Deprotection of the *S*-Trimethylacetamidomethyl (Tacm) Group Using Silver Tetrafluoroborate: Application to the Synthesis of Porcine Brain Natriuretic Peptide-32 (pBNP-32)[1,2]

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Silver tetrafluoroborate ($AgBF_4$) in trifluoroacetic acid (TFA) has been found to cleave the *S*-trimethylacetamidomethyl (Tacm) group or the *S*-acetamidomethyl (Acm) group without affecting other functional groups in a peptide chain. A newly isolated porcine brain natriuretic peptide-32 (pBNP-32) was synthesized by the combined use of the *S*-Tacm group and $AgBF_4$ deprotection. The synthetic pBNP-32 was obtained in better yield by the $AgBF_4$ procedure than by the standard I_2 procedure. The synthetic pBNP-32 has the highest chick rectum relaxant activity among the known members of the atrial natriuretic peptide–brain natriuretic peptide (ANP–BNP) families.

Somatostatin was also synthesized by the Fmoc-based solid-phase method using *S*-Tacm and $AgBF_4$. In this synthesis, the recently developed reagent tetrafluoroboric acid (HBF_4) was applied to cleave the peptide from the resin.

Keywords peptide synthesis; porcine brain natriuretic peptide-32; *S*-trimethylacetamidomethylcysteine; silver tetrafluoroborate deprotection; tetrafluoroboric acid deprotection; chick rectum relaxant activity; somatostatin

Recently, we have developed the *S*-trimethylacetamidomethyl (Tacm) group as an *S*-protecting group for cysteine.[3-5] This protecting group has chemical properties similar to those of the *S*-acetamidomethyl (Acm) group,[6] but is less susceptible to air-oxidation than the *S*-Acm group. This characteristic property of the *S*-Tacm group is expected to be advantageous for the syntheses of relatively large peptides. Porcine brain natriuretic peptide (pBNP), a 26-residue peptide containing one disulfide bridge, was successfully synthesized using this protecting group.[3,5] However, in the course of our synthetic studies of complex peptides, we have observed that partial oxidation of Met to Met(O) and/or modification of Trp occurs during the removal of the *S*-Tacm group with I_2 in aqueous AcOH.

We therefore sought a milder method for removal of the *S*-Tacm group and found that silver tetrafluoroborate ($AgBF_4$) in trifluoroacetic acid (TFA) could remove the *S*-Tacm group without affecting other functional groups in the peptide chain. The usefulness of this new *S*-deprotecting reagent was demonstrated by the solution-phase synthesis of newly isolated Met-containing porcine brain natriuretic peptide-32 (pBNP-32) (**1**),[7] the structure of which was determined by Sudoh *et al.*[8] As shown in Fig. 1, pBNP-32 consists of 32 amino acids, extending the *N*-terminus of pBNP,[9] and has remarkable sequence homology to mammalian α-atrial natriuretic peptides (α-ANPs).[10-12] This peptide elicits similar biological activities to those of α-ANPs, *i.e.* regulation of the homeostatic balance of body fluid and blood pressure.

In this paper, we describe the usefulness of $AgBF_4$ as an *S*-deprotecting reagent and present experimental details of the synthesis of pBNP-32 using this new reagent.[7] We also describe the solid-phase synthesis of a Trp-containing peptide, somatostatin, using the *S*-Tacm group and $AgBF_4$.[7] In this synthesis, the recently developed deprotecting reagent tetrafluoroboric acid (HBF_4)[13] has been applied to cleave the peptide from the resin.

Results and Discussion

Removal of the *S*-Tacm Group Using $AgBF_4$ In the deprotection of the *S*-protecting group with monovalent silver ions, the anion counterpart plays an important role since silver ions, in the form of nitrate[14] or trifluoromethanesulfonate,[15] remove several *S*-protecting groups, but in the form of acetate[16] they do not. We have employed silver ions in the form of tetrafluoroborate and examined the usefulness of this reagent for the removal of *S*-protecting groups.

Boc–Cys(Tacm)–OH in TFA was treated with $AgBF_4$ (10 eq) in the presence of anisole (2 eq) in an ice-bath for 60 min. After treatment with dithiothreitol (DTT, 20 eq) at 25 °C, the regenerated cysteine was quantified by using an amino acid analyzer. As shown in Table I, cysteine was quantitatively recovered from this derivative. Under the same conditions, *S*-Acm was also cleaved quantitatively.

Monovalent silver ion in the form of tetrafluoroborate

TABLE I. Cysteine Regenerated after Treatment (4 °C, 1 h) with $AgBF_4$ (10 eq)–Anisole (2 eq) in TFA

Protected cysteine derivative	Regenerated cysteine (%)[a]
Boc–Cys(Tacm)–OH	107
Boc–Cys(Acm)–OH	93
Boc–Cys(Bam)–OH	87
Boc–Cys(MBzl)–OH	87
Boc–Cys(Tmb)–OH	73
Boc–Cys(Bu^t)–OH	0
Boc–Cys(MeBzl)–OH	0

a) Determined by amino acid analysis after DTT (20 eq) treatment.

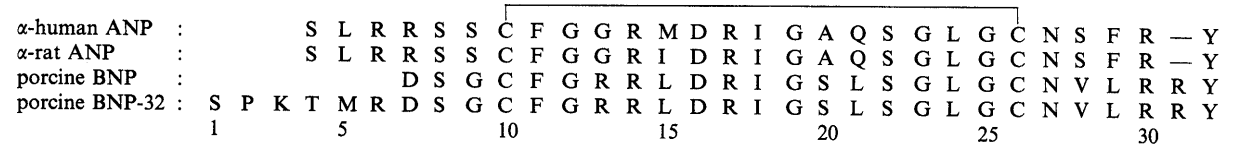

Fig. 1. Structures of α-Human and α-Rat ANP, pBNP and pBNP-32

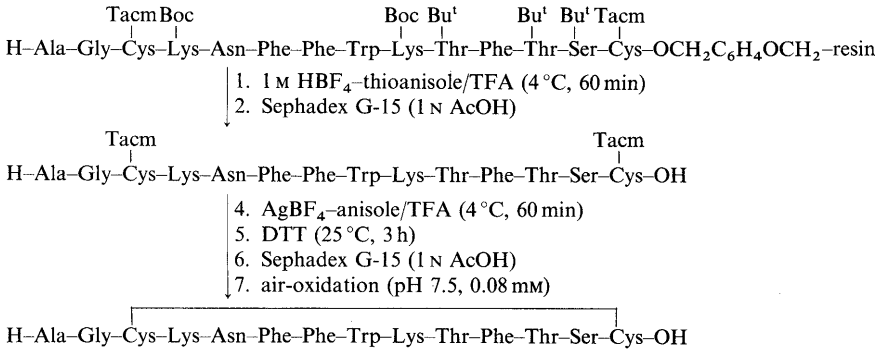

Fig. 2. Deprotection and Disulfide Bond Formation for Somatostatin

is moderately activated compared with other forms, and three other *S*-protecting groups, benzamidomethyl (Bam),[17] 4-methoxybenzyl (MBzl)[18] and 2,4,6-trimethylbenzyl (Tmb),[19] were incompletely cleaved (87%, 87% and 73%, respectively) by this reagent. On the other hand, silver trifluoromethanesulfonate[15] cleaved Bam and MBzl groups completely under the same conditions. *tert*-Butyl (Bu^t)[20] and 4-methylbenzyl (MeBzl)[21] were not affected. The results indicated that the mild reagent, $AgBF_4$ in TFA, is suitable for peptide synthesis using Cys (Tacm) or Cys (Acm) derivatives.

