Solution-Phase Synthesis of α-Human Atrial Natriuretic Peptide (α-hANP)[1)]

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α-Human atrial natriuretic peptide (α-hANP) was synthesized by assembling six peptide fragments in solution followed by deprotection with HF and subsequent air-oxidation. The trimethylbenzyl group was employed as an *S*-protecting group of cysteine. The HF–dimethylselenide–*m*-cresol system was employed as a final deprotecting reagent and, at the same time, as a reducing reagent of Met(O). Synthetic α-hANP elicited potent diuretic and natriuretic activity in rats.

Keywords solution-phase peptide synthesis; α-human atrial natriuretic peptide; hydrogen fluoride–dimethylselenide–*m*-cresol deprotection; Met(O) reduction; 2,4,6-trimethylbenzylcysteine; 2,2,2-trichloro-*tert*-butoxycarbonylhydrazine; diuretic activity; natriuretic activity

In 1984, Kangawa and Matsuo isolated α-human atrial natriuretic peptide (α-hANP) from human atrial extract by using an *in vitro* assay for the relaxant effect on the contractility of chick rectum and determined its amino acid sequence.[2)] This human atrial natriuretic peptide (α-hANP) is a 28-amino acid peptide with one disulfide bridge (Fig. 1) and has potent diuretic and natriuretic activities as well as vasorelaxant activity in rats. Following the structure determination, we have reported preliminarily the solution-phase synthesis of this peptide, in which a new deprotection procedure using dimethylselenide, a new substituted hydrazine, 2,2,2-trichloro-*tert*-butoxycarbonylhydrazine (Tcboc-$NHNH_2$), and a cysteine derivative, 2,4,6-trimethylbenzylcysteine [Cys(Tmb)], were employed.[3-5)] Several other research groups have also reported solid-phase[6)] or, preliminarily, solution-phase syntheses[7)] of this peptide. In this paper, we wish to present a detailed account of our total synthesis of α-hANP.

Our synthetic route to α-hANP is illustrated in Fig. 2. In the present synthesis, the 2,4,6-trimethylbenzyl (Tmb) group was employed as an *S*-protecting group of cysteine. This Tmb group was originally introduced by Brtnik *et al.*, in 1981,[8)] but has not been widely applied in practical peptide synthesis. They reported that the Tmb group was stable to TFA (23 °C, 2 h) and cleavable by HF–anisole (0 °C, 30 min). In particular, the Tmb group was reported to be recovered nearly quantitatively (96.1%) after refluxing with TFA for 1 h, whereas the 4-methoxybenzyl (MBzl) group, one of the most widely used *S*-protecting groups currently employed in peptide synthesis, is partially cleaved by TFA treatment at 0 °C for 2.5 h.[9)] In addition, Z(OMe)–Cys(Tmb)–OH was found to be less susceptible to sulfoxide formation as compared to Z(OMe)–Cys(MBzl)–OH; 11% of the latter was oxidized to the corresponding sulfoxide by vigorous stirring in DMF at 25 °C for 4 d, whereas only 3% of the former was oxidized to its sulfoxide. Because of these attractive features of the Tmb group as an *S*-protecting group, we decided to examine its usefulness in the present synthesis of α-hANP.

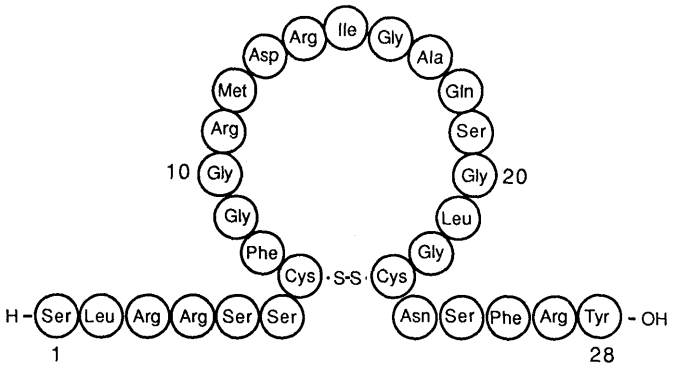

Fig. 1. Structure of α-Human Atrial Natriuretic Peptide (α-hANP)

In combination with the TFA-labile Z(OMe) group as a temporary N^{α}-protection, amino acid derivatives bearing protecting groups removable by HF were employed; *i.e.*, Asp(OBzl), Arg(Mts),[10)] and Cys(Tmb), mentioned above. Of these, the β-Bzl ester of the Asp residue (position 13) was employed since the Asp–Arg sequence (position 13—14) is known to be less susceptible to base catalyzed succinimide formation.[11-12)] In addition, Met(O) was employed to prevent partial alkylation during TFA-deprotection of the Z(OMe) group.[13)] To reduce Met(O) to Met, the HF–dimethylselenide system was employed as described later. Six peptide fragments were selected as building blocks to construct the entire peptide backbone of α-hANP as shown

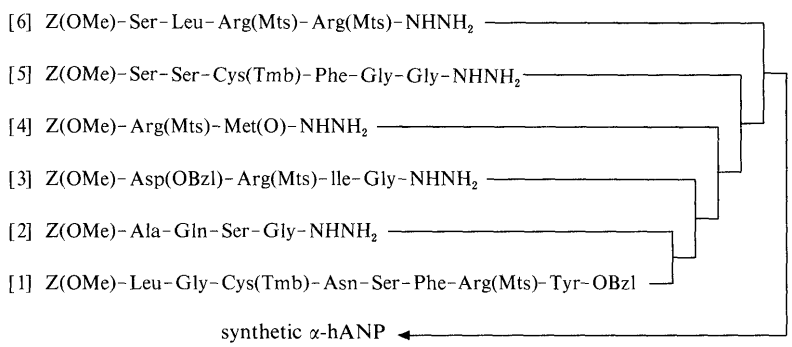

Fig. 2. Synthetic Route to α-hANP

in Fig. 2.

The C-terminal protected octapeptide ester, Z(OMe)–Leu–Gly–Cys(Tmb)–Asn–Ser–Phe–Arg(Mts)–Tyr–OBzl [1], was prepared according to the scheme illustrated in Fig. 3. Starting from H–Tyr–OBzl, Z(OMe)–Arg(Mts)–OH was coupled by the mixed anhydride procedure[14] and the resulting dipeptide ester, after TFA-treatment, was condensed with the known dipeptide hydrazide, Z(OMe)–Ser–Phe–$NHNH_2$,[15] *via* the azide[16] to give Z(OMe)–Ser–Phe–Arg(Mts)–Tyr–OBzl. Z(OMe)–Asn–OH and Z(OMe)–Cys(Tmb)–OH were introduced successively onto a TFA-treated sample of this tetrapeptide ester *via* the corresponding Np ester,[17] followed by azide condensation of Z(OMe)–Leu–Gly–$NHNH_2$ to give the protected C-terminal octapeptide ester [1]. The purity of fragment [1] was ascertained by thin-layer chromatography (TLC), elemental analysis and amino acid analysis after 6 N HCl hydrolysis, as was done with other fragments.

Fragment [2], Z(OMe)–Ala–Gln–Ser–Gly–$NHNH_2$, was prepared in a stepwise manner starting with H–Gly–OBzl, onto which Ser, Gln and Ala were condensed successively by the azide, the Np ester, and the mixed anhydride methods, respectively. The resulting tetrapeptide ester was converted to [2] by the usual hydrazine treatment (Fig. 4).

Fragment [3], Z(OMe)–Asp(OBzl)–Arg(Mts)–Ile–Gly–$NHNH_2$, was prepared with the aid of the substituted hydrazine,[3,4] Tcboc–$NHNH_2$, as shown in Fig. 5. The availability of Tcboc–$NHNH_2$ was also described previously in the synthesis of adrenorphin-methorphamide.[18] A dipeptide hydrazide, Z(OMe)–Arg(Mts)–Ile–$NHNH_2$, prepared by the mixed anhydride procedure followed by hydrazine treatment, was coupled with H–Gly–NHNH–Tcboc by the azide procedure, and the resulting tripeptide derivative, after TFA-treatment, was condensed with Z(OMe)–Asp(OBzl)–OH *via* the Np ester. From the resulting tetrapeptide, Z(OMe)–Asp(OBzl)–Arg(Mts)–Ile–Gly–NHNH–Tcboc, the Tcboc group was removed by treatment with Zn–AcOH to afford [3].

