Synthesis of Cysteine-Containing Polypeptide Using a Peptide Thioester Containing a Cys(Acm) Residue

Toru Kawakami, Shuji Kogure, and Saburo Aimoto*

Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565

(Received June 17, 1996)

A method was developed for the preparation of a cysteine-containing polypeptide, using a Cys(Acm)-containing peptide thioester obtained by a Boc solid-phase method. To demonstrate the utility of the developed method, human adrenomedullin consisting of 52 amino acid residues was synthesized. A peptide thioester, Boc–[Cys(Acm)^{16}]-adrenomedullin(1—19)-SCH_2CH_2CO-β-Ala–NH_2, and [Cys(Acm)^{21}, Lys(Boc)^{25,36,38,46}]-adrenomedullin(20—52) were prepared by conventional solid-phase methods. These peptide segments were condensed in the presence of silver ions, an additive, and a base. When HOBt or HOOBt was used as an additive, the segment coupling proceeded with negligible decomposition of Acm groups to give the desired product. A method for the removal of *S*-Acm groups from cysteine residues under basic conditions in the presence of silver ions and water, is also described.

Prior studies in this Laboratory[1—5] have involved studies of protein synthesis utilizing peptide thioesters as building blocks. The synthetic procedure, however, becomes complicated for proteins which contain one or more cysteine residue(s), since it becomes necessary to prepare a segment having benzyl-type protecting groups using an Npys solid-phase method, which are stable in the presence of silver ions. The acetamidomethyl (Acm) group[6] represents a more ideal blocking group for the mercapto group of cysteine residues, since it is more easily prepared using a solid-phase method by Boc-strategy. If we can find reaction conditions under which coupling reaction proceeds at a reasonable rate without decomposition of *S*-Acm groups, a simple and general method will be developed for the synthesis of such peptides.

This paper describes a method for the preparation of a cysteine-containing polypeptide, in which a Cys(Acm)-containing peptide thioester is used as a building block, and the application of this method to the synthesis of human adrenomedullin,[7] a peptide which contains 52 amino acid residues. Also described is a convenient method for the removal of Acm groups from a Cys(Acm)-containing peptide under basic conditions in the presence of silver ions and water.

Materials and Methods

Boc-amino acid derivatives, MBHA resin, and an authentic sample of human adrenomedullin were purchased from the Peptide Institute Inc. (Osaka). Fmoc-amino acid derivatives and Fmoc–NH–SAL–PEG-resin (NH_2–SAL=4-(α-amino-2,4-dimethoxybenzyl)phenoxymethyl)[8] were purchased from Watanabe Chemical Ind. Ltd. (Hiroshima). Amino acid derivatives used were of the L-configuration. The DMSO used for the segment coupling reaction was silylation grade (Pierce, Rockford, IL). Reversed-phase HPLC (RP-HPLC) was performed on Cosmosil 5C_{18}-AR (10×250 mm) (Nacalai Tesque, Kyoto) using a linear increasing gradient of acetonitrile in 0.1% aqueous TFA. Amino acids were analyzed on an L-8500 amino acid analyzer (Hitachi Ltd., Tokyo) after hydrolysis with 4 M# methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole at 110 °C for 24 h in an evacuated sealed tube. Peptide mass numbers were determined by FAB mass spectrometry using a JMS-HX100 (JEOL Ltd., Tokyo) equipped with a JMA-3100 mass data system, or by MALDI-TOF mass spectrometry using a Voyager^{TM}RP (PerSeptive Biosystems, Inc., Framingham, MA). Mass numbers were calculated as averages.

Abbreviations used are Acm, acetamidomethyl; Boc, *t*-butoxycarbonyl; Boc-OSu, *N*-*t*-butoxycarbonyloxysuccinimide; Br-Z, 2-bromobenzyloxycarbonyl; Bu^t, *t*-butyl; Bzl, benzyl; DCC, dicyclohexylcarbodiimide; DIEA, *N*,*N*-diisopropylethylamine; FAB, fast atom bombardment; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; HONp, *p*-nitrophenol; HOOBt, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine; HOSu, *N*-hydroxysuccinimide; MALDI-TOF, matrix assisted laser desorption ionization time-of-flight; MBHA, 4-methylbenzhydrylamine; NMP, 1-methylpyrolidin-2-one; Npys, 3-nitro-2-pyridinesulfenyl; PEG, polyethylene glycol; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; RP-HPLC, reversed-phase high performance liquid chromatography; SAL, super acid labile; TFA, trifluoroacetic acid; Tos, tosyl; Trt, triphenylmethyl.

Preparation of Boc–Gly–SCH_2CH_2CO–β-Ala–NH-Resin (1).[3] A solution of Boc-β-Ala (1.21 g, 6.38 mmol), HOBt·H_2O (1.05 g, 6.86 mmol), and DCC (0.95 g, 6.4 mmol) in NMP (13 mL) was stirred together for 50 min. The solution was then added to a neutralized MBHA resin (4.05 g, NH_2: 3.2 mmol) and this mixture was shaken for 3 h. The resulting resin was washed with DMF (2×1 min), 50% methanol in CH_2Cl_2 (3×3 min), and NMP (3×1 min), and then treated with an NMP solution containing 10% acetic anhydride and 5% DIEA (1×10 min). The resin thus obtained was washed with DMF (3×1 min) and then CH_2Cl_2 (3×1 min), treated with 50% TFA in CH_2Cl_2 (1×5 min and 1×20 min), washed with CH_2Cl_2 (3×1 min), treated with 5% DIEA in CH_2Cl_2(2 min), and then 5% DIEA in NMP (2 min), and then washed with NMP (3×1

