Solution-Phase Synthesis of Porcine Brain Natriuretic Peptide (pBNP) Using *S*-Trimethylacetamidomethylcysteine[1)]

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The hexadodecapeptide corresponding to the entire amino acid sequence of porcine brain natriuretic peptide (pBNP) was synthesized by assembling four segments in solution, followed by HF deprotection and subsequent oxidation to establish an intramolecular disulfide bridge. The synthesis using the newly developed *S*-trimethylacetamidomethylcysteine [Cys(Tacm)] derivative gave a better yield than that using the *S*-2,4,6-trimethylbenzylcysteine [Cys(Tmb)] derivative. The chick rectum relaxant activity of the synthetic pBNP was 2.9 times more potent than that of α-rat atrial natriuretic peptide (α-rANP).

Keywords solution-phase peptide synthesis; porcine brain natriuretic peptide; *S*-trimethylacetamidomethylcysteine; iodine-oxidation; *S*-protected cysteine sulfoxide; chick rectum relaxant activity

In 1988, Sudoh *et al.*[2)] determined the structure of a new 26-residue peptide, porcine brain natriuretic peptide (pBNP) isolated from porcine brain. The structure of pBNP, with an intramolecular disulfide linkage, is remarkably similar to but definitely distinct from that of α-atrial natriuretic peptide (α-ANP) (Fig. 1).[3-5)] This peptide exhibited similar biological activities to those of α-ANPs, *i.e.*, regulation of the homeostatic balance of body fluid and blood pressure. Following our synthetic studies on α-ANPs,[6-9)] we have synthesized pBNP in order to obtain a sufficient amount to examine its biological relationship with α-ANPs. A part of this work has been reported preliminarily.[10)] Solid phase synthesis of this peptide has also been reported preliminarily by Yajima *et al.*[11)] In this paper, we wish to present a detailed account of our solution-phase synthesis of the 26-residue peptide corresponding to the entire amino acid sequence of pBNP.

In the present synthesis, the newly developed *S*-trimethylacetamidomethyl (Tacm) group[10,12)] was employed as a cysteine S-protecting group to examine its usefulness in practical peptide synthesis. As described in the preceding paper,[10,12)] this *S*-Tacm group was stable under acidic conditions and removable with iodine. These properties are similar to those of the *S*-Acm group, but Cys(Tacm) was less susceptible to air-oxidation than Cys(Acm).[13)] Alternatively, the synthesis of pBNP using *S*-2,4,6-trimethylbenzylcysteine [Cys(Tmb)][6-9,14)] was carried out, and the yield and purity of each synthetic peptide were compared.

Synthesis of Each Peptide Segment In combination with the TFA-labile Boc group for N^{α}-protection, amino acid derivatives bearing protecting groups removable with HF[15)] were employed, *i.e.*, Asp(OcHex),[16)] Ser(Bzl), Arg(Tos), and Tyr(BrZ),[17)] except for the Cys-derivatives, mentioned above. Of these, Asp(OcHex) was employed to suppress the base-catalyzed succinimide formation, since the Asp–Ser sequence is very susceptible to this side reaction.[18)] The whole sequence was divided into four segments at Gly residue (Fig. 2) to avoid racemization during the coupling reaction using 1-ethyl-3-(3′-dimethylaminopropyl)-carbodiimide(water-soluble carbodiimide, WSC)[19)] plus 1-hydroxybenzotriazole (HOBt).[20)] Of these segments, segments [2]—[4] were synthesized by the use of phenacyl (Pac) ester[21)] at the C-terminus. Prior to condensation of each segment, removal of the Pac group was conducted with Zn–anthranilic acid in a mixture of DMF–pyridine.[22)]

The C-terminal segment [1], Boc–Cys(Tacm)–Asn–Val–Leu–Arg(Tos)–Arg(Tos)–Tyr(BrZ)–OBzl, was prepared in a stepwise manner according to the scheme illustrated in Fig. 3. Starting with a TFA-treated sample of Boc–