Fragment [4], Z(OMe)–Arg(Mts)–Met(O)–$NHNH_2$, was prepared by the condensation of Z(OMe)–Arg(Mts)–OH with H–Met(O)–OBzl *via* the mixed anhydride followed by usual hydrazine treatment.

Fragment [5], Z(OMe)–Ser–Ser–Cys(Tmb)–Phe–Gly–Gly–$NHNH_2$, was prepared starting from the known dipeptide, Z(OMe)–Gly–Gly–OMe,[19] as shown in Fig. 6. The mixed anhydride procedure was used to condense Z(OMe)–Phe–OH and Z(OMe)–Cys(Tmb)–OH successively to yield the protected tetrapeptide ester, Z(OMe)–Cys(Tmb)–Phe–Gly–Gly–OMe. This was, after TFA-treatment, condensed with the known dipeptide hydrazide, Z(OMe)–Ser–Ser–$NHNH_2$,[20] *via* the azide, and the resulting protected hexapeptide ester, Z(OMe)–Ser–Ser–Cys(Tmb)–Phe–Gly–Gly–OMe, was converted to the corresponding hydrazide [5] by the usual hydrazine treatment.

Fragment [6], Z(OMe)–Ser–Leu–Arg(Mts)–Arg(Mts)–$NHNH_2$, was prepared by condensation of two dipeptide units followed by usual hydrazine treatment as shown in Fig. 7. Z(OMe)–Arg(Mts)–Arg(Mts)–OBzl was prepared by the condensation of Z(OMe)–Arg(Mts)–OH with H–Arg(Mts)–OBzl[11] *via* the mixed anhydride, and Z(OMe)–Ser–Leu–OMe was prepared by the azide procedure. The latter dipeptide ester, after conversion to the corresponding hydrazide, was condensed with a TFA-treated sample of the former dipeptide ester *via* the azide.

The six peptide fragments [1]—[6], thus prepared, were

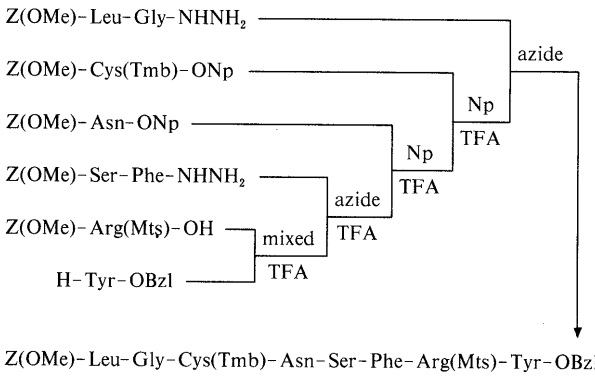

Fig. 3. Synthetic Scheme for the Protected Octapeptide Ester, Z(OMe)–(α-hANP 21—28)–OBzl [1]

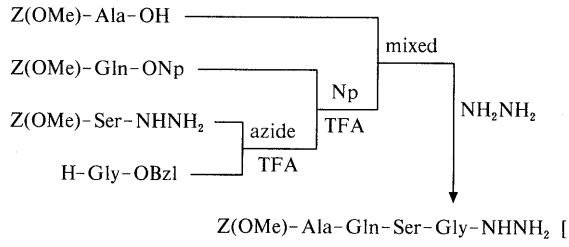

Fig. 4. Synthetic Scheme for the Protected Tetrapeptide Hydrazide, Z(OMe)–(α-hANP 17—20)–$NHNH_2$ [2]

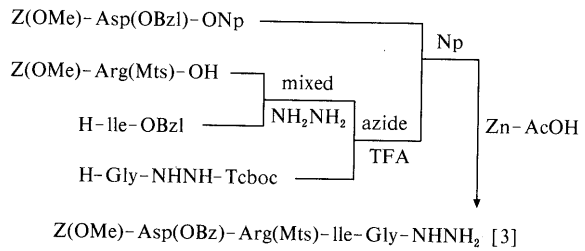

Fig. 5. Synthetic Scheme for the Protected Tetrapeptide Hydrazide, Z(OMe)–(α-hANP 13—16)–$NHNH_2$ [3]

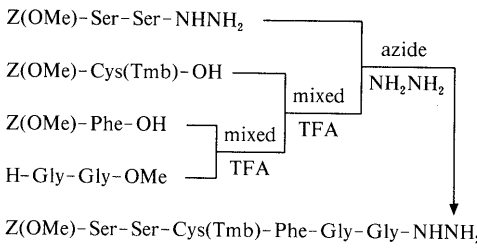

Fig. 6. Synthetic Scheme for the Protected Hexapeptide Hydrazide, Z(OMe)–(α-hANP 5—10)–$NHNH_2$ [5]

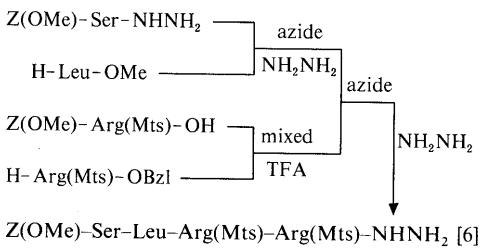

Fig. 7. Synthetic Scheme for the Protected Tetrapeptide Hydrazide, Z(OMe)–(α-hANP 1—4)–$NHNH_2$ [6]

then assembled successively by the azide procedure to minimize racemization.[16] Each condensation reaction was continued until the reaction mixture became negative to ninhydrin and the amount of the acyl component was increased from 1.5 to 3 eq as the chain elongation progressed. Each product was purified by simple precipitation from DMSO–DMF with MeOH. Throughout this synthesis, Phe was selected as the diagnostic amino acid in acid hydrolysis. By comparison of the recovery of Phe with those of newly incorporated amino acids after acid hydrolysis, satisfactory incorporation of each fragment was ascertained (Table I). The homogeneity of the protected α-hANP and every intermediate was further ascertained by TLC and elemental analysis.

It has been shown that HF deprotection in the presence of 2-mercaptopyridine[21] or dimethylsulfide,[22] or thioanisole-mediated deprotection with TFMSA[23] can partially reduce Met(O) to Met. However, thiol treatment is still required for the complete reduction of Met(O) and it takes rather long time. Recently, we have shown that selenide compounds in TFMSA/TFA can reduce Met(O) more effectively than sulfide compounds such as dimethylsulfide.[24] Thus, in order to eliminate a separate reduction step of Met(O), we examined the usefulness of several selenide compounds as well as sulfide compounds in a high concentration of HF as reducing reagents. As a model experiment, Met(O) was treated with HF in the presence of the selenide or sulfide compounds listed in Table II at 0 °C for 1 h. The regenerated methionine and the starting sulfoxide compound were detected by ninhydrin on TLC and the amount of each compound was determined quantitatively with a TLC-scanner at 570 nm. As shown in Table II, selenide compounds in HF reduced Met(O) to Met more effectively (59—100% conversion) than sulfide compounds (0—33% conversion). Among the selenide compounds examined, dimethylselenide (Me_2Se) in HF gave quantitative conversion of Met(O) to Met in combination with *m*-cresol or thiophenol. It seems likely that dimethyl selenoxide, generated during the conversion of Met(O) to Met, might be reduced back to dimethylselenide by thiophenol or scavenged by *m*-cresol, since thiophenol and *m*-cresol themselves have no reductive potency in HF.

