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Synthesis of Reaper, a Cysteine-Containing Polypeptide, Using a Peptide Thioester in the Presence of Silver Chloride as an Activator

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Abstract

Reaper was synthesized by condensing two peptide segments, one of which was a partially protected peptide thioester, Boc-[Lys(Boc)^{20}]-reaper(1—32)-SCH_2CH_2CO-β-Ala-NH_2, and the other a peptide which contained an Cys(Acm) residue, H-[Cys(Acm)^{49}, Lys(Boc)^{52,56,59,62}]-reaper(33—65)-OH. The condensation proceeded without loss of the Acm group when silver chloride was used as an activator of thioester moiety. This result indicates that silver chloride is a generally applicable activating reagent to the preparation of polypeptides which contain Cys(Acm) residues.

Keywords: *Peptides and polypeptides; Thioesters; Silver and compounds; Condensations.*

Part of an ongoing research project in this laboratory involves the development of a method for polypeptide synthesis, in which partially protected *S*-alkyl peptide thioesters (peptide thioesters) are used as building blocks [1–3]. The method has been applied to the synthesis of adrenomedullin, in which *S*-acetamidomethyl cysteine (Cys(Acm))-containing peptide segments,[1] prepared via the Boc solid-phase method, are used as building blocks [4].

In the synthesis of adrenomedullin, the condensation reaction proceeded without significant decomposition of the Acm groups in the presence of silver nitrate and 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine (HOObt). Under the same condensation conditions, however,

[1] **abbreviations**
Abbreviations used are Acm, acetamidomethyl; Boc, *t*-butoxycarbonyl; Boc-OSu, *N*-*t*-butoxycarbonyloxysuccinimide; Bom, benzyloxymethyl; Br-Z, 2-bromobenzyloxycarbonyl; Bzl, benzyl; Cl-Z, chlorobenzyloxycarbonyl; DCC, dicyclohexylcarbodiimide; DIEA, *N*,*N*-diisopropylethylamine; DTT, dithiothreitol; Fmoc, 9-fluorenylmethoxycarbonyl; cHex, cyclohexyl; HOBt, 1-hydroxybenzotriazole; HOObt, 3,4-dihydro-4-oxo-3-hydroxy-1,2,3-benzotriazine; MALDI-TOF MS, matrix assisted laser desorption ionization time-of-flight mass spectroscopy; Pam, phenylacetamidomethyl; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; RP-HPLC, reversed-phase high performance liquid chromatography; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; Tos, tosyl; Trt, triphenylmethyl.

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the unexpected removal of the Acm group was observed in the synthesis of reaper, which contains one cysteine residue. This phenomenon was further examined by synthesizing several model peptides, and led to our observation that the stability of the Acm group toward silver ions was greatly affected by the amino acid residues in the immediate vicinity of the Cys(Acm) residue and that among several different silver compounds such as silver nitrate, silver fluoride, silver thiocyanate and silver chloride, only silver chloride promotes the condensation while retaining the intact Acm group in the presence of HOObt [5]. This paper describes the utility of the combination of silver chloride and HOObt for the synthesis of Cys-containing polypeptides through the synthesis of reaper [6], along with comparison of the effect of silver chloride and silver nitrate.

The amino acid sequence of reaper is shown in Figure 1, in which an arrow indicates the site of segment condensation. The Trp^{32} residue was chosen as the C-terminal amino acid residue in a peptide thioester, since there is no Gly residue suitable for the segment condensation site.

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1                                                                  20
Met-Ala-Val-Ala-Phe-Tyr-Ile-Pro-Asp-Gln-Ala-Thr-Leu-Leu-Arg-Glu-Ala-Glu-Gln-Lys-
                                                                   40
Glu-Gln-Gln-Ile-Leu-Arg-Leu-Arg-Glu-Ser-Gln-Trp-Arg-Phe-Leu-Ala-Thr-Val-Val-Leu-
                                                 ↑                 60
Glu-Thr-Leu-Arg-Gln-Tyr-Thr-Ser-Cys-His-Pro-Lys-Thr-Gly-Arg-Lys-Ser-Gly-Lys-Tyr-
                          65
Arg-Lys-Pro-Ser-Gln-OH
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Figure 1. Amino acid sequence of reaper. An arrow indicates the site of segment condensation.