1 M=1 mol dm^{-3}.

min). Trt-$SCH_2CH_2CO_2H$ (2.23 g, 6.4 mmol), HOBt·H_2O (1.05 g, 6.86 mmol), and DCC (0.95 g, 6.4 mmol) in NMP (13 mL) was mixed for 30 min and the resulting solution was added to the resin. The suspension was then shaken for 20 h. This cycle was repeated one additional time by shaking for 4 h with freshly activated Trt-$SCH_2CH_2CO_2H$. The resin was washed with solvents, treated with acetic anhydride according to the procedure described above, washed with DMF (3×1 min) and then CH_2Cl_2 (3×1 min), and finally dried in vacuo overnight to give the Trt-SCH_2CH_2CO–β-Ala–NH-resin (5.05 g). An aliquot of the resin (2.0 g) was treated with a mixture of TFA, water, phenol, 1,2-ethanedithiol (87.5 : 5 : 5 : 2.5, v/v, reagent K[9]) for 30, 30, and 60 min. The resin was washed with CH_2Cl_2 (3×1 min), treated with 5% DIEA in CH_2Cl_2 (2 min), then 5% DIEA in NMP (2 min), and then washed with NMP (3×1 min). A solution of Boc–Gly (0.91 g, 5.2 mmol), HOBt·H_2O (0.84 g, 5.5 mmol), and DCC (0.77 g, 5.5 mmol) in NMP (11mL) was stirred for 50 min. This solution was then added to the resin and the mixture was shaken for 14 h. The reaction with Boc–Gly was repeated one additional time (20 h). Then resin was treated as described above to give the Boc–Gly–SCH_2CH_2CO–β-Ala–NH-resin (**1**) (1.95 g, Gly: 0.47 mmol g^{-1}).

Preparation of Boc–[Cys(Acm)[16]]-Adrenomedullin(1—19) –SCH_2CH_2CO–β-Ala–NH_2 (4). Starting from the Boc–Gly–SCH_2CH_2CO–β-Ala–NH-resin (**1**) (0.59 g, 0.28 mmol), we prepared 1.21 g of a protected peptide resin corresponding to the sequence of adrenomedullin(1—19), Boc–Tyr(Br-Z)–Arg (Tos)– Gln– Ser(Bzl)– Met– Asn– Asn– Phe– Gln– Gly– Leu– Arg (Tos)– Ser(Bzl)– Phe– Gly– Cys(Acm)– Arg(Tos)– Phe– Gly– SCH_2CH_2CO–β-Ala–NH-resin (**2**), on a peptide synthesizer 430A (Applied Biosystems Inc., Foster City, CA.). The 0.5 mmol standard protocol of system software version 1.40 NMP/HOBt *t*-Boc was employed. End capping by acetic anhydride was performed after each amino-acid introduction reaction. An aliquot of the resin (96 mg) was treated with HF (1.7 mL), anisole (0.15 mL), and 1,4-butanedithiol (0.15 mL) at 0 °C for 90 min. After evaporation of the HF, ether (10 mL) was added to the mixture, and the precipitate obtained was washed with ether (10 mL × 2), then extracted with 25% aqueous acetonitrile (20 mL) to give crude peptide (44 mg) after freeze-drying. This was purified on RP-HPLC to yield [Cys(Acm)[16]]-Adrenomedullin(1—19)–SCH_2CH_2CO–β-Ala–NH_2 (**3**) (16 mg, 4.5 μmol, 20% based on Gly in the starting resin): MS (FAB) Found: *m/z* 2498.4 $(M+H)^+$. Calcd for $(M+H)^+$: 2498.9. Amino acid analysis: $Asp_{2.03}$ $Ser_{1.74}Glu_{2.03}Gly_3Cys_{nd}Met_{0.85}Leu_{1.00}Tyr_{0.96}$(Phe+β-Ala)$_{3.31}Arg_{3.02}$.

To a solution of the thioester **3** in DMSO (0.20 mL) were added Boc–OSu (3.0 mg, 14 μmol) and DIEA (6.0 μL). After stirring for 6 h, the peptide was isolated by RP-HPLC to give Boc–[Cys(Acm)[16]]-Adrenomedullin(1—19)–SCH_2CH_2CO–β-Ala–NH_2 (**4**) (12 mg, 2.8 μmol, 13% based on Gly in the starting resin **1**): MS (FAB) Found: *m/z* 2598.6 $(M+H)^+$. Calcd for $(M+H)^+$: 2598.0. Amino acid analysis: $Asp_{2.09}Ser_{1.87}Glu_{2.10}Gly_{3.02}Cys_{nd}Met_{1.01}Leu_1Tyr_{1.01}$(Phe+β-Ala)$_{3.29}Arg_{2.98}$.

Preparation of [Cys(Acm)[21], Lys(Boc)[25,36,38,46]]-Adrenomedullin(20—52) (7). Starting from Fmoc–NH–SAL–PEG-resin (0.40 g, NH: 0.096 mmol), we prepared 0.90 g of a protected peptide resin corresponding to the sequence of adrenomedullin-(20—52), Fmoc–Thr(Bu^t)–Cys(Acm)–Thr(Bu^t)–Val–Gln(Trt)–Lys (Boc)–Leu–Ala–His(Trt)–Gln(Trt)–Ile–Tyr(Bu^t)–Gln(Trt)–Phe–Thr(Bu^t)– Asp(OBu^t)– Lys(Boc)– Asp(OBu^t)– Lys(Boc)– Asp (OBu^t)– Asn(Trt)– Val– Ala– Pro– Arg(Pmc)– Ser(Bu^t)– Lys(Boc)– Ile–Ser(Bu^t)–Pro–Gln(Trt)–Gly–Tyr(Bu^t)–NH–SAL–PEG-resin (**5**), on a peptide synthesizer 433 A (Applied Biosystems Inc., Foster City, CA.). The 0.1 mmol standard protocol of FastMoc 0.25 ΩMonPrevPk was employed. An aliquot of the resin (270 mg) was treated with reagent K[9] (10 mL) for 4 h. The reaction mixture was poured into cold ether (200 mL), and the resulting precipitate was washed with ether (5 mL × 2), then extracted with 25% aqueous acetonitrile to give crude peptide (104 mg) after freeze-drying, which was purified by RP-HPLC to yield Fmoc–[Cys(Acm)[21]]-adrenomedullin(20—52) (**6**) (40 mg, 9.8 μmol, 26% based on the amino group in the starting resin): MS (FAB) Found: *m/z* 4074.1 $(M+H)^+$. Calcd for $(M+H)^+$: 4074.6. Amino acid analysis: $Asp_{4.02}Thr_{2.47}Ser_{1.87}Glu_{4.12}Pro_{1.78}Gly_{1.06}Ala_{2.04}Cys_{nd}Val_{1.93}Ile_{1.80}$ $Leu_1Tyr_{1.97}Phe_{1.06}Lys_{3.97}His_{1.11}Arg_{1.02}$.