Solid-Phase Synthesis of Somatostatin As Trp is susceptible to modification during oxidative cleavage of the *S*-Acm group with I_2 in aqueous AcOH,[22] we selected somatostatin, a 14-residue peptide containing one Trp residue, as a model peptide. The amounts of Trp modification during the removal of the *S*-Tacm group with $AgBF_4$ and I_2 were compared by high-performance liquid chromatography (HPLC).

The Fmoc-based solid-phase method[23] was employed to avoid Trp-modification during the peptide chain construction. Fmoc-amino acid derivatives bearing protecting groups based on *tert*-butanol were employed, together with Cys(Tacm). The *C*-terminal residue, Fmoc–Cys(Tacm)–OH,[4] was loaded on the *p*-alkoxybenzyl alcohol resin[24] by the diisopropylcarbodiimide (DIPCDI)[25]–4-dimethylaminopyridine (DMAP)[26] procedure. The combination of piperidine treatment[27] and the DIPCDI plus 1-hydroxybenzotriazole (HOBt)[28] procedure served to elongate the peptide chain manually, according, in principle, to the automated program proposed by Sheppard *et al.*[29] The resin became negative to the ninhydrin test[30] after a single coupling at every condensation step. The amino acid composition of the protected somatostatin resin thus assembled was in fairly good agreement with that predicted by theory after acid hydrolysis with 12 N HCl–propionic acid (1:1).[31]

The protected somatostatin resin was treated with the recently developed deprotecting reagent 1 M tetrafluoroboric acid (HBF_4)–thioanisole/TFA[13] in the presence of two additional scavengers, *m*-cresol and EDT,[32] in an ice-bath for 60 min to remove all protecting groups except for two *S*-Tacm groups and to cleave the peptide from the resin. After gel-filtration on Sephadex G-15, the di-Tacm–somatostatin in TFA was treated with $AgBF_4$ (40 eq) in the presence of anisole (10 eq) in an ice-bath for 60 min. The Ag-peptide salt was then treated with DTT (80 eq) at 25 °C for 3 h. The product, after gel-filtration on Sephadex G-15, was subjected to air-oxidation at pH 7.5 to form the disulfide bond. The purification of the oxidized peptide was carried out by fast protein liquid chromatography (FPLC) on a YMC-ODS AQ-300 (S-50) column to give a homogeneous peptide in 39% yield.

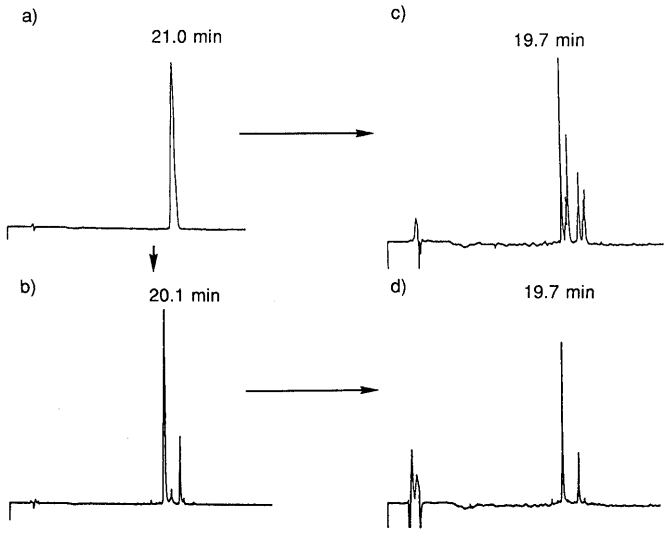

Fig. 3. HPLC Profile of Synthetic Somatostatin

a) Tacm form. b) SH form. c) Crude somatostatin using I_2. d) Crude somatostatin using air-oxidation.

For comparison, oxidative cleavage of the *S*-Tacm group with I_2 in 50% aqueous AcOH was performed in essentially the same manner as described for the *S*-Acm group.[22] As shown in Fig. 3, the crude product obtained using I_2 in aqueous AcOH gave a more complex elution pattern on HPLC than that obtained using air-oxidation after $AgBF_4$–DTT treatment.

Solution-Phase Synthesis of pBNP-32 (1) In order to demonstrate the usefulness of the $AgBF_4$ procedure in practical peptide synthesis, we have undertaken the solution-phase synthesis of pBNP-32, which is an *N*-terminal six amino-acids-extended form of pBNP. In this synthesis, N^{α}-Boc protection and side-chain protecting groups based on benzyl alcohol were employed as in the case of our previous synthesis of pBNP,[5] for which the standard I_2 procedure was employed to oxidatively cleave the *S*-Tacm group.

To construct the peptide backbone, two segments were selected (Fig. 4). The *N*-terminal segment (**2**), which covered the *N*-terminal-extended 6 amino acids, was newly synthesized. The *C*-terminal segment (**3**) was an intermediate