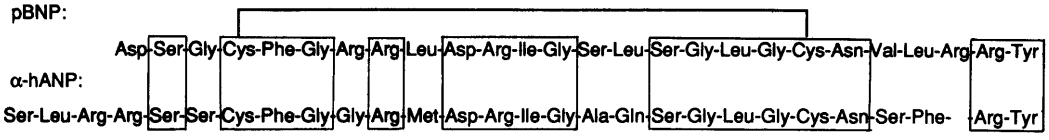

Fig. 1. Structures of pBNP and α-Human ANP

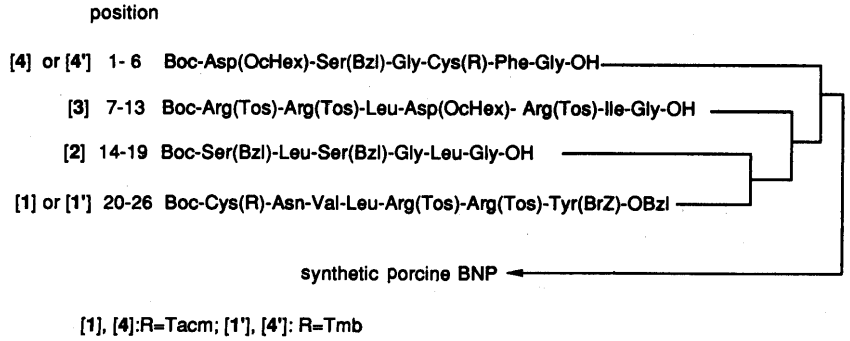

Fig. 2. Synthetic Route to pBNP

Tyr(BrZ)–OBzl, two Boc–Arg(Tos)–OH and Boc–Leu–OH were coupled by the mixed anhydride (MA) procedure,[23] Boc–Val–OH was introduced by the Su active ester procedure,[24] Boc–Asn–OH by the Np active ester procedure,[25] and Boc–Cys(Tacm)–OH by the WSC plus HOBt procedure to give the segment [1]. The purity of the protected heptapeptide ester thus obtained was ascertained by thin-layer chromatography (TLC), elemental analysis, and amino acid analysis after 6 N HCl hydrolysis, as was done with other segments.

Segment [2], Boc–Ser(Bzl)–Leu–Ser(Bzl)–Gly–Leu–Gly–OH, was synthesized according to the scheme illustrated in Fig. 4. The three necessary dipeptide units, Boc–Ser(Bzl)–Leu–OBzl, Boc–Ser(Bzl)–Gly–OH and Boc–Leu–Gly–OH were each prepared by the Su active ester procedure. Of these, Boc–Ser(Bzl)–Gly–OH was characterized as the CHA salt. Boc–Leu–Gly–OH was reacted with phenacyl bromide *via* the Cs salt to obtain the corresponding Pac ester[26] and then the ester, after TFA-treatment, was condensed with Boc–Ser(Bzl)–Gly–OH by the NB active ester procedure.[27] The resulting tetrapeptide derivative was coupled with Boc–Ser(Bzl)–Leu–$NHNH_2$, prepared by hydrazinolysis of Boc–Ser(Bzl)–Leu–OBzl, by the azide[28] procedure and treated with Zn–anthranilic acid in a mixture of DMF–pyridine to give [2].

Segment [3], Boc–Arg(Tos)–Arg(Tos)–Leu–Asp(OcHex)–Arg(Tos)–Ile–Gly–OH, was synthesized in a stepwise manner starting with Boc–Ile–Gly–OPac, which was

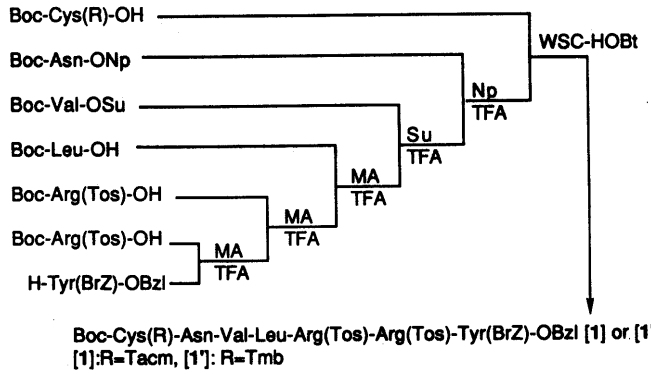

Fig. 3. Synthetic Scheme for the Protected Heptapeptide Ester, Segment [1] or [1']