To a solution of peptide **6** (30 mg, 3.6 μmol) in DMSO (0.40 mL) were added Boc–OSc (18 mg, 86 μmol) and DIEA (12 μL). After stirring for 2 h, ether was added to the reaction mixture. The resulting precipitate was washed with ether (2 mL × 3), and then dissolved in DMSO (0.40 mL). To the solution was added piperidine (20 μL), followed by stirring for 1 h. To the mixture was added acetic acid (12 μL), and the peptide was isolated by RP-HPLC to give [Cys(Acm)[21], Lys(Boc)[25,36,38,46]]-adrenomedullin-(20—52) (**7**) (21 mg, 3.6 μmol, 17% based on the amino group in the starting resin) after freeze-drying: MS (FAB) Found: *m/z* 4251.4 $(M+H)^+$. Calcd for $(M+H)^+$: 4252.9. Amino acid analysis: $Asp_{3.88}Thr_{2.60}Ser_{1.74}Glu_{4.00}Pro_{1.76}Gly_1Ala_{1.93}Cys_{nd}Val_{1.94}Ile_{1.83}$ $Leu_{0.94}Tyr_{1.91}Phe_{1.00}Lys_{3.78}His_{1.03}Arg_{0.98}$.

Analysis of Products in the Coupling Reaction Mixtures. $AgNO_3$ (0.05 mg, 0.30 μmol), an additive (HONp, HOSu, HOBt·H_2O, or HOOBt) (3.0 μmol), and DIEA (0.35 μL, 2.0 μmol) were dissolved in DMSO (10 μL) and the resulting solution was stirred for 1 h. To this mixture was added the solutions of each of two peptides, **4** and **7**, in DMSO (5.0 μL, 20 mM, 0.10 μmol each). The mixture was stirred in the dark. After 4 h, the reaction mixture was analyzed by RP-HPLC (column: Cosmosil $5C_{18}$ AR (10×250 mm) using the following elution conditions: linear increase of acetonitrile concentration from 20 to 50% in 0.1% aq TFA over 30 min at a flow rate of 2.5 mL min^{-1}). Components in the reaction mixture were isolated, and each of the isolated materials was subjected to mass measurement.

Synthesis of Human Adrenomedullin. A solution of $AgNO_3$ (0.88 mg, 5.1 μmol), HOOBt (8.4 mg, 52 μmol), and DIEA (6.0 μL, 34 μmol) in DMSO (0.20 mL) was stirred for 1 h, and to the mixture was added a solution of peptide segments **4** (7.0 mg, 1.7 μmol) and **7** (10.0 mg, 1.7 μmol) in DMSO (0.15 mL). After stirring for 24 h, a product was isolated by RP-HPLC (column: Cosmosil $5C_{18}$ AR (10×250 mm) using the following elution conditions: linear increase of acetonitrile concentration from 20 to 50% in 0.1% aq TFA over 30 min at a flow rate of 2.5 mL min^{-1}) to give the peptide **8** (12.5 mg, 1.25 μmol, 73%) after freeze-drying: MS (MALDI-TOF) Found: *m/z* 6671.6 $(M+H)^+$. Calcd for $(M+H)^+$: 6673.6. Amino acid analysis: $Asp_{5.92}Thr_{2.55}Ser_{3.50}Glu_{6.19}Pro_{1.80}Gly_4Ala_{2.47}Cys_{nd}Val_{1.95}Met_{0.67}$ $Ile_{1.87}Leu_{2.01}Tyr_{2.96}Phe_{3.96}Lys_{3.89}His_{0.99}Arg_{4.01}$.

An aliquot of the peptide **8** (6.6 mg, 0.72 μmol) was treated with TFA containing 5% 1,4-butanedithiol (60 μL) for 1 h. A peptide was precipitated with ether. The precipitate was washed with ether (2 mL × 3), and then lyophilized from aqueous acetonitrile to give peptide **9**: MS (MALDI-TOF) Found: *m/z* 6174.6 $(M+H)^+$. Calcd for $(M+H)^+$: 6174.0.

The powder obtained was dissolved in H_2O (0.20 mL) to which was added a solution of $AgNO_3$ (0.62 g, 3.6 μmol) and DIEA

(1.9 μL, 11 μmol) in DMSO (70 μL). After stirring for 2 h, a mixture of 1 M hydrochloric acid and DMSO (1 : 1, v/v, 3.0 mL) was added to the reaction mixture, and the mixture was stirred for 24 h. The major main product was isolated by RP-HPLC (column: Cosmosil $5C_{18}$ AR (10×250 mm) using the following elution conditions: Linear increase of acetonitrile concentration from 20 to 50% in 0.1% aq TFA over 30 min at a flow rate of 2.5 mL min^{-1}) to give adrenomedullin (3.6 mg, 0.41 μmol, 57% based on peptide **8**) after freeze-drying: MS (MALDI-TOF) Found: *m/z* 6027.8 $(M+H)^+$. Calcd for $(M+H)^+$: 6029.8. Amino acid analysis: $Asp_{6.03}Thr_{2.89}Ser_{3.69}Glu_{6.06}Pro_{1.87}Gly_4Ala_{2.28}Cystine_{0.87}Val_{1.88}Met_{0.87}Ile_{1.77}Leu_{1.98}Tyr_{2.88}Phe_{3.82}Lys_{3.68}His_{1.02}Arg_{3.97}$.

Results

The amino acid sequence of human adrenomedullin is shown in Fig. 1, in which an arrow indicates the site of segment coupling.