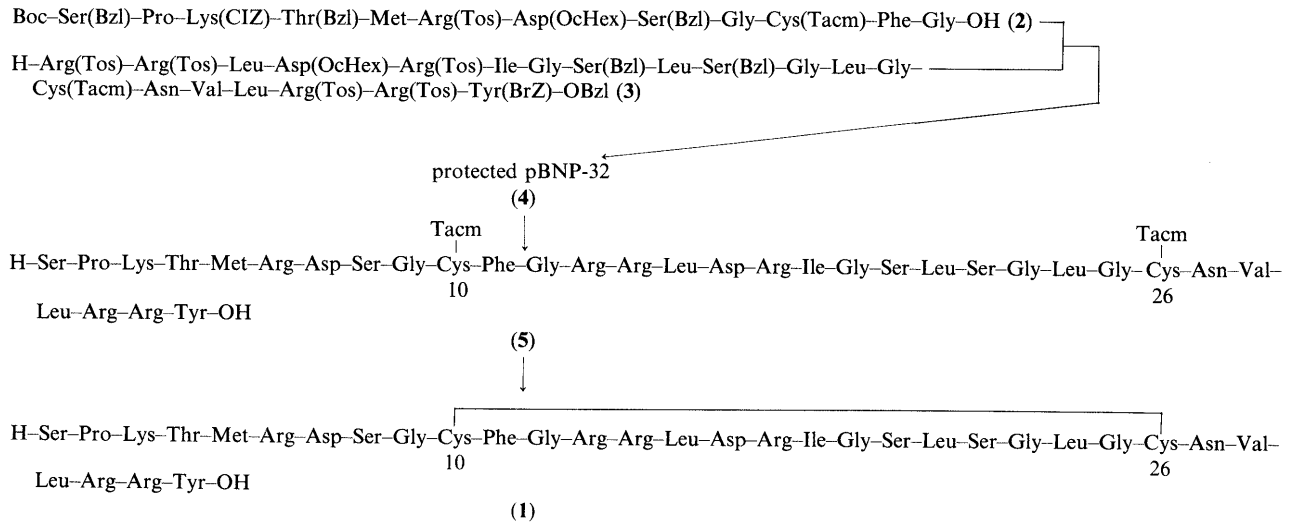

Fig. 4. Synthetic Route to pBNP-32

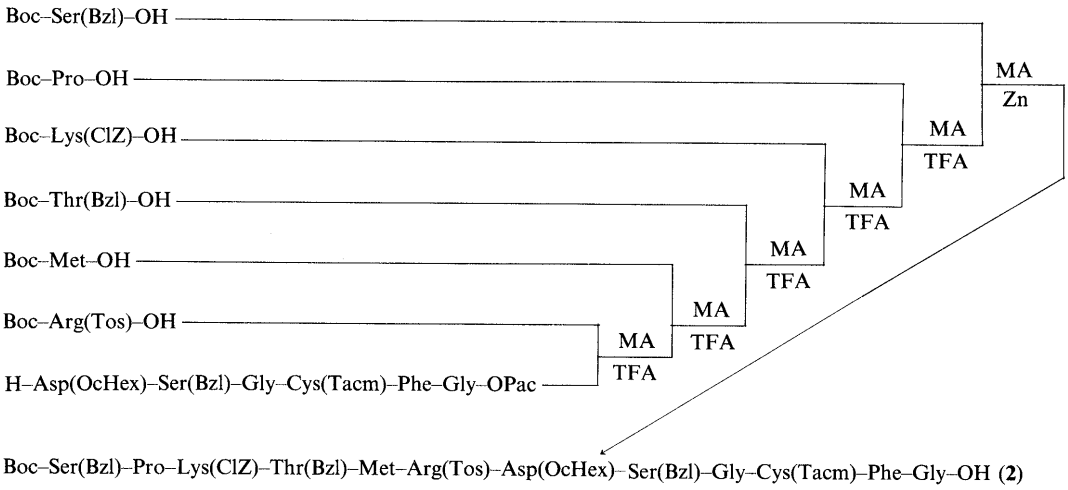

Fig. 5. Synthetic Scheme for the Protected Dodecapeptide Carboxylic Acid, Boc–(pBNP-32, 1—12)–OH (2)

peptide derivative used in our previous synthesis of pBNP.[5] For the formation of the disulfide bond, air-oxidation after the $AgBF_4$ or I_2 procedure was employed and amounts of Met (O)-peptide during both treatments were compared by HPLC.

The *N*-terminal segment (**2**) was prepared in a stepwise manner starting with a TFA-treated sample of Boc–Asp(OcHex)–Ser(Bzl)–Gly–Cys(Tacm)–Phe–Gly–OPac[5] (Fig. 5). The mixed anhydride (MA)[33] procedure was employed to introduce the respective amino acid residues. After incorporation of the Met residue, the N^{α}-Boc group was removed by TFA in the presence of anisole containing 2% EDT to suppress the *S*-alkylation of Met.[34] From the resulting protected dodecapeptide phenacyl (Pac) ester, the Pac group was removed by using Zn–anthranilic acid in a mixture of DMF–pyridine.[35] The purity of this segment thus obtained was ascertained by thin-layer chromatography (TLC), elemental analysis, and amino acid analysis after acid hydrolysis with 6 N HCl.

The *N*-terminal segment (**2**) and the *C*-terminal segment (**3**) were assembled using 1-ethyl-3-(3′-dimethylaminopropyl)-carbodiimide (water-soluble carbodiimide, WSC)[36] plus HOBt (Fig. 4). The reaction proceeded smoothly and the completion of the coupling reaction was checked by

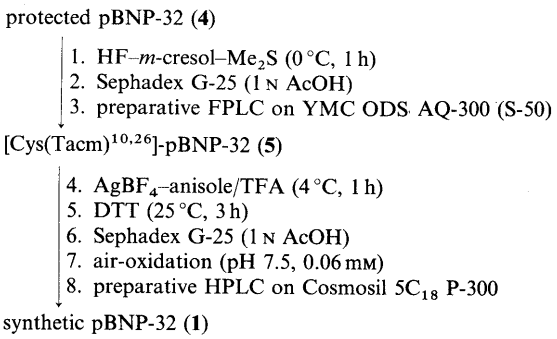

Fig. 6. Deprotection and Purification of pBNP-32

means of the ninhydrin test. The protected pBNP-32 (**4**) was purified by simple precipitation with DMF and EtOH to give a single spot on TLC. Satisfactory coupling was confirmed by an amino acid analysis using Leu as a diagnostic amino acid. The homogeneity of the protected pBNP-32 (**4**) was further confirmed by elemental analysis.

Deprotection and subsequent purification were carried out according to the scheme shown in Fig. 6. The fully protected peptide (**4**) was treated with HF[37] in the presence of *m*-cresol and dimethylsulfide[38] in an ice-bath for 60 min

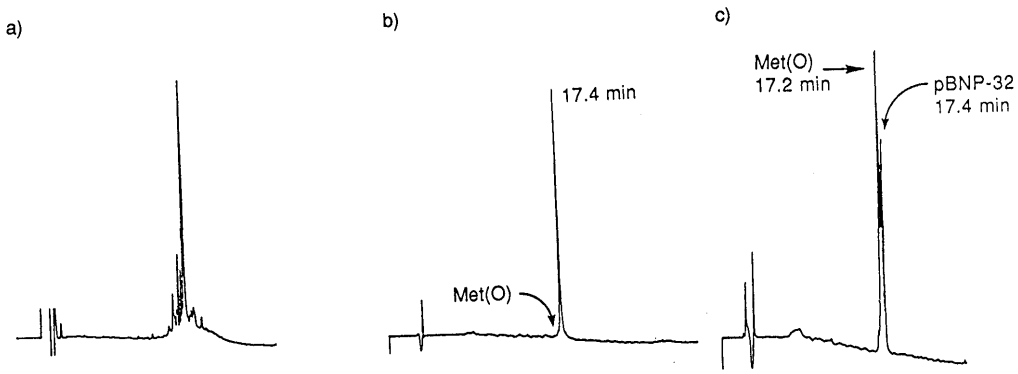

Fig. 7. HPLC of Synthetic pBNP-32

a) Crude air-oxidized sample. b) Purified sample. c) Crude I_2-oxidized sample.