From the experimental data described above, the HF–Me_2Se–*m*-cresol system was selected as the most effective reducing reagent of Met(O) and, at the same time, as the final deprotecting reagent in the present synthesis (Fig. 8). The protected α-hANP was treated with HF in the presence of Me_2Se and *m*-cresol in an ice-bath for 60 min. The deprotected peptide was dissolved in 0.2 N AcOH. The pH of the solution was adjusted to 8.0 with 5% NH_3 to reverse the possible N→O shift at three Ser residues[25] and then incubated with 2-mercaptoethanol. This thiol treatment seems to be effective to ensure the complete reduction of the possible disulfide aggregated peptide. After removal of the reducing reagent by gel-filtration of Sephadex G-25, the product was air-oxidized by the high dilution method at pH 7.5 to form the intramolecular disulfide bond. The progress of the reaction was monitored by using the Ellman test[26] and the value became constant after 120 h. The crude product obtained after lyophilization was purified by gel-filtration on Sephadex G-25 followed by preparative reverse-phase high performance liquid chromatography (HPLC) on a TSK-gel ODS-120T column using gradient elution with acetonitrile (20—40%) in 0.3% TFA (Fig. 9a). The purified peptide thus obtained exhibited a single peak on an analytical HPLC column (Nucleosil $5C_{18}$) (Fig. 9b) and a single spot on TLC in different solvent systems. Its purity was further confirmed by amino acid analysis after acid hydrolysis. In addition, synthetic α-hANP was proved

TABLE I. Amino Acid Ratios in 6 N HCl Hydrolysate of the Protected α-hANP and Its Intermediates

	Positions						
	21—28	17—28	13—28	11—28	5—28	1—28	
Asp	1.01	1.03	2.14	2.03	1.99	2.03	(2)
Ser	0.90	1.90	1.87	1.83	3.61	4.12	(5)
Glu		1.21	1.21	1.21	1.16	1.17	(1)
Gly	0.95	2.17	3.49	3.36	5.40	5.40	(5)
Ala		1.17	1.30	1.18	1.16	1.17	(1)
Cys	0.11	0.66	0.22	0.39	1.17	0.28	(1)
Met[a]				0.78	0.78	0.81	(1)
Ile			1.19	1.13	1.10	1.13	(1)
Leu	0.96	0.98	1.04	0.99	0.98	1.99	(2)
Tyr	0.99	0.97	0.91	0.93	0.84	0.90	(1)
Phe	1.00	1.00	1.00	1.00	2.00	2.00	(2)
Arg	1.03	1.03	2.19	2.97	2.96	4.96	(5)
Rec. (%)	78	97	81	90	85	73	

a) Met + Met(O). Numbers in parentheses are theoretical values.

TABLE II. Reductive Potency of Selenide or Sulfide Compounds in a High Concentration of HF

Additives	Equivalent[a]	Regenerated methionine (%)
m-Cresol	10	0
Thiophenol	10	0
Ph_2S	40	0
Thioanisole	40	15
Me_2S	10	0
Me_2S	40	33
Me_2S + EDT	10 : 10	0
Me_2S + thiophenol	10 : 10	8
Me_2S + *m*-cresol	40 : 10	33
Ph_2Se	40[b]	32
Selenoanisole	40[b]	100
Me_2Se	10	59
Me_2Se	40	87
Me_2Se + EDT	10 : 10	73
Me_2Se + thiophenol	10 : 10	100
Me_2Se + *m*-cresol	40 : 10	100

a) The reaction was continued for 1 h at 0 °C. *b*) The reaction mixture was colored red.

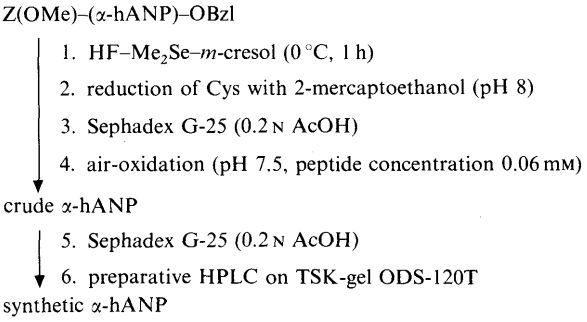

Fig. 8. Deprotection and Purification of α-hANP

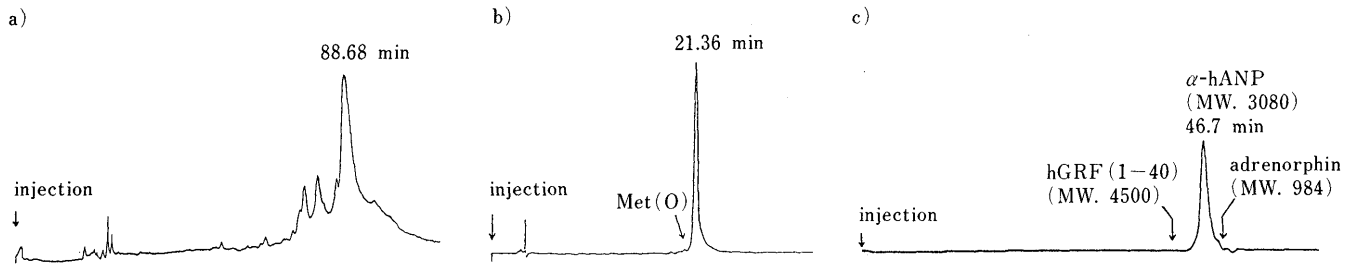

Fig. 9. HPLC of Synthetic α-hANP

a) Gel-filtered sample. *b*) HPLC-purified sample. *c*) Gel-permeation chromatography of purified sample.

to be a monomer by using gel-permeation HPLC (Fig. 9c).

When injected intravenously into the assay rats, our synthetic peptide elicited dose-dependent diuretic and natriuretic activity[3,4] with nearly the same magnitude as the literature value.[2]

In the present synthesis, the usefulness of the Tmb group as an *S*-protecting group and of the HF–Me_2Se–*m*-cresol system as a final deprotecting reagent as well as an effective reducing reagent of Met(O) was confirmed. These methodological improvements will be applied to the syntheses of other natriuretic peptides and their analogs in a subsequent paper.

Experimental

An azide was prepared according to Honzl and Rudinger[16] with isoamyl nitrite and a mixed anhydride was prepared according to Vaughan and Osato[14] with isobutyl chloroformate.

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 solvents. Procedure B: For purification of protected peptides less soluble in AcOEt, the crude product was triturated with ether and 5% citric acid. The resulting powder was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O and recrystallized or precipitated from appropriate solvents.

TLC of products obtained in this series was performed on silica gel (Kieselgel 60 F_{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–AcOH (9:1:0.5), Rf_3 $CHCl_3$–MeOH (10:0.5), Rf_4 *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2), Rf_5 $CHCl_3$, RF_6 *n*-BuOH–AcOH–pyridine–H_2O (30:20:6:24).

Analytical HPLC was conducted with a Hitachi 655A. Preparative HPLC and gel-permeation HPLC were conducted with Shimadzu LC-4A and LC-5A instruments, respectively.

Z(OMe)–Cys(Tmb)–OH H–Cys(Tmb)–OH[8] (5.0 g, 19.7 mmol) was dissolved in H_2O (25 ml) containing Et_3N (6.0 ml, 43.4 mmol), and Z(OMe)–N_3 (4.91 g, 23.7 mmol) in THF (25 ml) was added to the above ice-chilled solution. After being stirred at 5 °C overnight, the solution was washed with ether and the aqueous phase was neutralized with 5% citric acid. The resulting powder was recrystallized from DMF and ether; yield 4.59 g (56%), mp 153—158 °C, $[\alpha]_D^{20}$ −37.5° (*c*=0.6, DMF), Rf_1 0.56. *Anal.* Calcd for $C_{22}H_{27}NO_5S$: C, 63.29; H, 6.25; N, 3.36. Found: C, 63.49; H, 6.54; N, 3.41.

Z(OMe)–Cys(Tmb)(O)–OH A solution of Z(OMe)–Cys(Tmb)–OH (0.50 g, 1.20 mmol) in a mixture of AcOEt–H_2O (1:1, 20 ml) was stirred in the presence of $NaBO_3 \cdot 4H_2O$ (203 mg, 1.32 mmol) at room temperature for 24 h. The solution was acidified with citric acid. The separated AcOEt layer was washed with H_2O–NaCl, dried over Na_2SO_4 and concentrated. The residue was recrystallized from MeOH and ether; 0.31 g (61%), mp 146—148 °C, $[\alpha]_D^{20}$ −58.6° (*c*=0.4, MeOH), Rf_1 0.32. *Anal.* Calcd for $C_{22}H_{27}NO_6S \cdot 1/4H_2O$: C, 60.32; H, 6.32; N, 3.19. Found C, 60.37; H, 6.29; N, 3.23.

