2 (Fig. S1(b))), and suppression of the hydrolysis of **8** was attempted using hexafluoroisopropanol (HFIP)[13]. The attempted reaction of **1** with $CuCl_2$ (40 mM)–1.2 M Gn·HCl–20 mM Tris·HCl in HFIP–$H_2O$ (4:1, v/v) gave **4** as the desired main product; however, a considerable amount of deletion peptides was formed (entry 3 (Fig. S1(c))). Replacement of HFIP with EtOH resulted in the formation of oxidized compounds as the major products (entry 4 (Fig. S1(d))). Nucleophilic attack of guanidine on the intermediary imine formed during the ring-opening of Thz is related to the formation of the deletion peptides.[14] Accordingly, omission of guanidine from the reaction was attempted but this led to an immiscible two-phase liquid mixture. The addition of ethylene glycol (EG), a hydroxy radical scavenger,[15] led to an incremental improvement in the homogeneity of the reagent mixture. Subsequently, the reaction of **1** in 100 mM Tris·HCl in HFIP–EG–$H_2O$ (8:1:1, (V/V)) in the presence of 40 mM $CuCl_2$ gave a homogeneous solution and afforded **4** in 75% yield with suppression of the formation of oxidized by-products (entry 5 (Figs. 3(b) and S1(e))). Replacement of $H_2O$ with HFIP, which could function as an acid catalyst for conversion of **7** to **8** or **8** to **4**,[13,16] was apparently indispensable for suppression of the by-product formation shown in Fig. 2; however, a non-negligible amount of oxidized by-product still was produced.

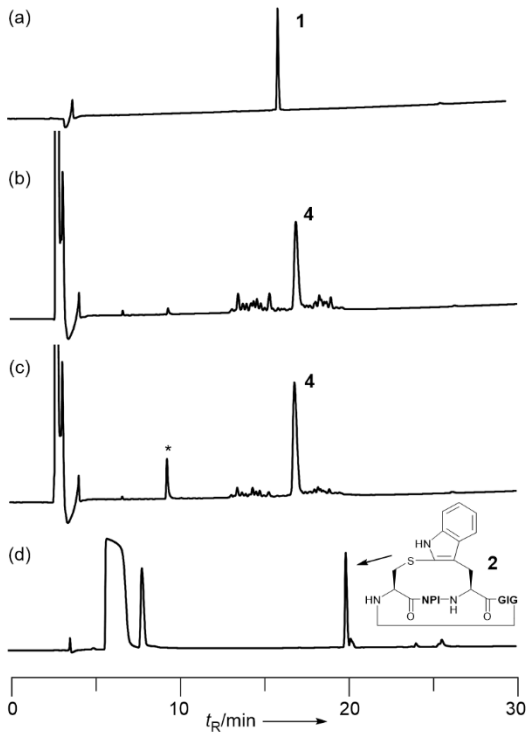


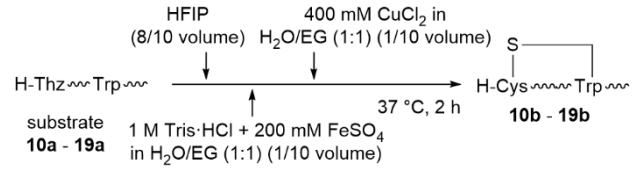

**Fig. 3.** HPLC examination of the reactions listed in Table 1 and subsequent lactam formation of the resulting **4**. (a) Purified substrate **1**. (b) Entry 5. (c) Entry 6. (d) Lactam formation. Analytical HPLC conditions: linear gradient of 0.1% TFA/$CH_3CN$ in 0.1% TFA/$H_2O$, 5% to 65% over 30 min; detection at 220 nm; Column temperature 60 °C.