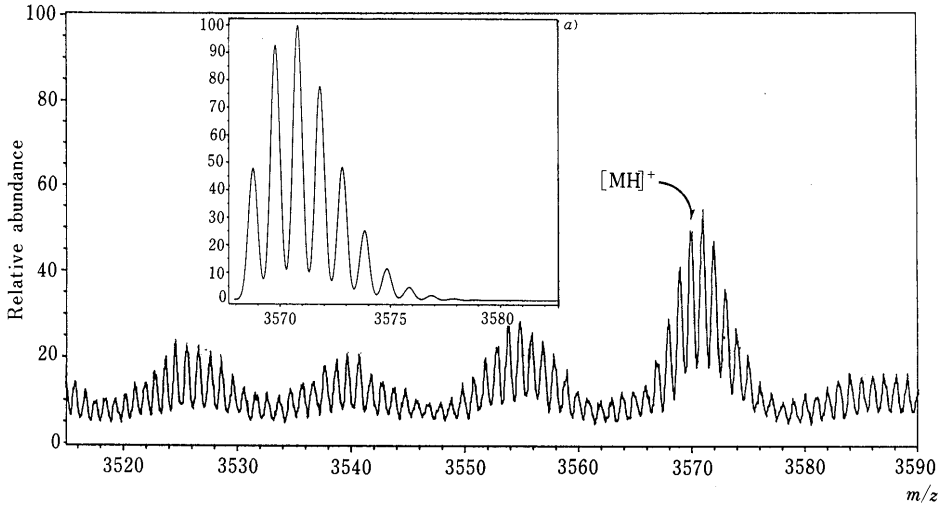

Fig. 8. FAB-MS of Synthetic pBNP-32

a) The theoretical isotopic mass distribution for $C_{149}H_{251}N_{52}O_{44}S_3$ is shown in the box.

to remove all protecting groups except for two Tacm groups. The di-Tacm-pBNP-32 (**5**) was dissolved in H_2O and the pH of this solution was adjusted to 8 with 5% NH_4OH to reverse any possible N→O shift at the Ser and Thr residues.[39] This solution was gel-filtered on Sephadex G-25 and the product was partially purified by FPLC on a YMC-ODS AQ-300 column.

The FPLC-purified sample in TFA was treated with $AgBF_4$ (40 eq) in the presence of anisole (10 eq) to remove the two *S*-Tacm groups followed by DTT (80 eq) treatment as described above. After gel-filtration on Sephadex G-25, the reduced peptide was subjected to air-oxidation at pH 7.5. The progress of the reaction was monitored by HPLC and the disappearance of the starting reduced peptide was confirmed (Fig. 7a). The crude air-oxidized peptide was purified by preparative HPLC on a Cosmosil $5C_{18}$P-300 using a gradient of MeCN in 0.1% aqueous TFA to give a homogeneous peptide in 10.5% yield (calculated from protected peptide). The purified pBNP-32 (**1**) exhibited a single peak on analytical HPLC (YMC AM-302) (Fig. 7b) and was proved to be a monomer by gel-permeation HPLC (YMC Pack Diol-60). Its acid hydrolysate gave amino acid ratios in good agreement with the theoretical values. In addition, it gave the mass value $[MH]^+$ of 3569.9 (theoretical value: 3569.819) and the base peak of 3570.9 (theoretical value: 3570.820) in the molecular ion region in the fast atom bombardment mass spectrum (FAB-MS) (Fig. 8).

In an alternative oxidative cleavage of di-Tacm-pBNP-32 (**5**) using I_2 in 90% aqueous AcOH, we observed a large amount of the Met(O) derivative on HPLC (Fig. 7c). The separation of this Met(O)-form on HPLC was rather difficult and the isolation yield of homogeneous peptide was fairly low (2.1%).

In a chick rectum relaxant assay system, our synthetic pBNP-32 showed approximately twice the activity of synthetic pBNP.

Conclusion

These excellent results show that $AgBF_4$ in TFA is a useful mild reagent for removal of the *S*-Tacm or the *S*-Acm group in peptide synthesis. Formation of Met(O) derivative and modification at Trp residues during the removal of the *S*-Tacm group were suppressed more effectively with $AgBF_4$ than in the standard I_2 procedure. It is noteworthy that synthetic pBNP-32 obtained in a highly purified form had approximately 5 times greater chick rectum relaxant activity than α-rat ANP. To our knowledge, synthetic pBNP-32 has the most potent chick rectum relaxant activity among the ANP–BNP families.

Experimental

General experimental procedures employed in this investigation were

essentially the same as described in connection with the synthesis of porcine BNP.[5]

Prior to the coupling reaction, the N^{α}-protecting Boc group was cleaved by TFA (*ca.* 10 ml per 1.0 g of a peptide) in the presence of anisole (2 eq or more) at ice-bath temperature for 1 h. The WSC plus HOBt condensation was performed at room temperature. The mixed anhydride condensation was performed using isobutyl chloroformate at ice-bath temperature for 3 h. To prepare 10 ml of the 1 M HBF_4–thioanisole in TFA system, 10 mmol each of the HBF_4–diethyl ether complex (Aldrich Chem. Co.) and thioanisole were dissolved in TFA and the total volume was adjusted to 10 ml with TFA.[13]

Unless otherwise mentioned, products were purified by one of the following two procedures. Procedure A: For purification of protected peptides soluble in AcOEt, the extract was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O–NaCl, then dried over Na_2SO_4 and concentrated. The residue was recrystallized from appropriate solvent. Procedure B: For purification of protected peptides less soluble in AcOEt, the crude product was triturated with ether–5% cirtic acid. The resulting powder was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, and recrystallized or re-precipitated from appropriate solvent.

TLC was performed on silica gel (Kiesel-gel $60F_{254}$, Merck). *Rf* values refer to the following v/v solvent systems: Rf_1 $CHCl_3$–MeOH–H_2O (8 : 3 : 1, lower phase), Rf_2 $CHCl_3$–MeOH (10 : 0.5), Rf_3 $CHCl_3$–MeOH (9 : 1).

Leucine-aminopeptidase (LAP, lot No. L-6007) was purchased from Sigma. Analytical HPLC was conducted with a Hitachi 655A. Preparative FPLC and HPLC were conducted with a Pharmacia FPLC system and a Shimadzu LC-4A, respectively. Amino acid analysis was conducted with a Hitachi L-8500. FAB-MS were obtained on a JEOL JMX-HX 110 double-focussing spectrometer, equipped with an FAB ion source and a data processor (JEOL DA-5000).

Deprotection of *S*-Protected Derivatives with $AgBF_4$ Boc–Cys(R)–OH (R = Tacm, Acm, Bam, MBzl, Tmb, Bu^t, or MeBzl; *ca.* 0.03 mmol) in TFA (*ca.* 1.0 ml) was treated with $AgBF_4$ (10 eq) in the presence of anisole (10 eq) at an ice-bath temperature. The solution was stirred for 60 min, and dry ether was added. The resulting precipitate was collected by centrifugation, then washed with ether and dried over KOH pellets *in vacuo*. The residue was dissolved in 1 N AcOH (1.0 ml) and DTT (10 eq) was added. The mixture was stirred at 25 °C for 60 min. After centrifugation, the supernatant was applied to an amino acid analyzer. Recovery of cysteine is listed in Table I.

Solid-Phase Synthesis of Protected Somatostatin *p*-Alkoxybenzyl alcohol type polystyrene resin (290 mg, 0.2 mmol) (substitution: 0.69 mmol/g, purchased from Watanabe Chem. Ind. Ltd.) was placed in a polypropylene column (1.6 × 8.0 cm) and Fmoc–Cys(Tacm)–OH[4] (2.5 eq), DIPCDI (2.5 eq) and DMAP (0.1 eq) in DMF (3 ml) were added. The column was vortexed at 25 °C for 2 h. After washing of the resin with DMF, the coupling procedure was repeated. The following Fmoc amino acids and derivatives (purchased from Watanabe Chem. Ind., Ltd.) were used to construct the peptide chain on the resin: Ala, Gly, Asn, Phe, Trp, Thr(Bu^t), Ser(Bu^t), and Lys(Boc). Each amino acid derivative (2.5 eq) was introduced with DIPCDI (2.5 eq) in the presence of HOBt (2.5 eq) according to the manual schedule described in the literature.[40] Every condensation was continued until the resin became negative to the Kaiser test.[30] After assembling the respective amino acids, the protected peptide resin was hydrolyzed in 12 N HCl–propionic acid (1 : 1, v/v). Amino acid ratios in the hydrolysate of the protected peptide resin (numbers in parentheses are theoretical): Asp 1.08 (1), Ser 0.30 (1), Thr 1.40 (2), Gly 1.02 (1), Ala 1.06 (1), Cys N. D. (1), Phe 3.00 (3), Lys 1.94 (2), Trp N. D. (1).