Sulfoxide Formation from Z(OMe)–Cys(Tmb)–OH and Z(OMe)–Cys(MBzl)–OH in DMF A solution of Z(OMe)–Cys(Tmb)–OH (21 mg, 0.05 mmol) in DMF (1 ml) was stirred vigorously at 25 °C for 4 d. An aliquot was spotted on a thin layer plate (5×10 cm), which was developed with $CHCl_3$–MeOH–H_2O (8:3:1, lower phase). Z(OMe)–Cys(Tmb)–OH (Rf_1 0.56) and its sulfoxide (Rf_1 0.32) were detected by HCl–ninhydrin, and the amount of each compound was measured by using a Shimadzu CS-910 chromatoscanner at 570 nm. The amount of Z(OMe)–Cys(Tmb)(O)–OH was 3%. For comparison, Z(OMe)–Cys(MBzl)–OH (20 mg, 0.05 mmol) in DMF (1 ml) was stirred as stated above. The amount of Z(OMe)–Cys(MBzl)(O)–OH[27] was 11%.

Z(OMe)–Arg(Mts)–Tyr–OBzl A mixed anhydride [prepared from 10.0 g (16.1 mmol) of Z(OMe)–Arg(Mts)–OH·CHA] in DMF (20 ml) was added to an ice-chilled solution of H–Tyr–OBzl [prepared from 8.25 g (19.3 mmol) of the tosylate] in DMF (50 ml) and the mixture was stirred in an ice-bath for 3 h. The solvent was removed by evaporation and the oily residue was dissolved in AcOEt. The organic phase was washed with 0.5 N HCl and H_2O–NaCl, dried over Na_2SO_4 and then evaporated. Trituration of the residue afforded a powder, which was purified by column chromatography on silica (4.5×25 cm) using $CHCl_3$–MeOH (10:0.5) as an eluant. The product was finally recrystallized from AcOEt and ether; yield 11.5 g (92%), mp 98—102 °C, $[\alpha]_D^{20}$ −3.2° (*c*=0.9, DMF), Rf_2 0.73, Rf_3 0.28. *Anal.* Calcd for $C_{40}H_{47}N_5O_9S$: C, 62.08; H, 6.12; N, 9.05. Found: C, 62.19; H, 6.16; N, 8.66.

Z(OMe)–Ser–Phe–Arg(Mts)–Tyr–OBzl Z(OMe)–Arg(Mts)–Tyr–OBzl (5.0 g, 6.46 mmol) was treated with TFA–anisole (10 ml–2.8 ml) in an ice-bath for 60 min, then TFA was removed by evaporation. The residue was washed with *n*-hexane, dried over KOH pellets *in vacuo* for 3 h and dissolved in DMF (20 ml) containing Et_3N (0.9 ml, 6.46 mmol). The azide [prepared from 3.34 g (7.75 mmol) of Z(OMe)–Ser–Phe–$NHNH_2$[15]] in DMF (10 ml) and Et_3N (1.29 ml, 9.30 mmol) were added to the above ice-chilled solution and the mixture, after being stirred at 5 °C for 14 h, was concentrated. The residue was purified by column chromatography on silica (3.5×20 cm) using $CHCl_3$–MeOH (20:0.5) as an eluant. The product was finally recrystallized from AcOEt and ether; yield 4.85 g (76%), mp 98—100 °C, $[\alpha]_D^{20}$ −5.1° (*c*=0.6, DMF), Rf_1 0.67. *Anal.* Calcd for $C_{52}H_{61}N_7O_{12}S$: C, 61.95; H, 6.10; N, 9.73. Found: C, 62.03; H, 6.08; N, 9.67.

Z(OMe)–Asn–Ser–Phe–Arg(Mts)–Tyr–OBzl The above protected tetrapeptide ester (4.50 g, 4.46 mmol) was treated with TFA–anisole (10 ml–2.4 ml) as stated above, and then dry ether was added. The resulting powder was washed with ether, dried over KOH pellets *in vacuo* for 3 h and dissolved in DMF (20 ml) together with Et_3N (0.62 ml, 4.46 mmol), Z(OMe)–Asn–ONp (2.05 g, 4.91 mmol) and NMM (0.49 ml, 4.46 mmol). After being stirred for 14 h, the solution was neutralized with AcOH and the solvent was removed by evaporation. Trituration of the residue with ether–H_2O afforded a powder, which was precipitated from DMF with EtOH; yield 3.95 g (79%), mp 171—173 °C, $[\alpha]_D^{20}$ −15.0° (*c*=0.6, DMF), Rf_1 0.63. *Anal.* Calcd for $C_{56}H_{67}N_9O_{14}S$: C, 59.93; H, 6.02; N, 11.23. Found: C, 59.79; H, 6.08; N, 11.14.

Z(OMe)–Cys(Tmb)–ONp Z(OMe)–Cys(Tmb)–OH (5.0 g, 12.0 mmol) and *p*-nitrophenol (1.83 g, 13.2 mmol) were dissolved in THF (50 ml). After addition of DCC (2.72 g, 13.2 mmol), the solution was stirred at room temperature overnight, and filtered. The filtrate was concentrated by evaporation and the product was purified by recrystallization from DMF and 2-propanol; yield 3.56 g (55%), mp 121 °C, $[\alpha]_D^{20}$ −25.5° (*c*=1.0, DMF), Rf_5 0.71. *Anal.* Calcd for $C_{28}H_{30}N_2O_7S$: C, 62.44; H, 5.61; N, 5.20. Found: C, 62.40; H, 5.61; N, 5.20.

Z(OMe)–Cys(Tmb)–Asn–Ser–Phe–Arg(Mts)–Tyr–OBzl Z(OMe)–Asn–Ser–Phe–Arg(Mts)–Tyr–OBzl (2.0 g, 1.78 mmol) was treated with TFA–anisole (4 ml–1 ml) as usual, then dry ether was added. The resulting powder was dried over KOH pellets *in vacuo* for 3 h, and dissolved in DMF (20 ml) together with Z(OMe)–Cys(Tmb)–ONp (1.15 g, 2.14 mmol) and Et_3N (0.23 ml, 1.78 mmol). After being stirred for 14 h, the solution was

neutralized with AcOH and the solvent was removed by evaporation. Trituration of the residue with H_2O afforded a powder, which was precipitated from DMF with EtOH; yield 2.01 g (83%), mp 200—203 °C, $[\alpha]_D^{20}$ −20.0° (c=0.8, DMF), Rf_1 0.51. *Anal.* Calcd for $C_{69}H_{84}N_{10}O_{15}S_2$: C, 61.04; H, 6.24; N, 10.32. Found: C, 60.85; H, 6.40; N, 10.30.

Z(OMe)–Leu–Gly–NHNH$_2$ Z(OMe)–Leu–Gly–OMe[28] (7.20 g, 19.7 mmol) was dissolved in MeOH (30 ml) and treated with 80% hydrazine hydrate (5.90 ml, 5 eq) for 24 h. The solvent was removed by evaporation. Trituration of the residue with H_2O and ether afforded a powder, which was precipitated from MeOH with ether; yield 5.97 g (83%), mp 108—112 °C, $[\alpha]_D^{20}$ −6.0° (c=0.7, DMF), Rf_1 0.57. *Anal.* Calcd for $C_{17}H_{26}N_4O_5$: C, 55.72; H, 7.15; N, 15.29. Found: C, 55.74; H, 7.15; N, 15.20.

Z(OMe)–Leu–Gly–Cys(Tmb)–Asn–Ser–Phe–Arg(Mts)–Tyr–OBzl, Z-(OMe)–(α-hANP 21—28)–OBzl [1] Z(OMe)–Cys(Tmb)–Asn–Ser–Phe–Arg(Mts)–Tyr–OBzl (2.01 g, 1.48 mmol) was treated with TFA–anisole (4 ml–1 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMF (5 ml) containing Et_3N (0.21 ml, 1.48 mmol). The azide [prepared from 0.65 g (1.78 mmol) of Z(OMe)–Leu–Gly–NHNH$_2$] in DMF (3 ml) and Et_3N (0.3 ml, 2.14 mmol) were added to the above ice-chilled solution and the mixture were stirred at 5 °C overnight. After evaporation of the solvent, the residue was treated with H_2O and the resulting powder was purified by precipitation from DMF with EtOH; yield 2.06 g (91%), mp 210—212 °C, $[\alpha]_D^{20}$ −11.4° (c=0.7, DMF), Rf_1 0.56. Amino acid ratios in 6 N HCl hydrolysate were listed in Table I. *Anal.* Calcd for $C_{77}H_{98}N_{12}O_{17}S_2$: C, 60.53; H, 6.47; N, 11.00. Found: C, 60.29; H, 6.69; N, 10.85.