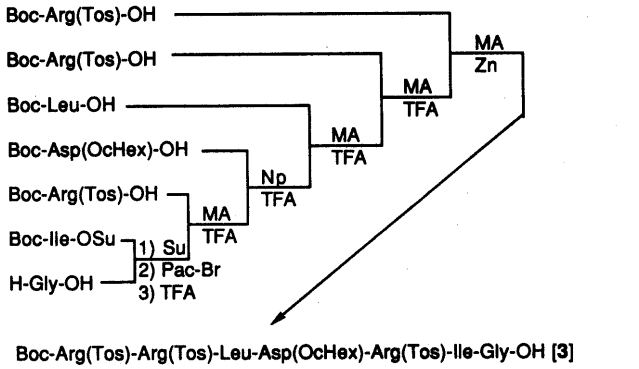

Fig. 5. Synthetic Scheme for the Protected Heptapeptide Carboxylic Acid, Segment [3]

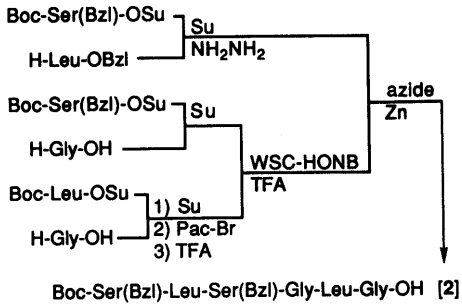

Fig. 4. Synthetic Scheme for the Protected Hexapeptide Carboxylic Acid, Segment [2]

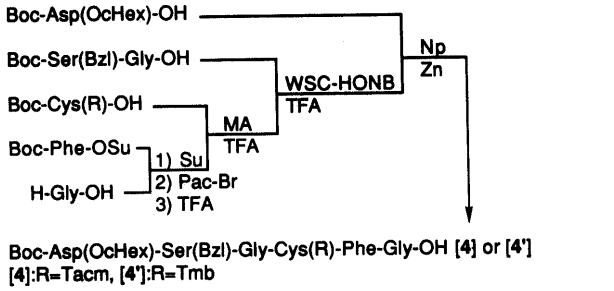

Fig. 6. Synthetic Scheme for the Protected Hexapeptide Carboxylic Acid, Segment [4] or [4']

TABLE I. Amino Acid Ratios in 6 N HCl Hydrolysates of Synthetic pBNP and Its Intermediates

	Protected intermediates								Synthetic pBNP	
	20—26		14—26		7—26		1—26			
	Tacm[a]	Tmb[b]	Tacm	Tmb	Tacm	Tmb	Tacm	Tmb	Tacm	Tmb
Asp	0.96	0.95	0.96	0.96	2.01	2.08	2.99	2.91	3.06	2.98 (3)[d]
Ser			1.64	1.74	1.67	1.63	2.67	2.53	2.86	2.64 (3)
Gly			1.99	1.97	3.09	3.10	5.17	4.97	4.95	5.01 (5)
Val	0.92	0.96	1.01	0.99	1.00	0.92	0.95	0.90	0.96	0.91 (1)
Cys	0.27	0.35	0.25	0.31	0.19	0.30	0.39	0.59	0.73	0.29 (1)
Ile					1.04	1.19	1.02	1.14	0.98	1.05 (1)
Leu[c]	1.00	1.00	3.00	3.00	4.00	4.00	4.00	4.00	4.00	4.00 (4)
Tyr	1.03	1.08	1.02	1.00	0.97	0.85	0.96	0.87	0.95	0.95 (1)
Phe							0.92	0.99	0.98	0.97 (1)
Arg	1.97	2.16	2.00	1.99	5.13	5.46	4.94	5.14	5.08	5.04 (5)
Recovery (%)	73	82	91	84	85	83	91	71	90	80

a) Synthesized using Cys(Tacm). *b*) Synthesized using Cys(Tmb). *c*) Diagnostic amino acid. *d*) Numbers in parentheses are theoretical values.

prepared by the Su condensation of Boc–Ile–OH and H–Gly–OH followed by the reaction with phenacyl bromide *via* the Cs salt. The mixed anhydride and Np active ester procedure were employed to introduce the respective amino acid residues. From the resulting protected heptapeptide ester, the Pac group was easily removed by Zn treatment as described above to give [3] (Fig. 5).