Preparation of a Cys(Acm)-Containing Peptide Thioester 4. The *N*-terminal peptide thioester segment, Boc–[Cys(Acm)^{16}]-Adrenomedullin(1—19)–SCH_2CH_2CO–β-Ala–NH_2 (**4**), was prepared according to the scheme shown in Fig. 2. The peptide was prepared by a Boc solid-phase method using a Boc derivative of an *S*-acetamidomethyl cysteine for a cysteine residue. Starting from an MBHA resin, we manually prepared Boc–Gly–SCH_2CH_2CO–β-Ala–MBHA resin (**1**). This resin was used for the preparation of a protected peptide resin **2** corresponding to the sequence of adrenomedullin(1—19). After completion of chain elongation cycles, the protected peptide resin **2** was treated with anhydrous HF containing 1,4-butanedithiol and anisole to give the crude product. The crude material was purified by RP-HPLC to give peptide **3**. The *N*-terminal amino group of the peptide was protected by a Boc group with Boc–OSu in the presence of DIEA to give the partially protected peptide thioester **4** in 13% yield, based on the Gly residue in the starting resin **1**. The mass number and amino acid analysis data were in good agreement with expected values.

Preparation of a Peptide 7. Another peptide segment, [Cys (Acm)^{21},Lys(Boc)^{25,36,38,46}]-adrenomedullin(20—52) (**7**), was prepared by an Fmoc solid-phase method according to the scheme shown in Fig. 3. A protected peptide resin **5** corresponding to the sequence of adrenomedullin-(20—52) was prepared starting from the Fmoc–NH–SAL–PEG-resin.[8] The peptide resin was treated with reagent K[9] to give a crude product, which was purified by RP-HPLC to give Fmoc–[Cys(Acm)^{21}]-adrenomedullin(20—52) (**6**). To the side-chain amino groups, Boc groups were introduced by treatment with Boc–OSu in the presence of DIEA. The peptide obtained was treated with 5% piperidine to give the peptide **7** in 17% yield, based on the amino group in the starting resin, after purification by RP-HPLC.

Analysis of Products in the Coupling Reaction Mixtures. The segment condensations were carried out according to the scheme shown in Fig. 4. The RP-HPLC elution profiles of the reaction mixtures after 4 h stirring are shown in Fig. 5 (A—D). All the peaks observed were isolated and their mass numbers were measured. When HONp was used as an active ester component, no peak corresponding to pep-

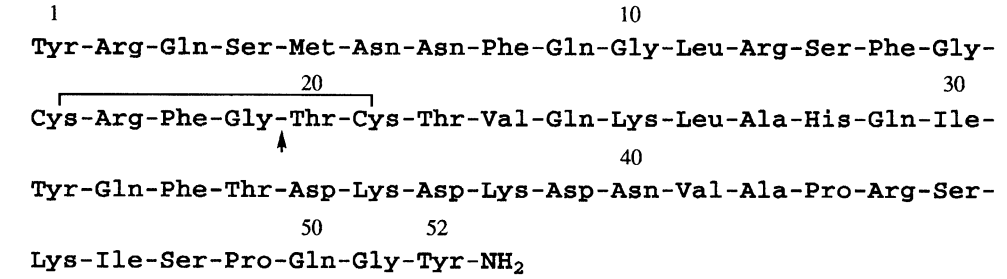

Fig. 1. Amino acid sequence of human adrenomedullin. An arrow indicates the site of segment coupling.

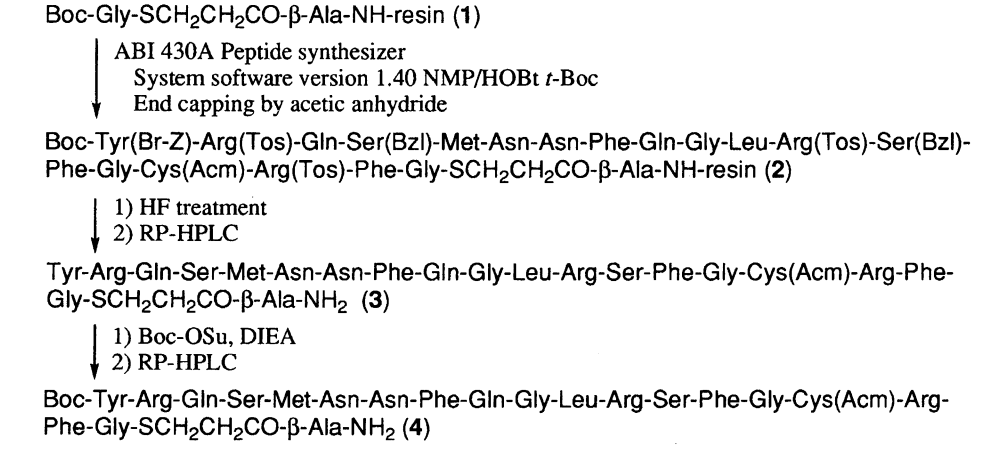

Fig. 2. Synthetic route for Boc–[Cys(Acm)^{16}]-adrenomedullin(1—19)–SCH_2CH_2CO–β-Ala–NH_2 (**4**).

Fmoc-NH-SAL-PEG-resin

↓ ABI 433A Peptide synthesizer
FastMoc 0.25 ΩMonPrevPk
End capping by acetic anhydride

Fmoc-Thr(Bu^t)-Cys(Acm)-Thr(Bu^t)-Val-Gln(Trt)-Lys(Boc)-Leu-Ala-His(Trt)-Gln(Trt)-Ile-Tyr(Bu^t)-Gln(Trt)-Phe-Thr(Bu^t)-Asp(OBu^t)-Lys(Boc)-Asp(OBu^t)-Lys(Boc)-Asp(OBu^t)-Asn(Trt)-Val-Ala-Pro-Arg(Pmc)-Ser(Bu^t)-Lys(Boc)-Ile-Ser(Bu^t)-Pro-Gln(Trt)-Gly-Tyr(Bu^t)-NH-SAL-PEG-resin (**5**)

↓ 1) Reagent K
2) RP-HPLC

Fmoc-Thr-Cys(Acm)-Thr-Val-Gln-Lys-Leu-Ala-His-Gln-Ile-Tyr-Gln-Phe-Thr-Asp-Lys-Asp-Lys-Asp-Asn-Val-Ala-Pro-Arg-Ser-Lys-Ile-Ser-Pro-Gln-Gly-Tyr-NH_2 (**6**)

↓ 1) Boc-OSu, DIEA
2) piperidine
3) RP-HPLC

Thr-Cys(Acm)-Thr-Val-Gln-Lys(Boc)-Leu-Ala-His-Gln-Ile-Tyr-Gln-Phe-Thr-Asp-Lys(Boc)-Asp-Lys(Boc)-Asp-Asn-Val-Ala-Pro-Arg-Ser-Lys(Boc)-Ile-Ser-Pro-Gln-Gly-Tyr-NH_2 (**7**)

Fig. 3. Synthetic route for [Cys(Acm)^{21}, Lys(Boc)^{25,36,38,46}]-adrenomedullin(20—52) (**7**).