Z(OMe)–Ser–Gly–OBzl The azide [prepared from 7.0 g (24.7 mmol) of Z(OMe)–Ser–NHNH$_2$] in DMF (10 ml) and Et_3N (4.11 ml, 29.6 mmol) were added to an ice-chilled solution of H–Gly–OBzl [prepared from 9.51 g (29.6 mmol) of the tosylate] in DMF (30 ml), and the mixture was stirred at 5 °C overnight. After evaporation of the solvent, the product was purified by procedure A, followed by precipitation from THF with ether; 6.89 g (67%), mp 95—97 °C, $[\alpha]_D^{20}$ +3.0° (c=0.7, DMF), Rf_1 0.89, Rf_3 0.33. *Anal.* Calcd for $C_{21}H_{24}N_2O_7$: C, 60.57; H, 5.81; N, 6.73. Found: C, 60.67; H, 5.78; N, 6.84.

Z(OMe)–Gln–Ser–Gly–OBzl Z(OMe)–Ser–Gly–OBzl (5.0 g, 12.0 mmol) was treated with TFA–anisole (10 ml–2.6 ml) and the N^{α}-deprotected peptide isolated as usual was dissolved in DMF (20 ml), together with Et_3N (3.34 ml, 24.0 mmol) and Z(OMe)–Gln–ONp (5.69 g, 13.2 mmol). After being stirred for 14 h, the solution was concentrated and the residue was purified by procedure B, followed by precipitation from DMF with MeOH; mp 198—200 °C, $[\alpha]_D^{20}$ +3.9° (c=0.5, DMF), Rf_1 0.12. *Anal.* Calcd for $C_{26}H_{32}N_4O_9$: C, 57.34; H, 5.92; N, 10.29. Found: C, 57.31; H, 5.98; N, 10.43.

Z(OMe)–Ala–Gln–Ser–Gly–OBzl The above tripeptide (3.0 g, 5.51 mmol) was treated with TFA–anisole (6.0 ml—1.8 ml) and the N^{α}-deprotected peptide was dissolved in DMF (30 ml) containing Et_3N (0.77 ml, 5.51 mmol). A mixed anhydride [prepared from 1.67 g (6.61 mmol) of Z(OMe)–Ala–OH] in DMF (20 ml) was added to the above ice-chilled solution and the mixture was stirred for 3 h. The solvent was removed by evaporation and the product was purified by procedure B, followed by precipitation from DMF with EtOH; yield 2.72 g (80%), mp 105—107 °C, $[\alpha]_D^{20}$ −3.6° (c=0.8, DMF), Rf_1 0.62. *Anal.* Calcd for $C_{29}H_{37}N_5O_{10}$: C, 56.57; H, 6.06; N, 11.38. Found: C, 56.71; H, 6.10; N, 11.58.

Z(OMe)–Ala–Gln–Ser–Gly–NHNH$_2$ [2] The above protected tetrapeptide ester (2.72 g, 4.42 mmol) in DMF (30 ml) was treated with 80% hydrazine hydrate (1.3 ml, 5 eq) for 24 h. The solvent was removed by evaporation and the resulting powder was purified by precipitation from DMSO–DMF (1 : 1) with EtOH; yield 2.29 g (96%), mp 216—218 °C, $[\alpha]_D^{20}$ −19.1° (c=0.7, DMSO), Rf_1 0.21. Amino acid ratios in 6 N HCl hydrolysate: Ser 0.90, Glu 1.02, Gly 1.00, Ala 1.02 (recovery of Gly 89%). *Anal.* Calcd for $C_{22}H_{33}N_7O_9$: C, 48.97; H, 6.17; N, 18.17. Found: C, 48.67; H, 6.38; N, 18.17.

Tcboc–NHNH$_2$ 2,2,2-Trichloro-1,1-dimethylethyl chloroformate (12.0 g, 50 mmol) in THF (50 ml) was added dropwise to an ice-chilled solution of 80% hydrazine hydrate (6.0 ml, 100 mmol) in THF (25 ml) at 0 °C. After being stirred for 2 h, the mixture was concentrated by evaporation. Trituration of the residue with 5% $NaHCO_3$ afforded a powder, which was purified by recrystallization from AcOEt and n-hexane; yield 8.27 g (70%), mp 123—125 °C, Rf_3 0.48. *Anal.* Calcd for $C_5H_9Cl_3N_2O_2$: C, 25.50; H, 3.85; N, 11.90. Found: C, 25.80; H, 3.77; N, 12.14.

Z(OMe)–Gly–NHNH–Tcboc Tcboc–NHNH$_2$ (9.89 g, 42.0 mmol) was added to the solution of a mixed anhydride [prepared from 10.0 g (42.0 mmol) of Z(OMe)–Gly–OH] in THF (200 ml) at 0 °C. After being stirred for 3 h, the solution was concentrated by evaporation. The product was purified by procedure A, followed by recrystallization from MeOH and n-hexane; yield 17.6 g (92%), mp 65—70 °C, Rf_1 0.67. *Anal.* Calcd for $C_{16}H_{20}Cl_3N_3O_6$: C, 42.07; H, 4.41; N, 9.20. Found: C, 42.18; H, 4.46; N, 9.03.

Z(OMe)–Arg(Mts)–Ile–NHNH$_2$ A mixed anhydride [prepared from 10.0 g (16.1 mmol) of Z(OMe)–Arg(Mts)–OH·CHA] was added to an ice-chilled solution of H–Ile–OBzl [prepared from 7.32 g (19.4 mmol) of the tosylate] in DMF (50 ml) and the mixture was stirred in an ice-bath for 3 h. The solvent was removed by evaporation and the product was purified by procedure A. The oily dipeptide ester thus obtained (Rf_3 0.32) was dissolved in MeOH (30.0 ml) and treated with 80% hydrazine hydrate (4.83 ml, 5 eq) for 24 h. The solvent was removed by evaporation. Trituration of the residue with H_2O–ether afforded a powder, which was precipitated from MeOH with ether; yield 7.24 g (70%), mp 140—143 °C, $[\alpha]_D^{20}$ +1.7° (c=0.6, DMF), Rf_2 0.46. *Anal.* Calcd for $C_{30}H_{45}N_7O_7S$: C, 55.62; H, 7.00; N, 15.14. Found: C, 55.52; H, 7.04; N, 14.99.

Z(OMe)–Arg(Mts)–Ile–Gly–NHNH–Tcboc Z(OMe)–Gly–NHNH–Tcboc (2.0 g, 4.38 mmol) was treated with TFA–anisole (4 ml–0.5 ml) as usual, and the N^{α}-deprotected peptide was dissolved in DMF (10 ml) containing Et_3N (0.61 ml, 4.38 mmol). The azide [prepared from 2.84 g (4.38 mmol) of Z(OMe)–Arg(Mts)–Ile–NHNH$_2$] in DMF (5 ml) and Et_3N (0.73 ml, 5.26 mmol) were added to the above ice-chilled solution and the mixture was stirred at 5 °C overnight. After evaporation of the solvent, the product was purified by procedure A, followed by column chromatography on silica (3 × 30 cm) using $CHCl_3$–MeOH (20 : 0.5) as an eluant. The product was finally recrystallized from AcOEt and ether; yield 1.94 g (49%), mp 140—142 °C, $[\alpha]_D^{20}$ +4.0° (c=0.5, DMF), Rf_1 0.65. *Anal.* Calcd for $C_{37}H_{53}Cl_3N_8O_{10}S$: C, 48.92; H, 5.88; N, 12.34. Found: C, 48.83; H, 5.81; N, 12.06.

Z(OMe)–Asp(OBzl)–Arg(Mts)–Ile–Gly–NHNH–Tcboc Z(OMe)–Arg(Mts)–Ile–Gly–NHNH–Tcboc (3.99 g, 4.39 mmol) was treated with TFA–anisole (8 ml–0.5 ml) and the N^{α}-deprotected peptide isolated as usual was dissolved in DMF (10 ml), together with Et_3N (0.61 ml, 4.39 mmol), Z(OMe)–Asp(OBzl)–ONp (2.33 g, 4.39 mmol) and NMM (0.48 ml, 4.39 mmol). After being stirred for 14 h, the solution was neutralized with AcOH and concentrated. Trituration of the residue with H_2O afforded a powder, which was precipitated from THF with ether; yield 2.94 g (60%), mp 137—139 °C, $[\alpha]_D^{20}$ −4.4° (c=0.9, DMF), Rf_1 0.72. *Anal.* Calcd for $C_{48}H_{64}Cl_3N_9O_{13}S$: C, 51.77; H, 5.79; N, 11.32. Found: C, 51.89; H, 5.75; N, 11.44.