The N-terminal segment [4], Boc–Asp(OcHex)–Ser(Bzl)–Gly–Cys(Tacm)–Phe–Gly–OH was prepared as shown in Fig. 6. Boc–Phe–Gly–OPac was synthesized by the Su condensation of Boc–Phe–OH with H–Gly–OH and subsequent esterification with phenacyl bromide *via* the Cs salt. This, after TFA treatment, was coupled with Boc–Cys(Tacm)–OH by the mixed anhydride procedure. A TFA-treated sample of this tripeptide derivative was condensed with Boc–Ser(Bzl)–Gly–OH by the NB ester procedure, and then with Boc–Asp(OcHex)–OH *via* the Np active ester. The Pac group of the hexapeptide derivative was cleaved with Zn to give [4].

Construction of Protected pBNP The protected peptide chain was constructed by the assembly of segments [1]—[4] (Fig. 2). Every segment condensation reaction using WSC–HOBt proceeded smoothly without encountering solubility problems, and a slight excess (1.2 eq) amount of each acyl component was employed. The protected pBNP and its intermediates were purified by simple precipitation from DMF with EtOH. Throughout this synthesis, Leu was used as a diagnostic amino acid in amino acid analyses. After each segment condensation, the recovery of Leu was compared with those of newly added amino acids to confirm satisfactory incorporation (Table I).

Deprotection by HF and Characterization of [Cys-(Tacm)^{4,20}]-pBNP Deprotection and subsequent purification of the protected pBNP prepared using Cys(Tacm) were conducted according to the scheme illustrated in Fig. 7. The fully protected pBNP was treated with HF in the presence of *m*-cresol and dimethyl sulfide[29] in an ice-bath for 60 min to remove all protecting groups except for two Tacm groups. The di-Tacm peptide was dissolved in H_2O and the pH of the solution was adjusted to 8 with 5% NH_4OH to reverse any possible N→O shift at three Ser residues.[30] The solution was desalted by gel-filtration on Sephadex G-25 using 1 N AcOH as an eluant. The product showed a fairly complex elution pattern on high performance liquid chromatography (HPLC), and thus further purification was conducted by ion-exchange chromatography on CM-cellulose using gradient elution with 0.01—

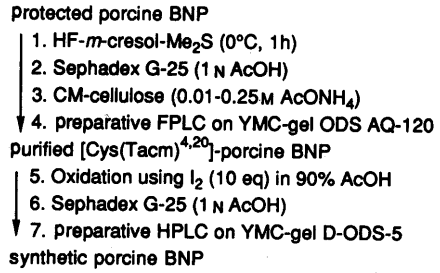

Fig. 7. Deprotection and Purification of pBNP

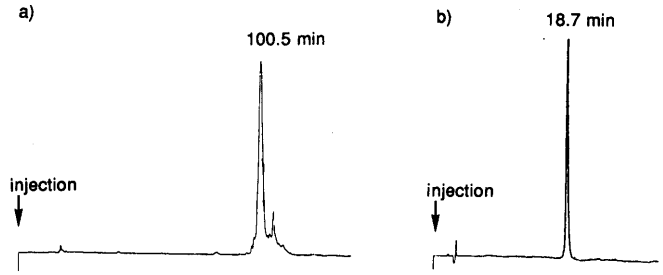

Fig. 8. HPLC Profile of Synthetic pBNP
a) Gel-filtered sample. b) HPLC-purified sample.