Boc-[Cys(Acm)^{16}]-Adrenomedullin(1–19)-SCH_2CH_2CO-β-Ala-NH_2 (**4**)

+

[Cys(Acm)^{21}, Lys(Boc)^{25,36,38,46}]-Adrenomedullin(20–52) (**7**)

↓ 1) $AgNO_3$, additive, DIEA
2) RP-HPLC

Boc-[Cys(Acm)^{16,21}, Lys(Boc)^{25,36,38,46}]-Adrenomedullin (**8**)

Fig. 4. Segment condensation of peptide thioester **4** with peptide **7**.

tide **8** was observed, as shown in Fig. 5 (A). Although the peak corresponding to peptide **7** disappeared, the large peak corresponding to peptide **4** remained in the reaction mixture. Three new peaks (**a**, **b**, and **c**) derived from peptides were observed. The mass number of peak **b** showed that it was the *p*-nitrophenyl ester derivative of peptide **4**. When HOSu was used as an active ester component, a peak corresponding to peptide **8** appeared, as shown in Fig. 5 (B). The peak of peptide **4** became small, and peaks **a**, **c**, **d**, and **e** appeared. In Fig. 5 (C), the elution profile of the reaction, in which HOBt[10] was used as an active ester component, is shown. The peaks observed corresponded to the starting materials and peptide **8**, the desired product. When HOOBt[11] was used as an active ester component, the coupling reaction proceeded almost completely without any accompanying side reactions, as shown in Fig. 5 (D). The isolated yield of peptide **8** by RP-HPLC was 69%. This procedure gave slightly better yields than the case in which HOBt was used as an additive. The mass numbers ($[M+H]^+$) of the peaks obtained by FAB mass measurement are as follows: peak **a**, *m/z* 2421.9; **b**, *m/z* 2562.9; **c**, *m/z* 4183.0; **d**, *m/z* 2439.0; **e**, *m/z* 2351.0. The peak corresponding to the peptide **8** showed a mass number of *m/z* 6671.6 by MALDI-TOF mass measurement. The calculated value for Boc–[Cys(Acm)^{16,21},Lys(Boc)^{25,36,38,46}]-adrenomedullin (**8**) is 6673.6. Amino acid analysis also showed good agreement with the calculated values.

Synthesis of Adrenomedullin. Partially protected peptide segments **4** and **7** were added to a mixture prepared from $AgNO_3$, HOOBt, and DIEA in DMSO; the resulting solution was stirred for 24 h. The RP-HPLC analysis of the reaction mixture showed a similar elution profile, as shown in Fig. 5 (D). Peptide **8** was obtained in 73% yield after isolation by RP-HPLC, followed by freeze-drying. The elution profile of the purified product is shown in Fig. 7 (A). The peptide **8** was converted to the corresponding native form according to the scheme shown in Fig. 6. Thus, Boc groups on the purified peptide **8** were removed by treating with TFA containing 5% 1,4-butanedithiol for 1 h (Fig. 7 (B)). Peptide **9** was washed with ether, and then lyophilized from aqueous acetonitrile. The resulting powder was dissolved into distd water, to which was added a DMSO solution of $AgNO_3$ and DIEA to remove Acm groups (Fig. 7 (C)). After stirring for 2 h, to the reaction mixture containing peptide **10** was added a mixture of 1 M hydrochloric acid and DMSO (1 : 1, v/v) to remove silver from silver thiolate as AgCl and to form a disulfide bond under the acidic DMSO conditions[12] (Fig. 7 (D)). Adrenomedullin was isolated in the native form by RP-HPLC in 57% yield based on the peptide **8**. The synthetic adrenomedullin and an authentic sample co-eluted as a single peak by RP-HPLC. The mass number and amino acid analysis data in good agreement with expected values.

Discussion

Adrenomedullin as a Model Compound. Adrenomedullin has two half-cystine residues and a suitable molecular size. Furthermore, a glycine residue exists between the two half-cystine residues at the middle of the molecule. Because of this, we can easily estimate the behavior of Acm groups during segment condensation by RP-HPLC analysis without the factor of racemization. This was the major reason for