We next attempted to reduce the involvement of **5** in the by-product-forming path by the use of an Fe(II) salt. The Fe(II) salt is expected to promote the reduction of the by-product-forming **5** by consumption of **6** through a Fenton-like reaction. The reaction of **1** in 40 mM $CuCl_2$–20 mM $FeSO_4$–100 mM Tris·HCl in HFIP–$H_2O$–EG (8:1:1, (v/v)) at 37 °C for 2 h proceeded efficiently, yielding **4** in 49% isolated yield (entry 6 (Figs. 3(c) and S2)). We fixed the found conditions optimum. Application of the same conditions to the reaction of the N-terminal Cys peptide **3** failed to give satisfactory results, indicating that the gradual formation of the free Cys residue is indispensable for a clean conversion (entry 7 (Fig. S1(f))).§ The requirement of the amino group of the N-terminal cysteine for production of tryptathionine was confirmed by the observation that the reaction of the N-acetyl substrate, Ac-Cys-**NPI**-Trp-**GIG**-OH (**9**), under optimum conditions affords no sulphenylated Trp (Fig. S3). Replacement of the Cu(II) salt with $FeCl_3$ in the reaction of **3** failed to produce the desired product **4**; instead, the disulphide dimer of **3** was produced almost quantitatively (Fig. S4).

Lactam formation of **4** by (benzotriazole-1-yloxy)tri-pyrrolidinophosphonium hexafluorophosphate (PyBOP)–*N,N*-diisopropylethylamine (DIPEA) in *N*-methylpyrrolidone (NMP) for 2 h at rt yielded the bicyclic peptide **2** in 71% isolated yield after HPLC purification (Figs. 3(d) and S5), which shows characteristic CD pattern of Amatoxin derivative (Fig. S6).[3b]

The wide applicability of the optimum conditions for formation of the Cys-Trp linkage was confirmed by the synthesis of various peptides (**10a**–**19a**) with either variable residue spacing between Cys and Trp or with amino acids sensitive to oxidation, as shown in Table 2. With the exception of the Met-containing peptide **15a**, reactions of other substrates, including the disulphide precursor **19a** of Apamin derivative proceeded almost quantitatively without significant by-products as judged by HPLC, giving the desired products in reasonable isolated yields (Figs S7 and S8(a-e, g, h), S9-12). Reaction of the Met-containing peptide **15a** was accompanied by the formation of the Met(O) peptide and minor amounts of unidentified by-products (Figs. S7 and S8 (f)).

**Table 2**. Summary of the application of the optimal conditions to various substrates[a]




| entry | Peptides | Products isolated yield (%) |
|---|---|---|
| 1 | H-Thz-**GA**-Trp-**R**-$NH_2$ (**10a**) | **10b** (62) |
| 2 | H-Thz-**GALRA**-Trp-**R**-NH2 (**11a**) | **11b** (63) |
| 3 | H-Thz-**G**-Phe-**L**-Trp-**R**-$NH_2$ (**12a**) | **12b** (63) |
| 4 | H-Thz-**G**-Lys-**L**-Trp-**R**-$NH_2$ (**13a**) | **13b** (79) |
| 5 | H-Thz-**G**-His-**L**-Trp-**R**-$NH_2$ (**14a**) | **14b** (62) |
| 6 | H-Thz-**G**-Met-**L**-Trp-**R**-$NH_2$ (**15a**) | **15b** (38) |
| 7 | H-Thz-**G**-Ser-**L**-Trp-**R**-$NH_2$ (**16a**) | **16b** (55) |
| 8 | H-Thz-**G**-Tyr-**L**-Trp-**R**-$NH_2$ (**17a**) | **17b** (51) |
| 9 | H-Thz-**YFQN**-Trp-**PRG**-$NH_2$ (Vasopressin analogue **18a**) | **18b** (76) |
| 10 | H-Thz-**N**-Cys-**KAPETL**-Trp-**AR**-Cys-**QQH**-$NH_2$ (Apamin analogue **19a**)[b] | **19b** (42) |

[a] A mixture of 200 mM $FeSO_4$ and 1 M Tris·HCl in $H_2O$ and EG (1:1, one-eighth volume of HFIP) was added to a solution of the peptide in HFIP. A white suspension was formed. Then, 400 mM $CuCl_2$ in $H_2O$ and EG (1:1, one-eighth volume of HFIP) was added, and the mixture was stirred at 37 °C for 2 h under aerobic conditions with the reaction changing to a green-yellow solution with a small amount of metal precipitate (final reaction mixture peptide (1 mM) in 40 mM $CuCl_2$, 20 mM $FeSO_4$ and 100 mM Tris·HCl in HFIP–$H_2O$–ethylene glycol (8:1:1, (v/v)). Addition of the same amount of 100 mM EDTA to the mixture quenched the reaction, and the resulting solution was used in the HPLC purification of the peptide. [b] Disulphide-bonding substrate was used.