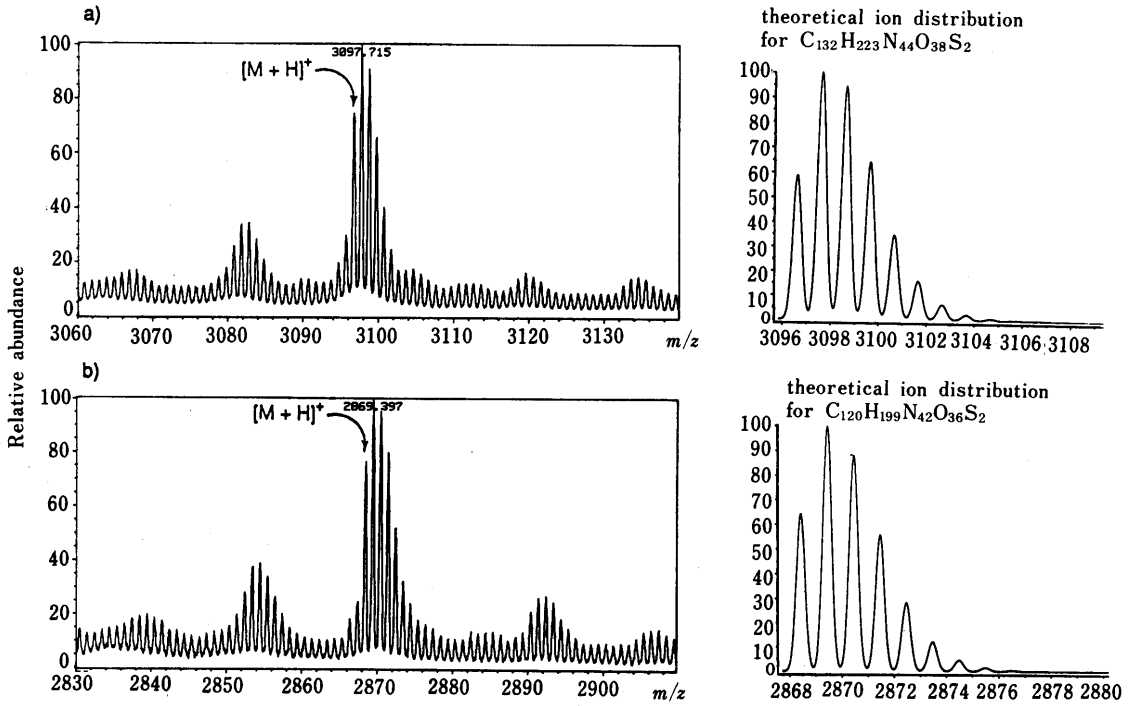

Fig. 9. FAB-MS of Synthetic pBNP
a) [Cys(Tacm)^{4,20}]-pBNP. b) Synthetic pBNP.

0.25 M $AcONH_4$, followed by fast protein liquid chromatography (FPLC) on a YMC-ODS-AQ 120 (S50) column. The purified peptide thus obtained exhibited a single peak on analytical HPLC. The purity of the di-Tacm peptide was further ascertained by amino acid analysis after aminopeptidase-M (AP-M) digestion. Quantitative recovery of Asp residues (1.95 for 2 residues) was obtained in this enzymatic digestion, which indicated that the purified di-Tacm peptide contains no aspartimide peptide. The $[MH]^+$ ion peak and the base peak of di-Tacm-pBNP in the fast atom bombardment-mass spectrum (FAB-MS) were observed at m/z: 3096.7 and m/z: 3097.7, respectively, in the molecular ion region [theoretical values: 3096.631 $(MH)^+$ and 3097.634 (base peak)]. The mass distribution of di-Tacm-pBNP in the molecular ion region was identical with the theoretical isotopic mass distribution (Fig. 9a).

Disulfide Bond Formation and Characterization of Synthetic pBNP Removal of the Tacm groups and formation of the intramolecular disulfide linkage were performed by the use of iodine in a dilute solution of the peptide (0.05 mM) in 90% AcOH.[31] After being stirred for 1 h at 25 °C, the product was gel-filtered on Sephadex G-25. The crude oxidized peptide was further purified by preparative HPLC on a YMC-D-ODS-5 column. The purified peptide thus obtained exhibited a single peak on an analytical HPLC column (Cosmosil $5C_{18}$) (Fig. 8b) and a single spot on TLC in different solvent systems. Its acid hydrolysate gave the amino acid ratios predicted by theory. The synthetic pBNP was proved to be a monomer by using gel-permeation HPLC on TSK-gel G2000-SW and FAB-MS. Synthetic pBNP gave the mass value $[MH]^+$ of 2868.4 (theoretical value: 2868.447) and a base peak of 2869.4 (theoretical value: 2869.449) in the molecular region on FAB-MS (Fig. 9b). The mass distribution of synthetic pBNP in the molecular region was identical with the theoretical isotopic mass distribution. These data confirmed that the synthetic peptide had the expected structure of pBNP.