Boc-[Lys(Boc) ^{20}]-reaper(1—32)-S CH_2CH_2CO-β-Ala-NH_2 (**1**) + H-[Cys(Acm) ^{49}, Lys(Boc)^{52,56,59,62}]-reaper(33—65)-OH (**2**)

↓ $AgNO_3$ or AgCl, HOObt, DIEA, DMSO

Boc-[Cys(Acm) ^{49}, Lys(Boc)^{20,52,56,59,62}]-reaper(1—65)-OH (**3**)

↓ TFA, 1,4-butanedithiol (95 : 5,*v/v*)

H-[Cys(Acm) ^{49}]-reaper(1—65)-OH (**4**)

↓ 1) $AgNO_3$, H_2O, DIEA, TFE, 2) DTT, HCl

Reaper protein

Scheme 1. Synthetic scheme of reaper

The reaper protein was synthesized based on the route shown in Scheme 1. When a peptide thioester, Boc-[Lys(Boc)^{20}]-reaper(1—32)-SCH_2CH_2CO-β-Ala-NH_2 (**1**),[2,3] and a

[2] Mass numbers were determined by MALDI-TOF MS using a Voyger^{TM}DE (PerSeptive Biosystems, Inc., Framingham, MA) and were calculated as an average.

[3] **Peptide thioester 1 was prepared as follows**: Starting from Trt-SCH_2CH_2CO-β-Ala-NH-resin [4], Boc-Trp(CHO) was introduced using DCC and HOBt by double coupling after treating with TFA containing water, *m*-cresol, thioanisole, and 1,2-ethanedithiol (reagent K [7]). After treatment with acetic anhydride, the peptide chain was elongated on a 430A peptide synthesizer (PE Applied Biosystems, Foster City, CA) using the standard Boc protocol to give the protected peptide resin, corresponding to the

peptide, H-[Cys(Acm)^{49}, Lys(Boc)^{52,56,59,62}]-reaper(33—65)-OH (**2**),[4] were condensed using silver nitrate as an activator, the condensation reaction proceeded well. The *S*-Acm group, however, was decomposed rapidly. The peak corresponding to the condensation product was split into two peaks after an 8 h reaction as shown in Fig. 1(A). The former portion of the main peak, **a**, consisted mainly of the desired product, Boc-[Cys(Acm)^{49}, Lys(Boc)$^{20,52,56.59,62}$]-reaper(1—65)-OH (**3**), as evidenced by MALDI-TOF MS analysis, and the latter part of the main peak, **b**, showed a mass number corresponding to a des-Acm product, Boc-[Lys(Boc)$^{20,52,56.59,62}$]-reaper(1—65)-OH (found, *m/z* 8282.2, calcd for $[M+H]^+$, 8282.6). After a 24 h reaction, the mass signal corresponding to the desired product disappeared, as evidenced by the disappearance of the peak **a**. These facts suggest that the Acm group was removed completely in 24 h.

When peptides **1** and **2** were condensed using silver chloride as an activator,[5] splitting of the main peak was not observed even after 24 h reaction as shown in Fig 1(B). The desired product **3** (MALDI-TOF MS: found, *m/z* 8350.7, Calcd for $[M+H]^+$, 8354.7), eluted at 31 min (peak **a**). No condensed product, whose *S*-Acm group was decomposed, was observed in peak **a**. A compound contained in peak **c** showed the same mass number as that of the desired product. This compound was identified as the epimerization product, Boc-[Cys(Acm)^{49}, Lys(Boc)$^{20,52,56.59,62}$, D-Trp^{32}]-reaper(1—65)-OH.[6] These results demonstrate the superiority of silver chloride over silver nitrate as an activator of the thioester moiety in Cys(Acm)-containing polypeptide synthesis, and hence its utility in practical polypeptide synthesis.