Having established the optimum reaction conditions, we studied linking of Trp and selenocysteine (Sec) using a Cu(II)-mediated protocol. Various elegant C-H selenylation methods of arenes,[17] including the umpolung[18] and radical[19] approaches, have been published recently. Difficult access to Sec-peptides with unprotected Se and the success using Thz as a protected cysteine led to preparation of the 1,3-selenazolidine-4-carbonyl (Sez)-peptide (H-Sez-**NPI**-Trp-**GIG**-OH (**20**)) as a Se-substituted amatoxin precursor.[20,21] Use of the optimized conditions with **20** afforded the desired 2-selenyl tryptophan-containing peptide **21** in 53% isolated yield (Figs. 4(b) and S13). Lactam formation of **21** by PyBOP–DIPEA in NMP afforded the Se analogue **22** of the Amatoxin derivative **2** which was isolated in 72% yield (Figs. 4(c) and S14). The isolated material exhibited a large negative Cotton effect at around 230 nm, characteristic of the Amatoxin derivative (**2**) (Fig. S15 vs S6).[3b]

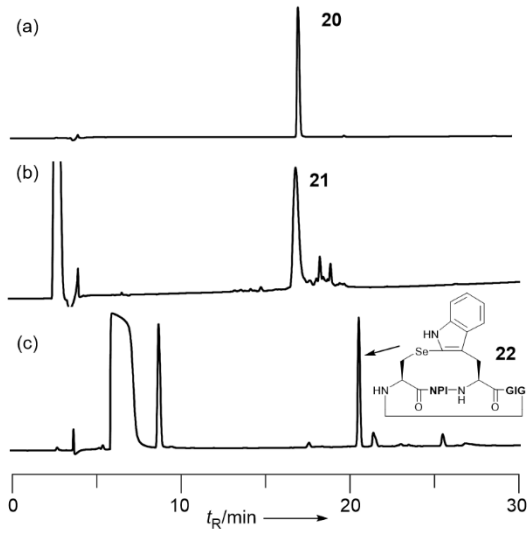



**Fig. 4.** HPLC of the C-H selenylation of the Trp in **20** followed by lactam formation. (a) Purified Sez-containing substrate **20**. (b) C-H Selenylation. (c) Lactam formation. Analytical HPLC conditions: linear gradient of 0.1% TFA/$CH_3CN$ in 0.1% TFA/$H_2O$, 5% to 65% over 30 min; detection at 220 nm; Column temperature 60 °C.

In conclusion, the ring-opening of 1,3-thiazolidine-4-carbonyl (Thz) or 1,3-selenazolidine-4-carbonyl (Sez) peptides and subsequent oxidation mediated by Cu(II) salts in the presence of chloride allows for the C-H sulphenylation or selenylation of Trp to form tryptathionine or its Se analogue. Evaluation of reaction conditions and investigation of the mechanism reveals that the use of Fe(II) salt as a Fenton reaction agent in HFIP is indispensable for full conversion. Optimum conditions were found that achieve the synthesis of several modified Trp-peptides, including a selenylated variation. The transformation proceeds through the umpolung of the nucleophilic Cys or Sec residue.

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## Conflicts of interest

There are no conflicts to declare.

## Notes and references

‡ During this research, the formation of thioether of the peptide sequence corresponding **1** (or **3**) was found to be quite difficult among several substrate peptides,[22] see Figs. S16 and S1(b).

§ Addition of formaldehyde in the reaction of **3** improved the reaction outcome, see Figs. S17 and S1(f).

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