Our synthetic pBNP showed 2.9 times more potent chick rectum relaxant activity than synthetic α-rat (r) ANP, in reasonable agreement with literature values.[2]

Alternative Synthesis of pBNP Using Cys(Tmb) Segments [1′] and [4′] were prepared using Cys(Tmb) instead of Cys(Tacm). Construction of the peptide chain was conducted in essentially the same manner as described for the synthesis using Cys(Tacm). Deprotection of the protected pBNP obtained here was carried out in the same manner as described above using HF–*m*-cresol–dimethyl sulfide. The deprotected peptide was dissolved in H_2O and oxidized with $K_3[Fe(CN)_6]$ (10 eq) to form the disulfide bridge by a high-dilution method. The crude oxidized peptide was purified with the same procedure as described in the former experiment. The purified peptide exhibited a single peak on analytical HPLC (Cosmosil $5C_{18}$) and possessed physicochemical properties identical with those of the pBNP synthesized using Cys(Tacm). However, the yield obtained here (6.8%, from the protected peptide) was about half of that in the experiment using Cys(Tacm) (12.0%).

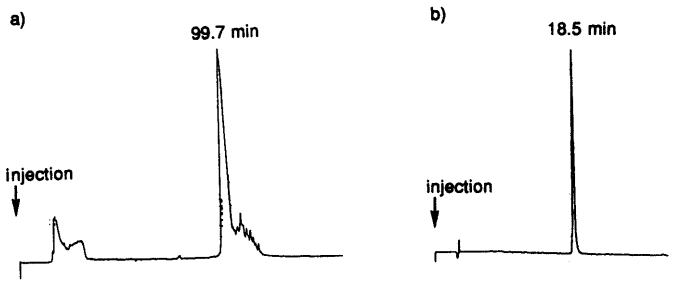

Fig. 10. HPLC Profile of Synthetic pBNP Using Cys(Tmb)
a) CM-purified sample. b) HPLC-purified sample.

Conclusion

We have synthesized biologically active pBNP by a solution-phase method using the new *S*-Tacm group. The synthetic pBNP was obtained in highly pure form and was identical with pBNP synthesized using the *S*-Tmb group. These results show the usefulness of the *S*-Tacm group in peptide synthesis.

Experimental

General experimental procedures employed in this investigation were essentially the same as those described in connection with the syntheses of α-human ANP[6] and α-rat ANP.[7]

Prior to the coupling reaction, the N^{α}-protecting group, Boc, 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 and active ester condensations were performed at room temperature. The azide condensation was performed at 4 °C and the MA condensation was performed using isobutyl chloroformate at 0 °C for 3 h.

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–5% citric acid. The resulting powder was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, and recrystallized or reprecipitated from appropriate solvents.

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), Rf_4 *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2), Rf_5 *n*-BuOH–AcOH–pyridine–H_2O (30:20:6:24).

AP-M (lot. No 2513445) was purchased from Merck. Analytical HPLC was conducted with a Hitachi 655A instrument. Preparative FPLC and HPLC were conducted with a Pharmacia FPLC system and a Shimadzu LC-4A, respectively. 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).

Boc–Tyr(BrZ)–OBzl Boc–Tyr(BrZ)–OH (4.00 g, 8.09 mmol) was esterified with benzyl bromide (1.06 ml, 8.98 mmol) and DCHA (1.93 ml, 9.71 mmol). After being stirred overnight at room temperature, the mixture was concentrated. The product was purified by procedure A, to give an oily residue: yield 4.20 g (89%), Rf_2 0.94, $[\alpha]_D^{19}$ −10.5° (c=0.4, DMF), FAB-MS m/z: 586 $[MH]^+$.

Boc–Arg(Tos)–Tyr(BrZ)–OBzl A mixed anhydride [prepared from 3.64 g (8.50 mmol) of Boc–Arg(Tos)–OH] in DMF (10 ml) was added to a TFA-treated sample of Boc–Tyr(BrZ)–OBzl (7.08 mmol) in DMF (30 ml) containing Et_3N (0.98 ml, 7.08 mmol) and the mixture, after being stirred for 3 h, was concentrated. The product was purified by procedure A, followed by column chromatography on silica using $CHCl_3$–MeOH (20:0.5) as an eluant. The product was triturated with *n*-hexane to give a powder: yield 4.65 g (69%), Rf_3 0.33, mp 69—70 °C, $[\alpha]_D^{19}$ −11.9° (c=0.5, DMF). *Anal.* Calcd for $C_{42}H_{48}BrN_5O_{10}S$: C, 56.37; H, 5.41; N, 7.83. Found: C, 56.09; H, 5.46; N, 7.87.