H-Ala-Gly-Cys(Tacm)-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys(Tacm)-OH, [Cys(Tacm)^{3,14}]-Somatostatin The protected peptide resin (100 mg) was treated with 1 M HBF_4–thioanisole in TFA (9.0 ml) in the presence of *m*-cresol (298 μl) and EDT (716 μl) in an ice-bath for 60 min. Dry ether was added to precipitate the product, then ether was removed by decantation. The crude product was extracted with 4 N AcOH (3 ml × 2) and the resin was removed by filtration. The filtrate was applied to a column of Sephadex G-15 (3.6 × 60 cm), which was eluted with 4 N AcOH. The fractions corresponding to the main peak (tube Nos. 18—25, 5.7 ml each, monitored by measuring the UV absorption at 280 nm) were combined and the solvent was removed by lyophilization to give a powder: 35.2 mg (67%). Amino acid ratios after LAP digestion (numbers in parentheses are theoretical): Thr 2.24 (2), Ser 1.02 (1), Gly 1.00 (1), Ala 1.00 (1), Phe 3.12 (3), Lys 2.03 (2), Trp 0.91 (1) [Asn (1) and Cys(Tacm) (2) were not determined (recovery of Gly 75%)].

Synthesis of Somatostatin Using $AgBF_4$ The above di-Tacm peptide (7.2 mg) was treated with $AgBF_4$ (31 mg, 40 eq) in the presence of anisole (4.3 μl) in TFA (4 ml) at ice-bath temperature for 60 min, then dry ether was added. The resulting powder was collected by centrifugation and dissolved in 1 N AcOH (4 ml), and DTT (98 mg, 80 eq) was added. The solution was stirred at 25 °C for 3 h and the supernatant was applied to a column of Sephadex G-15 (2.1 × 15 cm) using 1N AcOH as an eluant. The fractions corresponding to the front main peak (tube Nos. 2—7, 5.7 ml each, monitored by measuring the UV absorption at 280 nm) were combined and diluted with water (100 ml). The pH of this solution was adjusted to 7.5 with 5% NH_4OH and the solution was kept standing at 25 °C. The progress of air-oxidation was monitored by HPLC (Fig. 3b, d). After 2 d, the entire solution was lyophilized to give a powder. The air-oxidized peptide in 1 N AcOH (*ca.* 4 ml) was subjected to FPLC on a YMC ODS-AQ-300 (1.5 × 50 cm) column, which was eluted with a linear gradient of 60% MeCN/0.1% aqueous TFA (0—100%, 400 min) in 0.1% aqueous TFA at the flow rate of 3.0 ml/min. The eluate corresponding to the main peak, monitored by measuring the UV absorption at 280 nm, was collected and lyophilized to give a white fluffy powder; yield 2.5 mg (39%). The synthetic peptide exhibited a single peak on analytical HPLC and the retention time of this peptide was identical with that of an authentic sample (purchased from the Protein Research Foundation, Osaka, Japan). Amino acid ratios after LAP digestion (numbers in parentheses are theoretical): Thr 2.02 (2), Ser 0.98 (1), Gly 1.00 (1), Ala 0.97 (1), Cys 0.89 (1), Phe 2.80 (3), Lys 1.85 (2), Trp 0.86 (1) [Asn (1) was not determined (recovery of Gly 72%)].

Alternative Synthesis of Somatostatin Using I_2 The di-Tacm somatostatin (2.1 mg) was dissolved in 50% aqueous AcOH (10 ml) and 20% I_2/EtOH (20 eq) was added. This solution was stirred at 25 °C for 1 h. The reaction was stopped by adding excess ascorbic acid, and the solvent was removed by evaporation. The residue was dissolved in H_2O (*ca.* 2 ml), washed twice with ether (*ca.* 4 ml), and lyophilized. The purity of this crude peptide was determined by HPLC (Fig. 3c) and amino acid analysis after LAP digestion: Thr 1.82 (2), Ser 0.98 (1), Gly 1.00 (1), Ala 1.04 (1), Cys 0.40 (1), Phe 2.37 (3), Lys 1.52 (2), Trp 0.20 (1) [Asn (1) was not determined (recovery of Gly 46%)].

Boc-Arg(Tos)-Asp(OcHex)-Ser(Bzl)-Gly-Cys(Tacm)-Phe-Gly-OPac A mixed anhydride [prepared from 0.78 g (1.83 mmol) of Boc–Arg(Tos)–OH] in DMF (5 ml) was added to an ice-chilled solution of a TFA-treated sample of Boc–Asp(OcHex)–Ser(Bzl)–Gly–Cys(Tacm)–Phe–Gly–OPac[5] (1.80 g, 1.66 mmol) in DMF (10 ml) containing Et_3N (0.23 ml, 1.66 mmol). The mixture was stirred for 3 h and concentrated. The product was purified by procedure A and recrystallized from THF with ether; yield 1.94 g (84%), mp 132—134 °C, $[\alpha]_D^{26}$ −27.2° (*c* = 0.2, DMF), Rf_2 0.31. *Anal.* Calcd for $C_{68}H_{91}N_{11}O_{17}S_2 \cdot H_2O$: C, 57.64; H, 6.61; N, 10.87. Found: C, 57.27; H, 6.38; N, 10.84.

Boc-Met-Arg(Tos)-Asp(OcHex)-Ser(Bzl)-Gly-Cys(Tacm)-Phe-Gly-OPac A mixed anhydride [prepared from 0.38 g (1.53 mmol) of Boc–Met–OH] in DMF (5 ml) was added to an ice-chilled solution of a TFA-treated sample of the above heptapeptide ester (1.94 g, 1.39 mmol) in DMF (10 ml) containing Et_3N (0.19 ml, 1.53 mmol). The product was purified by procedure B and recrystallized from THF with ether; yield 2.00 g (94%), mp 175—178 °C, $[\alpha]_D^{26}$ −16.1° (*c* = 0.6, DMF), Rf_2 0.24. *Anal.* Calcd for $C_{73}H_{100}N_{12}O_{18}S_3 \cdot H_2O$: C, 56.64; H, 6.64; N, 10.86. Found: C, 56.76; H, 6.65; H, 10.73.