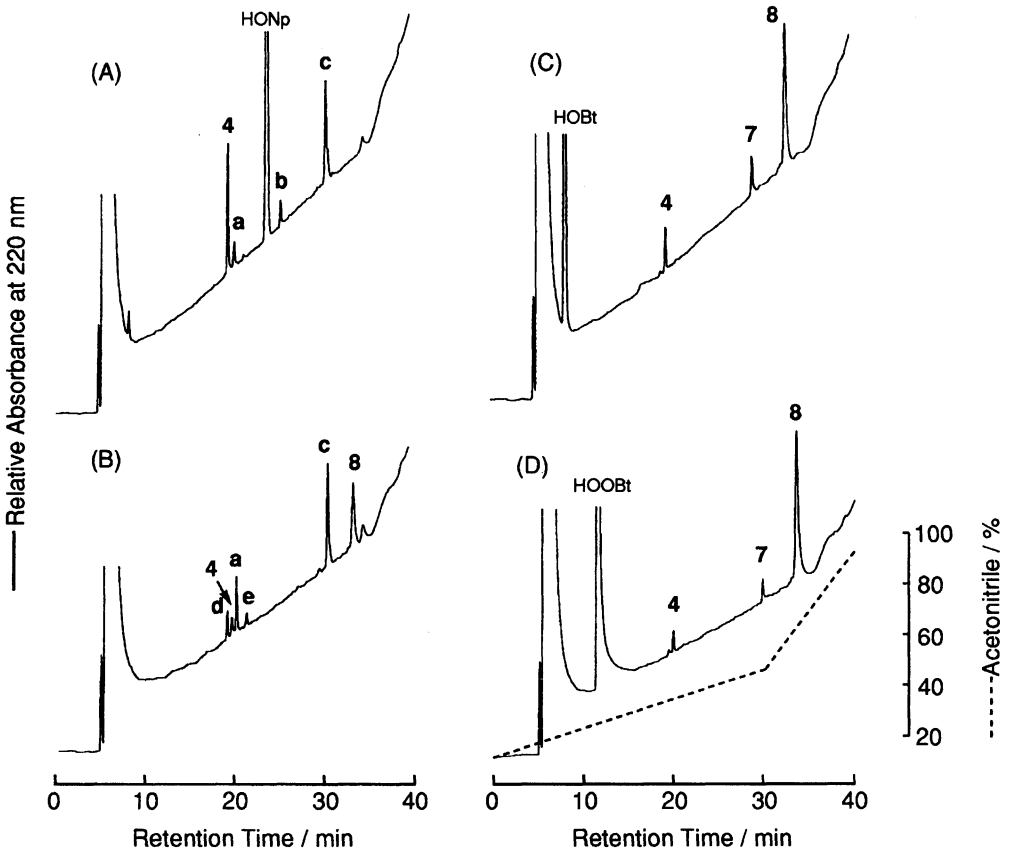

Fig. 5. RP-HPLC elution profiles of the reaction mixtures of segment condensations of peptide thioester **4** with peptide **7** under four different reaction conditions. Panels from A to D show the reaction in which HONp (panel A), HOSu (panel B), HOBt (panel C), or HOOBt (panel D) was used as an additive, respectively. Elution conditions: Column: Cosmosil $5C_{18}$ AR (10×250 mm). Linear increase of acetonitrile concentration from 20 to 50% in 0.1% aq trifluoroacetic acid over 30 min at a flow rate of 2.5 mL min^{-1}.

choosing adrenomedullin as the synthetic target.

Design of a Peptide Segments Containing a Cysteine Residue. In our preceding paper,[5] we utilized benzyl type groups such as 4-methylbenzyl or 2,4,6-trimethylbenzyl groups for the protection of mercapto groups of cysteine residues because of their silver-ion-resistant nature. A peptide thioester containing these protecting groups is prepared only by an Npys solid-phase method. Though this solid-phase method has some attractive features: That peptide chain elongation can be carried out under mild conditions and that the removal of Npys group can be easily monitored by the yellow color of the Npys group, improvement in the yield of peptide thioester is required in the method.

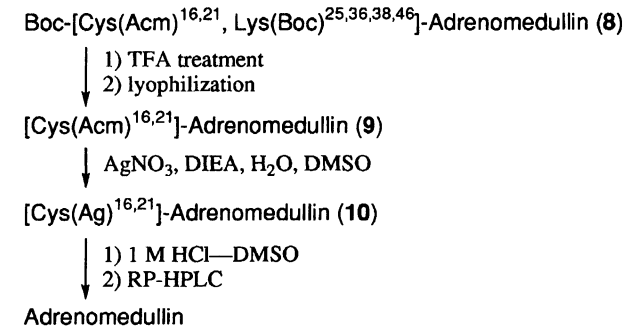

Fig. 6. A scheme for the preparation of the native form of adrenomedullin from partially protected adrenomedullin **8**.

On the other hand, an acetamidomethyl (Acm) group[6] for the protective group on a cysteine residue is stable under HF-treatment conditions. Thus, *S*-Acm cysteine-containing peptide thioesters can be easily prepared by a Boc solid-phase method. It is known, however, that the Acm group on a cysteine residue is removed by a silver ion-containing reagent, such as silver trifluoromethanesulfonate (AgOTf) in trifluoroacetic acid.[13] The *S*-Acm group is generally thought to be removed, to some extent, in the presence of silver ions under a wide range of conditions. If coupling conditions can be found under which Acm groups are quite stable in the presence of silver ions and segment condensation proceeds at a reasonable rate, cysteine-containing proteins can then be prepared as easily as non-cysteine containing protein by using Cys(Acm)-containing building blocks.

The peptide segments **4** and **7** were synthesized using the Acm group as a protective group on the cysteine residues without any problems. The peptide thioester segment, Boc–[Cys(Acm)^{16}]-adrenomedullin(1—19)–SCH_2CH_2CO–β-Ala–NH_2 (**4**), was prepared by a Boc solid-phase method and the other peptide segment, [Cys(Acm)^{21}, Lys(Boc)^{25,36,38,46}]-adrenomedullin(20—52) (**7**), was done by an Fmoc solid-phase method. Peptide **7**, of