Z(OMe)–Asp(OBzl)–Arg(Mts)–Ile–Gly–NHNH$_2$ [3] The above protected tetrapeptide (2.94 g, 2.64 mmol) dissolved in DMF–AcOH (10 ml–3 ml) was treated with Zn powder (1.7 g) at room temperature overnight. The solution was filtered, the filtrate was concentrated and 2% EDTA (30 ml) was added. The resulting powder was washed with H_2O, and then precipitated twice from DMF with EtOH; yield 1.74 g (73%), mp 136—138 °C, $[\alpha]_D^{20}$ −11.7° (c=0.6, DMF), Rf_1 0.74. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.01, Gly 1.00, Ile 0.95, Arg 1.05 (recovery of Gly 81%). *Anal.* Calcd for $C_{43}H_{59}N_9O_{11}S \cdot H_2O$: C, 55.65; H, 6.63; N, 13.58. Found: C, 55.92; H, 6.55; N, 13.44.

Z(OMe)–Met(O)–OBzl Z(OMe)–Met(O)–OH (5.0 g, 15.2 mmol) was dissolved in DMF (100 ml), together with DCHA (3.32 ml, 16.7 mmol). After addition of benzyl bromide (1.99 ml, 16.7 mmol), the solution was stirred overnight and the residue was purified by procedure A, followed by precipitation from MeOH with ether; yield 5.21 g (82%), mp 67—68 °C, $[\alpha]_D^{20}$ −17.8° (c=0.5, DMF), Rf_1 0.73. *Anal.* Calcd for $C_{21}H_{25}N_1O_6S$: C, 60.12; H, 6.01; N, 3.34. Found: C, 60.24; H, 6.08; N, 3.44.

Z(OMe)–Arg(Mts)–Met(O)–NHNH$_2$ [4] Z(OMe)–Met(O)–OBzl (4.68 g, 11.2 mmol) was treated with TFA–anisole (7 ml–1.2 ml), then TFA was removed by evaporation. The residue was washed with n-hexane, dried over KOH pellets *in vacuo* for 3 h and dissolved in DMF (20 ml) containing Et_3N (1.56 ml, 11.2 mmol). A mixed anhydride [prepared from 8.33 g (13.4 mmol) of Z(OMe)–Arg(Mts)–OH·CHA] in DMF (30 ml) was added to the above ice-chilled solution and the mixture was stirred for 3 h. The solvent was removed by evaporation and the product was purified by procedure A. The oily dipeptide ester thus obtained (Rf_3 0.21) was dissolved in MeOH (30 ml) and treated with 80% hydrazine hydrate (3.4 ml, 5 eq) for 24 h. The solvent was removed by evaporation and the residue was purified by column chromatography on silica (4.3 × 13 cm) using $CHCl_3$–MeOH (9 : 1) as an eluant, followed by precipitation from THF with ether to give an amorphous powder; yield 6.32 g (83%), mp 90—95 °C, $[\alpha]_D^{20}$ +1.7° (c=0.9, DMF), Rf_1 0.53. Amino acid ratios in 6 N HCl hydrolysate: Met 0.79, Arg 1.00 (recovery of Arg 79%). *Anal.* Calcd for $C_{29}H_{43}N_7O_8S_2$: C, 51.08; H, 6.36; N, 14.38. Found: C, 50.80; H, 6.43; N,

14.14.

Z(OMe)–Phe–Gly–Gly–OMe Z(OMe)–Gly–Gly–OMe[19] (5.0 g, 16.1 mmol) was treated with TFA–anisole (10 ml–1.7 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMF (20 ml) containing Et_3N (2.24 ml, 16.1 mmol). A mixed anhydride [prepared from 5.83 g (17.7 mmol) of Z(OMe)–Phe–OH] in DMF (50 ml) was added to the above ice-chilled solution and the mixture, after being stirred for 3 h, was concentrated. Trituration of the residue with 5% $NaHCO_3$–ether afforded a powder, which was purified by precipitation from MeOH with ether; yield 5.54 g (75%), mp 93—95 °C, $[\alpha]_D^{20}$ −17.9° (c=0.8, DMF), Rf_1 0.85, Rf_3 0.26. *Anal.* Calcd for $C_{23}H_{27}N_3O_7$: C, 60.38; H, 5.95; N, 9.19. Found: C, 60.43; H, 6.20; N, 9.17.

Z(OMe)–Cys(Tmb)–Phe–Gly–Gly–OMe The above protected tripeptide ester (2.0 g, 4.37 mmol) was treated with TFA–anisole (4 ml–1 ml) and the N^{α}-deprotected peptide was dissolved in DMF (20 ml) containing Et_3N (0.8 ml, 5.77 mmol). A mixed anhydride [prepared from 2.01 g (4.81 mmol) of Z(OMe)–Cys(Tmb)–OH] in DMF (10 ml) was added to the ice-chilled solution and the mixture, after being stirred for 3 h, was concentrated. Trituration of the residue with 5% citric acid afforded a powder, which was purified by precipitation from DMF with EtOH; yield 2.06 g (68%), mp 185—187 °C, $[\alpha]_D^{20}$ −25.7° (c=0.5, DMF), Rf_1 0.77. *Anal.* Calcd for $C_{36}H_{44}N_4O_8S$: C, 62.41; H, 6.40; N, 8.09. Found: C, 62.50; H, 6.61; N, 8.14.

Z(OMe)–Ser–Ser–Cys(Tmb)–Phe–Gly–Gly–OMe The above protected tetrapeptide ester (2.06 g, 2.97 mmol) was treated with TFA–anisole (5 ml–1 ml) and the N^{α}-deprotected peptide was dissolved in DMF (20 ml) containing Et_3N (0.41 ml, 2.97 mmol). The azide [prepared from 1.32 g (3.56 mmol) of Z(OMe)–Ser–Ser–$NHNH_2$[20]] in DMF (5 ml) and Et_3N (0.59 ml, 4.27 mmol) were added to the above ice-chilled solution and the mixture, after being stirred at 5 °C overnight, was concentrated. Trituration of the residue with 5% citric acid–ether afforded a powder, which was precipitated from DMF with 2-propanol; yield 1.52 g (59%), mp 185—187 °C, $[\alpha]_D^{20}$ −28.3° (c=0.6, DMF), Rf_1 0.58. *Anal.* Calcd for $C_{42}H_{54}N_6O_{12}S$: C, 58.18; H, 6.28; N, 9.69. Found: C, 58.05; H, 6.23; N, 9.86.

Z(OMe)–Ser–Ser–Cys(Tmb)–Phe–Gly–Gly–$NHNH_2$ [5] The above protected hexapeptide ester (1.52 g, 1.75 mmol) was dissolved in DMF–MeOH (1 : 1, 20 ml) and treated with 80% hydrazine hydrate (0.53 ml, 5 eq) for 24 h. The solvent was removed by evaporation, and the resulting powder was washed with H_2O, followed by precipitation from DMF with EtOH; yield 1.31 g (86%), mp 193—195 °C, $[\alpha]_D^{20}$ −30.0° (c=0.6, DMF), Rf_1 0.39. Amino acid ratios in 6 N HCl hydrolysate: Ser 1.44, Gly 2.00, Cys 0.45, Phe 0.98 (recovery of Gly 91%). *Anal.* Calcd for $C_{41}H_{54}N_8O_{11}S$: C, 56.80; H, 6.28; N, 12.93. Found: C, 56.61; H, 6.32; N, 12.89.