Boc-Thr(Bzl)-Met-Arg(Tos)-Asp(OcHex)-Ser(Bzl)-Gly-Cys(Tacm)-Phe-Gly-OPac A mixed anhydride [prepared from 269 mg (0.66 mmol) of Boc–Thr(Bzl)–OH] in DMF (5 ml) was added to an ice-chilled solution of a TFA-treated sample of the above octapeptide ester (0.83 g, 0.55 mmol) in DMF (10 ml) containing Et_3N (76 μl, 0.55 mmol). The product was purified by procedure B and recrystallized from THF with ether; yield 0.88 g (93%), mp 184—186 °C, $[\alpha]_D^{26}$ −19.6° (*c* = 0.3, DMF), Rf_2 0.21. *Anal.* Calcd for $C_{84}H_{113}N_{13}O_{20}S_3 \cdot 6H_2O$: C, 55.15; H, 6.88; N, 9.96. Found: C. 55.19; H, 6.35; N, 10.00.

Boc-Lys(ClZ)-Thr(Bzl)-Met-Arg(Tos)-Asp(OcHex)-Ser(Bzl)-Gly-Cys(Tacm)-Phe-Gly-OPac A mixed anhydride [prepared from 250 mg (0.60 mmol) of Boc–Lys(ClZ)–OH] in DMF (5 ml) was added to an ice-chilled solution of a TFA-treated sample of the above nonapeptide ester (0.87 g, 0.50 mmol) in DMF (10 ml) containing Et_3N (70 μl, 0.50 mmol). The product was purified by procedure B and recrystallized from DMF with ether; yield 0.83 g (82%), mp 188—191 °C, $[\alpha]_D^{26}$ −12.2° (*c* = 0.5, DMF), Rf_3 0.64. *Anal.* Calcd for $C_{98}H_{130}ClN_{15}O_{23}S_3 \cdot 6H_2O$: C, 55.36; H, 6.73; N, 9.88. Found C, 55.35; H, 6.32; N, 10.11.

Boc-Pro-Lys(ClZ)-Thr(Bzl)-Met-Arg(Tos)-Asp(OcHex)-Ser(Bzl)-Gly-Cys(Tacm)-Phe-Gly-OPac A mixed anhydride [prepared from 104 mg (0.48 mmol) of Boc–Pro–OH] in DMF (4 ml) was added to an ice-chilled solution of a TFA-treated sample of the above decapeptide ester

(0.81 g, 0.40 mmol) in DMF (10 ml) containing Et_3N (56 μl, 0.40mmol). The product was purified by procedure B and recrystallized from DMF with ether; yield 0.83 g (82%), mp 187—191 °C, $[\alpha]_D^{26}$ −32.0° (c=0.2, DMF), Rf_3 0.53. *Anal.* Calcd for $C_{103}H_{137}ClN_{16}O_{24}S_3 \cdot 7H_2O$: C, 55.20; H, 6.79; N, 10.00. Found: C, 55.11; H, 6.13; N, 9.92.

Boc–Ser(Bzl)–Pro–Lys(ClZ)–Thr(Bzl)–Met–Arg(Tos)–Asp(OcHex)–Ser(Bzl)–Gly–Cys(Tacm)–Phe–Gly–OPac A mixed anhydride [prepared from 104 mg (0.46 mmol) of Boc–Ser(Bzl)–OH] in DMF (4 ml) was added to an ice-chilled solution of a TFA-treated sample of the above undecapeptide ester (0.82 g, 0.38 mmol) in DMF (10 ml) containing Et_3N (53 μl, 0.38 mmol). The product was purified by procedure B and recrystallized from DMF with ether; yield 0.77 g (87%), mp 200—202 °C, $[\alpha]_D^{26}$ −26.1° (c=0.4, DMF), Rf_3 0.31. *Anal.* Calcd for $C_{113}H_{148}ClN_{17}O_{26}S_3 \cdot 7H_2O$: C, 56.12; H, 6.75; N, 9.84. Found: C, 56.14; H, 6.35; N, 10.09.

Boc–Ser(Bzl)–Pro–Lys(ClZ)–Thr(Bzl)–Met–Arg(Tos)–Asp(OcHex)–Ser(Bzl)–Gly–Cys(Tacm)–Phe–Gly–OH, Boc–(pBNP-32, 1—12)–OH (2) The above dodecapeptide derivative (0.75 g, 0.33 mmol) in a mixture of DMF–pyridine (5 : 1, 30 ml) was treated with Zn powder (0.66 g, 9.81 mmol) in the presence of anthranilic acid (1.35 g, 9.81 mmol) at 50°C for 3 h. The solution was filtered, the filtrate was concentrated *in vacuo*, and the residue was precipitated with 2% EDTA. The resulting powder was washed with H_2O and reprecipitated from DMF with ether; yield 0.60 g (84%), mp 200—202 °C (dec.), $[\alpha]_D^{26}$ −32.7° (c=0.3, DMF), Rf_1 0.83. *Anal.* Calcd for $C_{105}H_{142}ClN_{17}O_{25}S_3 \cdot 7H_2O$: C, 54.82; H, 6.83; N, 10.35. Found: C, 54.81; H, 6.39; N, 10.89. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.02 (1), Thr 0.82 (1), Ser 1.77 (2), Pro 0.88 (1), Gly 2.00 (2), Met 0.82 (1), Cys 0,10 (1), Phe 0.98 (1), Lys 0.91 (1), Arg 1.01 (1), (Recovery of Gly 96%).

Boc–Ser(Bzl)–Pro–Lys(ClZ)–Thr(Bzl)–Met–Arg(Tos)–Asp(OcHex)–Ser(Bzl)–Gly–Cys(Tacm)–Phe–Gly–Arg(Tos)–Arg(Tos)–Leu–Asp–(OcHex)–Arg(Tos)–Ile–Gly–Ser(Bzl)–Leu–Ser(Bzl)–Gly–Leu–Gly–Cys(Tacm)–Asn–Val–Leu–Arg(Tos)–Arg(Tos)–Tyr(BrZ)–OBzl, Protected pBNP-32 (4) Boc–(pBNP-32, 1—12)–OH (**2**) (355 mg, 0.16 mmol), HOBt (30 mg, 0.19 mmol) and WSC·HCl (44 mg, 0.23 mmol) were successively added to an ice-chilled solution of a TFA-treated sample of Boc–Arg(Tos)–Arg(Tos)–Leu–Asp(OcHex)–Arg(Tos)–Ile–Gly–Ser(Bzl)–Leu–Ser(Bzl)–Gly–Leu–Gly–Cys(Tacm)–Asn–Val–Leu–Arg(Tos)–Arg(Tos)–Tyr(BrZ)–OBzl[5] (**3**) (0.45 g, 0.12 mmol) in DMF (20 ml) containing Et_3N (16 μl, 0.12 mmol). The mixture was stirred for 48 h and concentrated. The product was purified by procedure B and reprecipitated from DMF with EtOH; yield 0.49 g (72%), mp 227 °C (dec.), $[\alpha]_d^{26}$ −31.3° (c=0.6, DMSO), Rf_1 0.81. Amino acid ratios in 6 N HCl hydrolysate: Asp 3.12 (3), Thr 0.92 (1), Ser 3.80 (4), Pro 0.91 (1), Gly 5.20 (5), Val 0.94 (1), Cys 0.28 (1), Met 0.89 (1), Ile 1.02 (1), Leu 4.00 (4), Tyr 0.97 (1), Phe 1.08 (1) Lys 1.00 (1), Arg 5.85 (6), (recovery of Leu 90%). *Anal.* Calcd for $C_{278}H_{384}BrClN_{54}O_{64}S_9 \cdot 8H_2O$: C, 55.14; H, 6.65; N, 12.49. Found: C, 54.97; H, 6.48; N, 12.53.