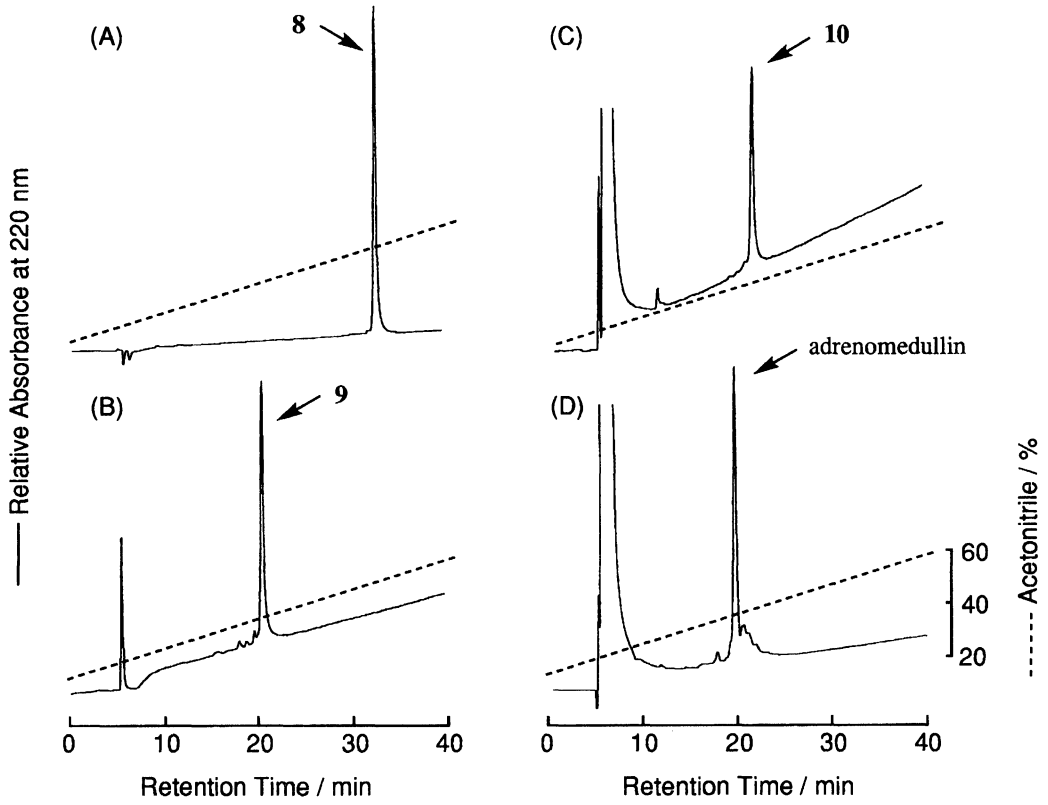

Fig. 7. RP-HPLC elution profiles of purified peptide **8** and reaction mixtures at each reaction step leading to the native form of adrenomedullin. Panel A, the elution profile of peptide **8**; Panel B, the elution profile of the reaction mixture of peptide **8** treated with TFA containing 5% 1,4-butanedithiol; Panel C, the reaction mixture of peptide **9** treated with $AgNO_3$, DIEA, H_2O, and DMSO; Panel D, the reaction mixture of peptide **10** treated with 1 M HCl–DMSO. Elution conditions: Column: Cosmosil $5C_{18}$ AR (10×251 mm). Linear increase of acetonitrile concentration from 20 to 60% in 0.1% aq trifluoroacetic acid over 40 min at a flow rate of 2.5 mL min^{-1}.

course, can be easily prepared by a Boc solid-phase method as well.

Segment Coupling. Thioester moieties are largely initially converted to the corresponding active esters in the presence of silver ions and additives. After this, peptide bond formation occurs between the active ester component and an amino component. To estimate the effect of the active ester component on the stability of Acm groups, we examined as additives four different reagents: HONp, HOSu, HOBt,[10] and HOOBt.[11]

For the case of HONp, which was successfully used in the synthesis of *c*-Myb(142—193)-NH_2,[1] no peak corresponding to a desired product was detected (Fig. 5 (A)). The mass number of peak **a** corresponds to that of a product that was formed by the elimination of thioester moiety from peptide **4**. Peak **b** showed the mass number of the *p*-nitrophenyl ester corresponding to peptide **4**. The mass number of peak **c** was equal to that of the peptide, whose Acm group had been removed from the peptide **7**.

HOSu was an efficient additive in the synthesis of several small proteins.[2—5] A desired product **8** was observed having a retention time of 33.2 min on the RP-HPLC (Fig. 5 (B)), whose mass number and amino acid analysis data were good agreement with expected values. Though the process of segment coupling was improved, several by-products were observed. Judging from the mass numbers, peaks **c** and **e** were corresponding to des-Acm peptides derived from peptides **7** and **4**, respectively. Peak **d** was estimated to be a product derived from hydrolysis of thioester in peptide **4**.

HOBt[10] and HOOBt[11] allowed efficient segment condensation without accompanying side reaction products. Decomposition of the Acm group was not observed. Judging from the elution profiles shown in Fig. 5 (C) and (D), we conclude that HOOBt was slightly more effective than HOBt as an additive. In a test reaction, product **8** was obtained in 69% yield after purification by RP-HPLC, when HOOBt was used as an additive. Though HOBt and HOOBt form highly reactive active esters, no serious side reaction occurred with potentially reactive side-chain functional groups. This is likely due to differences in reactivity of the active ester toward the amino group and the other reactive groups such as hydroxyl, imidazolyl, and carboxyl groups.

The stability of the Acm groups on the Cys residues under the coupling reaction conditions can be explained as follows: The thioether in Cys(Acm) as well as the thioester probably coordinate to silver ions under conditions of segment coupling. Thus, the carbon adjacent to the sulfur atom is activated toward nucleophiles. The carbonyl carbon of the thioester would be expected to be activated to a greater extent than the alkyl carbon of the thioether. If the nucleophilic-