Z(OMe)–Arg(Mts)–Arg(Mts)–OBzl H–Arg(Mts)–OBzl [prepared by TFA–anisole (7 ml–1.5 ml) treatment of Z(OMe)–Arg(Mts)–OBzl[11] (4.32 g, 7.07 mmol)] was dissolved in DMF (10 ml) containing Et_3N (0.98 ml, 7.07 mmol). A mixed anhydride [prepared from 5.26 g (8.49 mmol) of Z(OMe)–Arg(Mts)–OH·CHA] in DMF (20 ml) was added to the above ice-chilled solution and the mixture, after being stirred for 3 h, was concentrated. The product was purified by procedure A, followed by column chromatography on silica (4.3 × 15 cm) using $CHCl_3$–MeOH (10 : 0.5) as an eluant. The oily product was finally precipitated from AcOEt with ether; yield 5.72 g (85%), mp 96—100 °C, $[\alpha]_D^{20}$ +8.3° (c=0.6, DMF), Rf_1 0.63. *Anal.* Calcd for $C_{46}N_{60}N_8O_{10}S_2$: C, 58.21; H, 6.37; N, 11.81. Found: C, 58.28; H, 6.39; N, 11.57.

Z(OMe)–Ser–Leu–$NHNH_2$ The azide [prepared from 8.72 g (30.08 mmol) of Z(OMe)–Ser–$NHNH_2$] in DMF (20 ml) and Et_3N (5.14 ml, 37.0 mmol) were added to an ice-chilled solution of H–Leu–OMe [prepared from 5.60 g (30.8 mmol) of the hydrochloride] in DMF (20 ml) and the mixture was stirred at 5 °C overnight. After evaporation of the solvent, the product was purified by procedure A. The resulting oily product was dissolved in MeOH–DMF (1 : 1, 50 ml) and treated with 80% hydrazine hydrate (9.2 ml, 5 eq) for 24 h. The solvent was removed by evaporation and the residue was washed with H_2O, followed by precipitation from DMF with EtOH; yield 5.35 g (44%), mp 217—218 °C, $[\alpha]_D^{20}$ +0.7° (c=1.4, DMF), Rf_1 0.58. *Anal.* Calcd for $C_{18}H_{28}N_4O_6$: C, 54.53; H, 7.12; N, 14.13. Found: C, 54.26; H, 7.05; N, 13.84.

Z(OMe)–Ser–Leu–Arg(Mts)–Arg(Mts)–OBzl Z(OMe)–Arg(Mts)–Arg(Mts)–OBzl (3.0 g, 3.16 mmol) was treated with TFA–anisole (6 ml–1 ml) and the N^{α}-deprotected peptide was dissolved in DMF (5.0 ml) containing Et_3N (0.44 ml, 3.16 mmol). The azide [prepared from 1.38 g (3.48 mmol) of Z(OMe)–Ser–Leu–$NHNH_2$] in DMF (5 ml) and Et_3N (0.58 ml, 4.18 mmol) were added to the above ice-chilled solution and the mixture, after being stirred at 5 °C overnight, was concentrated. The product was purified by procedure A, followed by column chromatography on silica (3 × 3.6 cm) using $CHCl_3$–MeOH (20 : 0.5) as an eluant. The purified product was finally precipitated from AcOEt with ether; yield 2.37 g (65%), mp 104—110 °C, $[\alpha]_D^{20}$ −11.8° (c=0.8, DMF), Rf_1 0.84. *Anal.* Calcd for $C_{55}H_{76}N_{10}O_{13}S_2$: C, 57.47; H, 6.67; N, 12.19. Found: C, 57.28; H, 6.62; N, 11.97.

Z(OMe)–Ser–Leu–Arg(Mts)–Arg(Mts)–$NHNH_2$ [6] The above protected tetrapeptide (2.37 g, 2.06 mmol) was dissolved in MeOH (20 ml) and treated with 80% hydrazine hydrate (0.62 ml, 5 eq) for 24 h. The solvent was removed by evaporation. Trituration of the residue with H_2O–ether afforded a solid, which was precipitated from MeOH with ether; yield 2.06 g (96%), mp 122—130 °C, $[\alpha]_D^{20}$ −7.1° (c=0.7, DMF), Rf_1 0.69. Amino acid ratios in 6 N HCl hydrolysate: Ser 0.92, Leu 1.00, Arg 2.08 (recovery of Leu 88%). *Anal.* Calcd for $C_{49}H_{72}N_{12}O_{12}S_2$: C, 53.71; H, 6.76; N, 15.66. Found: C, 53.56; H, 6.87; N, 15.38.

Z(OMe)–Ala–Gln–Ser–Gly–Leu–Gly–Cys(Tmb)–Asn–Ser–Phe–Arg(Mts)–Tyr–OBzl, Z(OMe)–(α-hANP 17—28)–OBzl Z(OMe)–(α-hANP 21—28)–OBzl (2.06 g, 1.35 mmol) was treated with TFA–anisole (4 ml–1 ml) in an ice-bath for 60 min as usual, then dry ether was added. The precipitate was dried over KOH pellets *in vacuo* for 3 h, then dissolved in DMF (10 ml) containing Et_3N (0.19 ml, 1.35 mmol). The azide [prepared from 1.09 g (2.03 mmol) of fragment [2]] in DMSO–DMF (1 : 1, 10 ml) and Et_3N (0.34 ml, 2.44 mmol) were added to the above ice-chilled solution and the mixture was stirred at 5 °C overnight. H_2O (50 ml) was added and the resulting powder was purified by precipitation from DMF with 90% MeOH; yield 2.37 g (94%), mp 242—244 °C, $[\alpha]_D^{20}$ −7.1° (c=0.6, DMSO), Rf_4 0.85. *Anal.* Calcd for $C_{90}H_{119}N_{17}O_{23}S_2$: C, 57.77; H, 6.41; N, 12.73. Found: C, 57.56; H, 6.48; N, 12.51.

Z(OMe)–Asp(OBzl)–Arg(Mts)–Ile–Gly–Ala–Gln–Ser–Gly–Leu–Gly–Cys(Tmb)–Asn–Ser–Phe–Arg(Mts)–Tyr–OBzl, Z(OMe)–(α-hANP 13—28)–OBzl The above protected dodecapeptide ester (1.0 g, 0.53 mmol) was treated with TFA–anisole (3 ml–0.5 ml) and the N^{α}-deprotected peptide obtained as described above was dissolved in DMSO–DMF (1 : 1, 10 ml) containing Et_3N (74 μl, 0.53 mmol). The azide [prepared from 0.63 g (0.69 mmol) of fragment [3]] in DMF (5 ml) and Et_3N (116 μl, 0.83 mmol) were added to the above ice-chilled solution and the mixture was stirred at 5 °C overnight. H_2O (50 ml) was added and the resulting powder was purified by precipitation from DMSO–DMF (2 : 1) with MeOH; yield 1.12 g (81%), mp 237—240 °C, $[\alpha]_D^{20}$ −16.0° (c=0.5, DMSO), Rf_4 0.83. Amino acid ratios in 6 N HCl hydrolysate were listed in Table I. *Anal.* Calcd for $C_{124}H_{166}N_{24}O_{31}S_3 \cdot 2H_2O$: C, 56.82; H, 6.54; N, 12.83. Found: C, 56.79; H, 6.45; N, 12.95.

Z(OMe)–Arg(Mts)–Met(O)–Asp(OBzl)–Arg(Mts)–Ile–Gly–Ala–Gln–Ser–Gly–Leu–Gly–Cys(Tmb)–Asn–Ser–Phe–Arg(Mts)–Tyr–OBzl, Z-(OMe)–(α–hANP 11—28)–OBzl The above protected hexadecapeptide ester (1.12 g, 0.43 mmol) was treated with TFA–anisole (3 ml–0.4 ml) and the N^{α}-deprotected peptide ester was dissolved in DMSO–DMF (1 : 1, 10 ml) containing Et_3N (60 μl, 0.43 mmol). The azide [prepared from 443 mg (0.65 mmol) of fragment [4]] in DMF (5 ml) and Et_3N (108 μl, 0.78 mmol) were added to the above ice-chilled solution and the mixture was stirred at 5 °C overnight. H_2O (50 ml) was added and the resulting powder was purified by precipitation from DMSO–DMF (1 : 2) with MeOH; yield 1.20 g (90%), mp 226—228 °C, $[\alpha]_D^{20}$ −15.9° (c=0.7, DMSO), Rf_4 0.67. *Anal.* Calcd for $C_{144}H_{197}N_{29}O_{36}S_5 \cdot 2H_2O$: C, 55.67; H, 6.52; N, 13.08. Found: C, 55.62; H, 6.65; N, 13.21.