After the condensation reaction, dithiothreitol (DTT) was added to inactivate the silver ions and then ether was added to precipitate the crude peptide **3**. This crude product was treated with TFA containing 5% 1,4-butanedithiol to give H-[Cys(Acm)^{49}]-Reaper(1—65)-OH (**4**),[7] which was obtained in 70% yield after isolation by RP-HPLC. Peptide **4** was then treated with silver nitrate in the presence of DIEA and water in 2,2,2-trifluoroethanol (TFE) for 2 h to

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reaper sequence(1—32), Boc-Met-Ala-Val-Ala-Phe-Tyr(Br-Z)-Ile-Pro-Asp(OcHex)-Gln-Ala-Thr(Bzl)-Leu-Leu-Arg(Tos)-Glu(OBzl)-Ala-Glu(OBzl)-Gln-Lys(Cl-Z)-Glu(OBzl)-Gln-Gln-Ile-Leu-Arg(Tos)-Leu-Arg(Tos)-Glu(OBzl)-Ser(Bzl)-Gln-Trp(CHO)-SCH_2CH_2CO-β-Ala-NH-resin. After HF treatment and purification by RP-HPLC, H-reaper(1—32)-SCH_2CH_2CO-β-Ala-NH_2 was obtained in 9.8% yield based on β-Ala in the resin. This peptide was treated with Boc-OSu to give peptide **1** in 7.4% yield based on β-Ala in the resin after purification by RP-HPLC: MS (MALDI-TOF) Found: *m/z* 4221.1. Calcd for $[M+H]^+$, 4220.9. Amino acid analysis: $Asp_{1.02}Thr_{0.96}Ser_{0.94}Glu_{8.93}Ala_4Val_{0.95}Met_{1.05}Ile_{1.77}Leu_{4.07}Tyr_{1.06}(Phe+β\text{-}Ala)_{1.23}Lys_{1.07}Trp_{0.66}Arg_{2.90}$.

[4] **Peptide 2 was prepared as follows**: Peptide chain elongation was carried out on a 430A peptide synthesizer starting from a Boc-Gln-OCH_2-Pam resin, to give the protected peptide resin corresponding to the sequence of reaper(33—65), Fmoc-Arg(Pmc)-Phe-Leu-Ala-Thr(Bzl)-Val-Val-Leu-Glu(OBzl)-Thr(Bzl)-Leu-Arg(Tos)-Gln-Tyr(Br-Z)-Thr(Bzl)-Ser(Bzl)-Cys(Acm)-His(Bom)-Pro-Lys(Cl-Z)-Thr(Bzl)-Gly-Arg(Tos)-Lys(Cl-Z)-Ser(Bzl)-Gly-Lys(Cl-Z)-Tyr(Br-Z)-Arg(Tos)-Lys(Cl-Z)-Pro-Ser(Bzl)-Gln-OCH_2-Pam resin. After HF treatment and purification by RP-HPLC, Fmoc-[Cys(Acm)^{49}]-reaper(33—65)-OH was obtained in 12% yield based on Gln in the starting resin. After protection of the side chain amino group by introduction of a Boc group and deprotection of the Fmoc group with piperidine, peptide **2** was obtained in 5.4% yield based on Gln in the starting resin: MS (MALDI-TOF) Found: *m/z* 4309.7. Calcd for $[M+H]^+$, 4310.0. Amino acid analysis: $Thr_{3.71}Ser_{2.26}Glu_{2.86}Pro_{1.80}Gly_{1.92}Ala_{1.06}Cys_{nd}Val_{1.36}Leu_3Tyr_{2.18}Phe_{1.05}Lys_{4.02}His_{1.08}Arg_{4.15}$.

[5] **A typical procedure of the segment condensation**: Silver chloride (0.3 mg, 2 μmol) was added to a solution of peptide thioester **1** (4.0 mg, 0.75 μmol), peptide **2** (3.2 mg, 0.63 μmol), HOOBt (3.7 mg, 23 μmol), and DIEA (2.6 μL, 15 μmol) in DMSO (75 μL). The mixture was then allowed to stir at room temperature in the dark.

[6] Synthetic reaper was digested with V8 protease and analyzed by RP-HPLC and MALDI-TOF MS. All fragments could be identified and the Trp^{32} containing fragment (reaper(30—41)) was compared with the authentic peptide which was prepared by the Boc solid-phase synthesis. The ratio of peptide contents in peak **a** and peak **c** was 82:18.