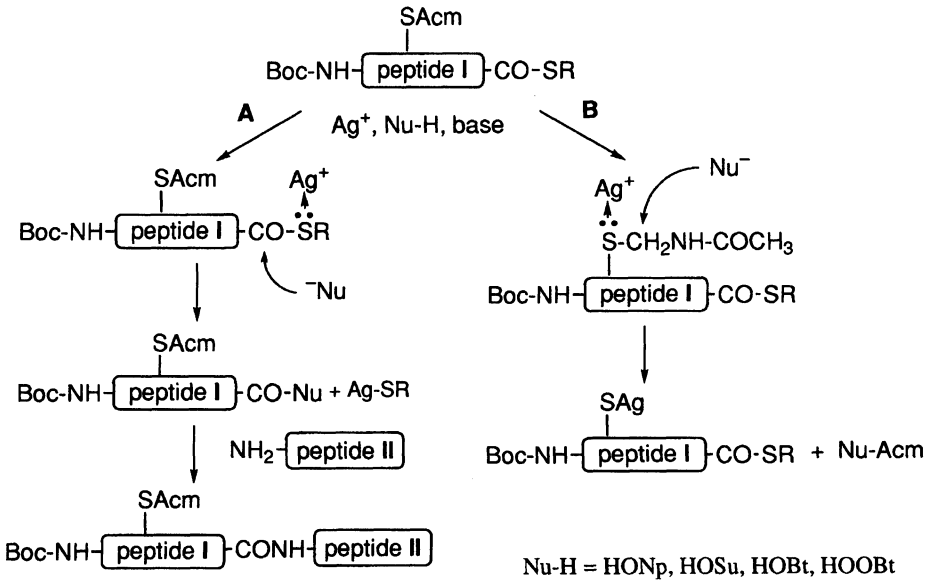

Fig. 8. The reaction model of the Cys(Acm)-containing thioester **I** with nucleophiles in the presence of silver ions. Path **A**: The thioester moiety is activated with a silver ion, and an active ester component (Nu^-) then attacks. The active ester obtained reacts with another peptide **II** to give the coupling product. Path **B**: The thioether moiety of the Cys(Acm) residue is activated with a silver ion resulting in the decomposition of the Cys(Acm) residue.

ity of an additive is sufficient strong to attack the activated thioester, and too weak to attack the thioether, the thioester selectively reacts with the additive. HOBt or HOOBt is strong enough to attack the thioester activated with a silver ion, but is too weak to attack the Acm group on a cysteine residue activated with a silver ion. Thus only path **A** in Fig. 8 is operative. On the other hand, HONp and HOSu are sufficiently strong to react with the Acm group on a cysteine residue activated with a silver ion. Thus, path **B** in Fig. 8 is operative. These hypotheses are supported by the fact that the coupling reaction in which HOOBt was added as an active ester component did not cause removal of an Acm group, but the removal occurred when piperidine was added to the same reaction mixture (data not shown). This indicates that silver ions coordinate even to the sulfur atoms on Cys(Am) residues, and that the Acm group reacts with strong nucleophile such as piperidine.

Synthesis of Adrenomedullin. The adrenomedullin synthesis clearly demonstrates that an easily available partially protected peptide thioester containing a Cys(Acm) residue is a useful building block for the thioester method. The segment condensation conditions that were established are useful for the practical preparation of a cysteine-containing polypeptide. Thus, the preparation of a cysteine-containing polypeptide has become basically as easy as that of polypeptides that contain no cysteine residues. If we develop conditions under which peptide thioesters containing Cys(Acm) residues can be successively condensed, the synthesis of cysteine-containing polypeptide with more than 100 amino acid residues will become routine.

In the adrenomedullin synthesis, we also developed a convenient route for the removal of *S*-Acm groups. On the basis of the observation that the *S*-Acm groups can be easily removed by a nucleophile when a sulfur atom in Cys(Acm) coordinates with a silver ion, a new method was developed. Initially [Cys(Acm)^{16,21}]-adrenomedullin (**9**) was treated with AgOTf in TFA[13] to remove Acm groups, but the Acm groups were not removed under these conditions. On the basis of the above observations, peptide **9** was treated with $AgNO_3$ and DIEA in H_2O and DMSO at room temperature, in which hydroxide ion acted as a nucleophile. Under the conditions, the Acm groups were deprotected within 2 h as shown in Fig. 7 (C). The [Cys(Ag)^{16,21}]-adrenomedullin (**10**) obtained was converted to the native form of adrenomedullin using a methodology described by Tamamura et al. (Fig. 7 (D)).[12]

In the conversion of peptide **8** to adrenomedullin, we attempted to isolate the silver thiolate **10** by RP-HPLC and to detect it by MALDI-TOF mass measurement. The peak corresponding to the silver thiolate **10** was collected, but the observed mass number coincided with that of the des-silver form of peptide **10**.

The authors express their thanks to Professor Yasutsugu Shimonishi and Dr. Toshifumi Takao of the Institute for Protein Research, Osaka University, for their help with FAB mass measurements. This research was partly supported by the Grant-in-Aid for Scientific Research on Priority Areas No. 06276102 from the Ministry of Education, Science, Sports and Culture.

References

1) H. Hojo and S. Aimoto, *Bull. Chem. Soc. Jpn.*, **64**, 111 (1991).

2) H. Hojo and S. Aimoto, *Bull. Chem. Soc. Jpn.*, **65**, 3055 (1992).

3) H. Hojo, Y. Kwon, Y. Kakuta, S. Tsuda, I. Tanaka, K. Hikichi, and S. Aimoto, *Bull. Chem. Soc. Jpn.*, **66**, 2700 (1993).

4) H. Hojo and S. Aimoto, *Bull. Chem. Soc. Jpn.*, **66**, 3004 (1993).

5) a) H. Hojo, S. Yoshimura, M. Go, and S. Aimoto, *Bull. Chem. Soc. Jpn.*, **68**, 330 (1995); b) Y. Kwon, R. Zhang, M. P. Bemquerer, M. Tominaga, H. Hojo, and S. Aimoto, *Chem. Lett.*, **1993**, 881.

6) D. F. Veber, J. D. Milkowski, S. L. Varga, R. G. Denkewalter, and R. Hirschmann, *J. Am. Chem. Soc.*, **94**, 5456 (1972).

7) K. Kitamura, K. Kangawa, M. Kawamoto, Y. Ichiki, S. Nakamura, H. Matsuo, and T. Eto, *Biochem. Biophys. Res. Commun.*, **192**, 553 (1993).

8) H. Rink, *Tetrahedron Lett.*, **28**, 3787 (1987).

9) D. S. King, C. G. Fields, and G. B. Fields, *Int. J. Peptide Protein Res.*, **36**, 255 (1990).

10) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).

11) W. König and R. Geiger, *Chem. Ber.*, **103**, 2034 (1970).

12) H. Tamamura, A. Otaka, J. Nakamura, K. Okubo, T. Koide, K. Ikeda, T. Ibuka, and N. Fujii, *Int. J. Peptide Protein Res.*, **45**, 312 (1995).

13) N. Fujii, A. Otaka, T. Watanabe, A. Okamachi, H. Tamamura, H. Yajima, Y. Inagaki, M. Nomizu, and K. Asano, *J. Chem. Soc., Chem. Commun.*, **1989**, 283.