Synthesis of pBNP-32 (1) Using $AgBF_4$ The above fully protected pBNP-32 (**4**) (100 mg) was treated with HF (*ca.* 4 ml) in the presence of *m*-cresol (100 μl) and dimethylsulfide (100 μl) at ice-bath temperature for 60 min. After evaporation of the excess HF, dry ether was added and the precipitate was dried over KOH pellets *in vacuo* for 30 min. The resulting powder was dissolved in H_2O (*ca.* 3 ml). The solution, after being adjusted to pH 8 with 5% NH_4OH, was stirred for 30 min in an ice-bath. The solution was applied to a column of Sephadex G-25 (2.7 × 63 cm), which was eluted with 1 N AcOH as an eluant. The fractions corresponding to the main peak (10.2 ml each, monitored by measuring the UV absorption at 280 nm, tube Nos. 12—19) were combined and lyophilized to give a powder. The gel-filtered sample was partially purified by preparative FPLC on YMC-gel ODS-AQ-300 (1.5 × 50 cm), which was eluted with a linear gradient of 60% MeCN/0.1% aqueous TFA (0—100%, 400 min) in 0.1% aqueous TFA (flow rate, 3.0 ml/min). The fractions corresponding to the main peak were collected and lyophilized to give **5** as a powder; 27.6 mg (65%).

The above FPLC-purified peptide (**5**) (27.6 mg) in TFA (*ca.* 4 ml) was treated with $AgBF_4$ (56 mg, 40 eq) in the presence of anisole (32 μl, 40 eq) at ice-bath temperature for 60 min, then dry ether was added. The resulting powder was dissolved in 1 N AcOH (*ca.* 4 ml) and DTT (90 mg, 80 eq) was added. This solution was stirred at 25 °C for 3 h and the supernatant was applied to a column of Sephadex G-25 (2.7 × 63 cm), which was eluted with 1 N AcOH. The fractions corresponding to the main peak (tube Nos. 11—18, 10.2 ml each, monitored by measuring the UV absorption at 280 nm) were combined and diluted with H_2O (125 ml). The pH of this solution was adjusted to 7.5 with 5% NH_4OH. The solution was kept standing at 25 °C and the progress of air-oxidation was monitored by HPLC. After 3 d, the entire solution was lyophilized to give a powder. The crude air-oxidized peptide was dissolved in H_2O (*ca.* 4 ml) and purified by HPLC on a Cosmosil $5C_{18}$ P-300 column (10 × 250 mm), which was eluted with a linear gradient of MeCN (20—40%, 90 min) in 0.1% aqueous TFA (flow rate: 4.0 ml/min). The eluate corresponding to the main peak was collected and lyophilized to give **1** as a fluffy white powder; yield 4.2 mg (10.5%, calculated from the protected peptide **4**), $[\alpha]_D^{26}$ −60° (c=0.1, 1 N AcOH), HPLC retention times: 17.4 min on an analytical YMC AM-302 (4.6 × 150 mm) column [gradient elution with MeCN (10—60%, 30 min) in 0.1% aqueous TFA, 0.7 ml/min] and 28.6 min on a gel-permeation YMC Pack Diol-60 (0.8 × 50 cm) column [eluted with 0.1 M phosphate buffer (pH 7.2): MeCN (4 : 1, v/v); flow rate, 0.5 ml/min, the retention time was between those of bovine insulin (24.6 min M.W. 5733) and adrenorphin (33.4 min, M.W. 984)], FAB-MS: observed m/z (relative intensity); 3566.758 (35), 3567.886 (53), 3568.893 (75), 3569.901 (91, MH^+), 3570.901 (100), 3571.917 (86), 3572.920 (65), 3573.944 (49), 3574.887 (37); theoretical mass values calculated for $C_{149}H_{251}O_{44}N_{52}S_3$ were 3569.819 (MH^+) and 3570.821 (base peak in the molecular ion region). Amino acid ratios in a 6 N HCl hydrolysate (numbers in parentheses are theoretical): Asp 3.04 (3), Thr 0.90 (1), Ser 3.64 (4), Pro 0.88 (1), Gly 5.05 (5), Val 0.94 (1), Cys 0.74 (1), Met 0.94 (1), Ile 0.94 (1), Leu 4.00 (4), Tyr 0.96 (1), Phe 0.94 (1), Lys 0.92 (1), Arg 5.98 (6), recovery of Leu 87%.

Alternative Synthesis of pBNP-32 (1) Using I_2 The di-Tacm pBNP-32 (**5**) (22.0 mg) was dissolved in 90% AcOH (100 ml) and 20% I_2/EtOH (20 eq) was added. The mixture was stirred at 25 °C for 1 h, then the reaction was stopped by adding excess ascorbic acid. The solvent was removed by evaporation and the residue was applied to a column of Sephadex G-25 (2.7 × 63 cm), which was eluted with 1 N AcOH. The fractions corresponding to the main peak (tube Nos. 13—21, 10.2 ml each, monitored by measuring the UV absorption at 280 nm) were collected and lyophilized to give a powder. The purity of this peptide was examined by HPLC on a YMC AM-302 (4.6 × 150 mm) column {retention times: 17.2 min [Met(O)^5-pBNP-32] (**1'**) and 17.4 min (**1**), eluted with MeCN (10–60%, 30 min) in 0.1% aqueous TFA; flow rate, 0.7 ml/min (Fig. 7c). The above crude peptide was further purified by HPLC on YMC R-ODS-5 column (2.15 × 25 cm), which was eluted with MeCN (20–40%, 100 min) in 0.3% aqueous TFA at the flow rate of 4.0 ml/min. The eluate corresponding to each peptide was collected and lyophilized to give **1** and **1'**as fluffy white powders; 0.7 mg and 2.4 mg (2.1% and 7.2%, respectively, calculated from the protected peptide **4**).