[7] **Peptide 4**: MS (MALDI-TOF) Found: *m/z* 7753.5. Calcd for $[M+H]^+$, 7754.0. Amino acid analysis: $Asp_{1.02}Thr_{4.43}Ser_{3.18}Glu_{11.8}Pro_{2.17}Gly_{1.88}Ala_{5.15}Cys_{nd}Val_{2.07}Met_{0.78}Ile_{1.77}Leu_7Tyr_{3.34}Phe_{2.12}Lys_{4.84}His_{0.99}Trp_{0.86}Arg_{7.01}$.

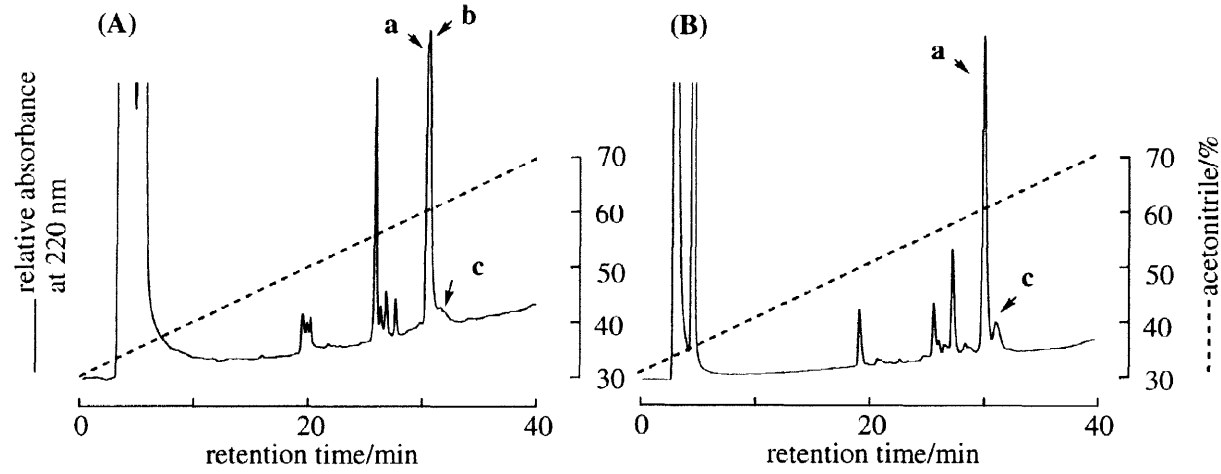

Figure 2. RP-HPLC elution profiles of the reaction mixture of peptide thioester **1** and peptide **2**. (A): Reaction mixture after an 8 h condensation reaction with silver nitrate as activator; (B): Reaction mixture after a 24 h condensation reaction with silver chloride as activator. The gradient is shown on panels and involved the use of aqueous acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 1.0 mL/min on Cosmosil $5C_{18}MS$ (4.6 x 250 mm, Nacalai Tesque, Inc., Kyoto).

remove the Acm group [4] Silver was removed from the peptide by adding DTT to the reaction mixture, followed by hydrochloric acid. The reaper thus obtained was isolated by RP-HPLC in 56% yield.[6,8]

In conclusion, segment condensation proceeded without loss of the Acm group when silver chloride was used as an activator of the thioester moiety. These data indicate that unlike silver nitrate, which is well soluble in DMSO, silver chloride can be used for the preparation of polypeptides by maintaining the silver ion concentration in the solution sufficiently low as to avoid its coordination to the sulfur atom in Cys(Acm).

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[8] **Synthetic reaper**: MS (MALDI-TOF) Found: *m/z* 7683.8. Calcd for $[M+H]^+$: 7682.9. Amino acid analysis: $Asp_{1.02}Thr_{4.57}Ser_{3.18}Glu_{12.1}Pro_{2.58}Gly_{1.93}Ala_{5.20}Cys_{nd}Val_{2.13}Met_{0.84}Ile_{1.82}Leu_7Tyr_{3.21}Phe_{2.11}Lys_{4.97}His_{1.01}Trp_{0.88}Arg_{7.15}$. The synthetic reaper was digested with V8 protease to reveal that [D-Trp^{32}]-reaper content was less than 1%.