Z(OMe)–Ser–Ser–Cys(Tmb)–Phe–Gly–Gly–Arg(Mts)–Met(O)–Asp(OBzl)–Arg(Mts)–Ile–Gly–Ala–Gln–Ser–Gly–Leu–Gly–Cys(Tmb)–Asn–Ser–Phe–Arg(Mts)–Tyr–OBzl, Z(OMe)–(α–hANP 5—28)–OBzl The above protected octadecapeptide ester (1.20 g, 0.39 mmol) was treated with TFA–anisole (3 ml–0.4 ml) and the N^{α}-deprotected peptide ester was dissolved in DMSO–DMF (1 : 1, 10 ml) containing Et_3N (54 μl, 0.39 mmol). The azide [prepared from 508 mg (0.59 mmol) of fragment [5]] in DMF (5 ml) and Et_3N (98 μl, 0.71 mmol) were added to the above ice-chilled solution and the mixture was stirred at 5 °C overnight. H_2O (50 ml) was added and the resulting powder was purified by precipitation from DMSO–DMF (2 : 1) with MeOH; yield 1.21 g (83%), mp 248—250 °C, $[\alpha]_D^{20}$ −13.6° (c=0.5, DMSO), Rf_4 0.78. *Anal.* Calcd for $C_{176}H_{239}N_{35}O_{44}S_6 \cdot H_2O$: C, 56.22; H, 6.46; N, 13.04. Found: C, 56.11; H, 6.51; N, 13.03.

Z(OMe)–Ser–Leu–Arg(Mts)–Arg(Mts)–Ser–Ser–Cys(Tmb)–Phe–Gly–Gly–Arg(Mts)–Met(O)–Asp(OBzl)–Arg(Mts)–Ile–Gly–Ala–Gln–Ser–Gly–Leu–Gly–Cys(Tmb)–Asn–Ser–Phe–Arg(Mts)–Tyr–OBzl, Protected α-hANP The above protected tetracosapeptide ester (600 mg, 0.16 mmol) was treated with TFA–anisole (3 ml–0.2 ml) and the N^{α}-deprotected peptide ester was dissolved in DMSO–DMF (1 : 1, 10 ml) containing Et_3N

(22 μl, 0.16 mmol). The azide [prepared from 343 mg (0.32 mmol) of fragment [6]] in DMF (3 ml) and Et_3N (53 μl, 0.38 mmol) were added to the above ice-chilled solution and the mixture was stirred at 5 °C overnight. H_2O (50 ml) was added and the resulting powder was purified by precipitation from DMSO–DMF (2 : 1) with MeOH; yield 694 mg (94%), mp 250—252 °C, $[\alpha]_D^{20}$ −16.0° (c = 0.1, DMSO), Rf_4 0.86. *Anal.* Calcd for $C_{215}H_{299}N_{45}O_{53}S_8 \cdot 3H_2O$: C, 55.26; H, 6.57; N, 13.49. Found: C, 55.12; H, 6.58; N, 13.12.

Reduction of Met(O) in a High Concentration of HF H–Met(O)–OH (5 mg, 0.03 mmol) was treated with HF (1 ml) in the presence of the additives listed in Table II in an ice-bath for 60 min. After evaporation of HF, TFA (2 ml) was added to the residue and an aliquot was spotted on thin layer plates (5 × 10 cm), which was developed with $CHCl_3$–MeOH–H_2O (8 : 3 : 1, lower phase). Methionine (Rf_1 0.28) and its sulfoxide (Rf_1 0.03) were detected by ninhydrin, and the amount of each compound was measured by using a Shimadzu CS-910 Chromatoscanner at 570 nm. The results are listed in Table II.

Synthetic α-hANP The protected α-hANP (200 mg) was treated with HF (10 ml) in the presence of Me_2Se (165 μl, 50 eq) and *m*-cresol (228 μl, 50 eq) in an ice-bath for 60 min. HF was removed by evaporation, dry ether was added and the precipitate was dried over KOH pellets *in vacuo* for 30 min. The deprotected peptide was dissolved in 0.2 N AcOH (2 ml). The solution, after being adjusted to pH 8 with 5% NH_3, was incubated with 2-mercaptoethanol (304 μl, 100 eq) under an argon atmosphere at 37 °C overnight. The above solution was applied to a column of Sephadex G-25 (1.6 × 140 cm), which was eluted with 0.2 N AcOH. The UV absorption at 280 nm was determined in each fraction (5.6 ml). The fractions corresponding to the front main peak (tube nos. 35—41) were combined and diluted with H_2O (500 ml). The pH of the solution was adjusted to 7.5 with 28% NH_3. The solution was kept standing at room temperature for 120 h, during which time the Ellman test value (absorption at 412 nm) dropped from 0.046 to 0.003. The entire solution was lyophilized and the residue was dissolved in 0.2 N AcOH (*ca.* 3 ml). This solution was applied to a column of Sephadex G-25 (1.6 × 140 cm) using 0.2 N AcOH as an eluant. Individual fractions (5.6 ml each) were collected and their absorption at 280 nm was determined. The fractions corresponding to the main peak (tube nos. 28—39) were combined and the solvent was removed by lyophilization to give a fluffy powder; yield 62 mg (46%).

The above gel-filtered sample (3 mg) was dissolved in 0.3% aqueous TFA containing 20% acetonitrile, and applied to a TSK-gel ODS-120T (21.5 × 300 nm) column, which was eluted with a linear gradient of acetonitrile (20—40%, 100 min) in 0.3% aqueous TFA at the flow rate of 5.0 ml/min. The eluate corresponding to the main peak (retention time 86.88 min, detected by UV absorption measurement) was collected (Fig. 9a). The rest of the sample (24 mg) was similarly purified and the combined eluates were evaporated at 25 °C *in vacuo* to remove acetonitrile. The resulting solution was then treated with Amberlite IRA-400 (acetate form, *ca.* 1 g) and lyophilized to give a white fluffy powder; yield 4.6 mg (17%) (overall yield calculated from the protected α-hANP, 8%). The purified peptide exhibited a single peak (retention time, 21.36 min) on a Nucleosil $5C_{18}$ (4.6 × 150 mm) column eluted with a linear gradient of acetonitrile (10—60%, 30 min) in 0.1% aqueous TFA at the flow rate of 0.7 ml/min, as shown in Fig. 9b. Synthetic α-hANP (MW, 3080) was eluted at 46.7 min on a gel-permeation HPLC column (TSK-gel G 2000SW, 7.5 × 600 mm), on which hGRF (MW, 4500) and adrenorphin (MW, 984), used as molecular weight standards, had retention times of 42.4 min and 47.8 min, respectively (Fig. 9c). $[\alpha]_D^{20}$ −78.2° (c = 0.1, 0.2 N AcOH), Rf_4 0.27, Rf_6 0.76. Amino acid ratios in 6 N HCl hydrolysate (numbers in parentheses are theoretical values): Asp 1.95 (2). Ser 4.14 (5), Glu 1.09 (1), Gly 4.88 (5), Ala 0.82 (1), Cys 0.65 (1), Met 0.82 (1), Ile 1.02 (1), Leu 2.02 (2), Tyr 0.92 (1), Phe 2.00 (2), Arg 4.99 (5) (recovery of Phe 79%). *Anal.* Calcd for $C_{127}H_{205}H_{45}O_{39}S_3 \cdot 6CH_3COOH \cdot 8H_2O$: C, 46.31; H, 6.91; N, 17.48. Found: C, 46.86; H, 6.61; N, 16.95

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References and Notes

1) Amino acid and peptide derivatives mentioned in this investigation are of the L-configuration. The following abbreviations are used: Z(OMe) = 4-methoxybenzyloxycarbonyl, Bzl = benzyl, Mts = mesitylene-2-sulfonyl, Tmb = 2,4,6-trimethylbenzyl, MBzl = 4-methoxybenzyl, DCC = *N*,*N*′-dicyclohexylcarbodiimide, Np = *p*-nitrophenyl, TFMSA = trifluoromethanesulfonic acid, TFA = trifluoroacetic acid, THF = tetrahydrofuran, DMF = *N*,*N*-dimethylformamide, DMSO = dimethyl sulfoxide, Tcboc = 2,2,2-trichloro-*tert*-butoxycarbonyl, NMM = *N*-methylmorpholine DCHA = dicyclohexylamine, EDTA = ethylenediaminetetraacetic acid.
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