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References and Notes

1) Presented in part at the 27th Symposium on Peptide Chemistry of Japan, Shizuoka, Oct. 1989; *Peptide Chemistry*, **1989**, 33.
2) Amino acids and peptides derivatives mentioned in this investigation are of the L-configuration. The following abbreviations are used: Acm = acetamidomethyl, Bam = benzamidomethyl, Boc = *tert*-butyloxycarbonyl, Bu^t = *tert*-butyl, Bzl = benzyl, BrZ = 2-bromobenzyloxycarbonyl, cHex = cyclohexyl, ClZ = 2-chlorobenzyloxycarbonyl, DMF = *N*,*N*-dimethylformamide, DMSO = dimethyl sulfoxide, DTT = dithiothreitol, EDT = ethane-1,2-dithiol, EDTA = ethylenediaminetetraacetic acid, Fmoc = 9-fluorenylmethyloxycarbonyl, HOBt = 1-hydroxybenzotriazole, MBzl = 4-methoxybenzyl, MeBzl = 4-methylbenzyl, MeCN = acetonitrile, Pac = phenacyl, Tacm = trimethylacetamidomethyl, TFA = trifluoroacetic acid, THF = tetrahydrofuran, Tmb = 2,4,6-trimethylbenzyl, Tos = *p*-toluenesulfonyl.
3) Y. Kiso, M. Yoshida, T. Kimura, Y. Fujiwara, and M. Shimokura, *Tetrahedron Lett.*, **30**, 1979 (1989).
4) Y. Kiso, M. Yoshida, Y. Fujiwara, T. Kimura, M. Shimokura, and K. Akaji, *Chem. Pharm. Bull.*, **38**, 673 (1990).
5) Y. Kiso, M. Yoshida, T. Kimura, Y. Fujiwara, M. Shimokura, and K. Akaji, *Chem. Pharm. Bull.*, **38**, 1192 (1990).
6) D. F. Veber, J. D. Milkowski, S. L. Varga, R. G. Denkewalter, and R. Hirschmann, *J. Am. Chem. Soc.*, **94**, 5456 (1972)
7) These results were outlined in preliminary paper; M. Yoshida, K. Akaji, T. Tatsumi, S. Iinuma, Y. Fujiwara, T. Kimura, and Y. Kiso, *Chem. Pharm. Bull.*, **38** 273 (1990).
8) T. Sudoh, N. Minamino, K. Kangawa, and H. Matsuo, *Biochem. Biophys. Res. Commun.*, **155**, 762 (1988).
9) T. Sudoh, K. Kangawa, N. Minamino, and H. Matsuo, *Nature* (London) **332**, 78 (1988).
10) K. Kangawa and H. Matsuo, *Biochem. Biophys. Res. Commun.*, **118**,

131 (1984).
11) T. G. Flynn, M. L. de Bold, and A. J. de Bold, *Biochem. Biophys. Res. Commun.*, **117**, 859 (1983).
12) K. Kangawa, A. Fukuda, and H. Matsuo, *Biochem. Biophys. Res. Commun.*, **121**, 585 (1984).
13) Y. Kiso, M. Yoshida, T. Tatsumi, Y. Fujiwara, T. Kimura, and K. Akaji, *Chem. Pharm. Bull.*, **37**, 3432 (1989); K. Akaji, M. Yoshida, T. Tatsumi, T. Kimura, Y. Fujiwara, and Y. Kiso, *J. Chem. Soc., Chem. Commun.*, **1990**, 288.
14) S. Guttmann, *Helv. Chim. Acta*, **49**, 83 (1966); H. T. Storey, J. Beacham, S. F. Cernosek, F. M. Finn, C. Yanaihara, and K. Hofmann, *J. Am. Chem. Soc.*, **94**, 6170 (1972).
15) 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.
16) O. Nishimura, C. Kitada, and M. Fujino, *Chem. Pharm. Bull.*, **26**, 1576 (1978).
17) P. K. Chakravarty and R. K. Olsen, *J. Org. Chem.*, **43**, 1270 (1978).
18) S. Akabori, S. Sakakibara, Y. Shimonishi, and Y. Nobuhara, *Bull. Chem. Soc. Jpn.*, **37** 433 (1964).
19) F. Brtnik, M. Krojidlo, T. Barth, and K. Jost, *Collect. Czech. Chem. Commun.*, **46**, 286 (1981); Y. Kiso, M. Shimokura, S. Hosoi, T. Fujisaki, Y. Fujiwara, and M. Yoshida, *J. Protein Chem.*, **6**, 147 (1987); M. Yoshida, M. Shimokura, Y. Fujiwara, T. Fujisaki, K. Akaji, and Y. Kiso, *Chem. Pharm. Bull.*, **38**, 382 (1990).
20) F. M. Callahan, G. W. Anderson, R. Paul, and J. E. Zimmermann, *J. Am. Chem. Soc.*, **85**, 201 (1963).
21) B. W. Erickson and R. B. Merrifield, *J. Am. Chem. Soc.*, **95**, 3750 (1973).
22) P. Sieber, B. Kamber, B. Riniker, and W. Rittel, *Helv. Chim. Acta*, **63**, 2358 (1980).
23) E. Atherton and R. C. Sheppard, "The Peptides," Vol. 9, ed. by S. Udenfriend and J. Meienhofer, Academic Press Inc., New York, 1987, p.1 and references cited therein.
24) S. S. Wang, *J. Am. Chem. Soc.*, **95**, 1328 (1973).
25) A. Tartar and J-C. Gesquire, *J. Org. Chem.*, **44**, 5000 (1979).
26) J. K. Chang, M. Shimidzu, and S. S. Wang, *J. Org. Chem.*, **41**, 3255 (1976).
27) L. A. Carpino and G. Y. Han, *J. Am. Chem. Soc.*, **92**, 5748 (1970); E. Atherton, C. J. Logan, and R. C. Sheppard, *J. Chem. Soc., Perkin Trans. 1*, **1981**, 538.
28) W. König and R. Geiger, *Chem. Ber.*, **103**, 788, 2024, 2034 (1970).
29) A. Dryland and R. C. Sheppard, *J. Chem. Soc., Perkin Trans. 1*, **1986**, 125; L. Cameron, M. Medal, and R. C. Sheppard, *J. Chem. Soc., Chem. Commun.*, **1987**, 270.
30) E. Kaiser, R. L. Colescott, C. S. Bossinger, and P. I. Cook, *Anal. Biochem.*, **34**, 595 (1970).
31) J. Scotchler, R. Lozier, and A. B. Robinson, *J. Org. Chem.*, **35**, 3151 (1970).
32) E. Jaeger, P. Thamm, S. Knof, and E. Wünsch, *Hoppe-Seyler's Z. Physiol. Chem.*, **359**, 1629 (1978); Y. Masui, N. Chino, and S. Sakakibara, *Bull. Chem. Soc. Jpn.*, **53**, 464 (1980); N. Fujii, S. Futaki, K. Yasumura, and H. Yajima, *Chem. Pharm. Bull.*, **32**, 2660 (1984).
33) J. R. Vaughan Jr. and R. L. Osato, *J. Am. Chem. Soc.*, **74**, 676 (1952).
34) J. J. Sharp, A. B. Robinson, and M. D. Kamen, *J. Am. Chem. Soc.*, **95**, 6097 (1973).
35) M. Neya, D. Hagiwara, T. Miyazaki, T. Nakamura, K. Hemmi, and M. Hashimoto, *J. Chem. Soc., Perkin Trans. 1*, **1989**, 2187.
36) T. Kimura, M. Takai, Y. Masui, T. Morikawa, and S. Sakakibara, *Biopolymers*, **20**, 1823 (1981).
37) S. Sakakibara and Y. Shimonishi, *Bull. Chem. Soc. Jpn.*, **38**, 1412 (1965).
38) J. P. Tam and R. B. Merrified, "The Peptides," Vol. 9 ed by S. Udenfriend and J. Meienhofer, Acedemic Press Inc., New York, 1987, p. 185.
39) S. Sakakibara, "Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins," Vol. 1, ed. by B. Weinstein, Marcel Dekker, New York, 1971, p. 51.
40) K. Akaji, N. Fujii, F. Tokunaga, T. Miyata, S. Iwanaga, and H. Yajima, *Chem. Pharm. Bull.*, **37**, 2661 (1989).