Boc–Arg(Tos)–Arg(Tos)–Tyr(BrZ)–OBzl A mixed anhydride [prepared from 1.11 g (2.60 mmol) of Boc–Arg(Tos)–OH] in DMF (10 ml) was added to an ice-chilled solution of a TFA-treated sample of the above dipeptide ester (1.94 g, 2.17 mmol) in DMF (10 ml) containing Et_3N (0.30 ml, 2.17 mmol). The mixture was stirred for 3 h and the solvent was removed by evaporation. The product was purified by procedure A,

followed by column chromatography on silica using $CHCl_3$–MeOH (30:0.5) and trituration with *n*-hexane: yield 1.26 g (50%), Rf_1 0.51, mp 95—96 °C, $[\alpha]_D^{19}$ −16.1° (c=0.4, DMF). *Anal.* Calcd for $C_{55}H_{66}BrN_9O_{13}S_2$: C, 54.81; H, 5.52; N, 10.46. Found: C, 54.53; H, 5.60; N, 10.47.

Boc–Leu–Arg(Tos)–Arg(Tos)–Tyr(BrZ)–OBzl A mixed anhydride [prepared from 0.29 g (1.14 mmol) of Boc–Leu–OH] in DMF (5 ml) was added to an ice-chilled solution of a TFA-treated sample of the above tripeptide ester (1.15 g, 0.95 mmol) in DMF (10 ml) containing Et_3N (0.13 ml, 0.95 mmol). The mixture was stirred for 3 h, and concentrated. The product was purified by procedure A and recrystallized from MeOH with ether: yield 1.14 g (91%), Rf_2 0.56, mp 98—99 °C, $[\alpha]_D^{17}$ −25.6° (c=0.4, DMF). *Anal.* Calcd for $C_{61}H_{77}BrN_{10}O_{14}S_2$: C, 54.82; H, 5.89; N, 10.63. Found: C, 54.91; H, 5.96; N, 10.48.

Boc–Val–Leu–Arg(Tos)–Arg(Tos)–Tyr(BrZ)–OBzl A mixture of Boc–Val–OSu (1.48 g, 4.72 mmol), Et_3N (0.66 ml, 4.72 mmol) and a TFA-treated sample of the above tetrapeptide ester (5.18 g, 3.93 mmol) in DMF (40 ml) was stirred overnight. The solvent was removed by evaporation and the product was purified by procedure A, followed by recrystallization from THF with ether: yield 5.15 g (92%), mp 105—107 °C, $[\alpha]_D^{17}$ −18.6° (c=0.5, DMF), Rf_2 0.29, Rf_3 0.57. *Anal.* Calcd for $C_{66}H_{86}BrN_{11}O_{15}S_2$: C, 55.92; H, 6.12; N, 10.87. Found: C, 55.63; H, 6.08; N, 11.01.

Boc–Asn–Val–Leu–Arg(Tos)–Arg(Tos)–Tyr(BrZ)–OBzl A mixture of Boc–Asn–ONp (1.49 g, 4.72 mmol), Et_3N (1.70 ml, 5.07 mmol) and a TFA-treated sample of the above pentapeptide ester (4.99 g, 3.52 mmol) was stirred overnight. The mixture was neutralized with AcOH and then concentrated *in vacuo*. The product was purified by procedure B, followed by recrystallization from THF with ether: yield 4.89 g (91%), mp 111—113 °C, $[\alpha]_D^{19}$ −14.6° (c=0.4, DMF), Rf_1 0.90, Rf_2 0.56. *Anal.* Calcd for $C_{70}H_{92}BrN_{13}O_{17}S_2$: C, 54.89; H, 6.05; N, 11.89. Found: C, 54.67; H, 6.05; N, 12.00.

Boc–Cys(Tacm)–Asn–Val–Leu–Arg(Tos)–Arg(Tos)–Tyr(BrZ)–OBzl, Boc–(pBNP 20—26)–OBzl [1] Boc–Cys(Tacm)–OH [prepared from 1.60 g (3.68 mmol) of the CHA salt], HOBt (0.62 g, 4.05 mmol) and WSC·HCl (0.85 g, 4.42 mmol) were added to a TFA-treated sample of the above hexapeptide ester (4.70 g, 3.07 mmol) in DMF (40 ml) containing Et_3N (0.43 ml, 3.07 mmol) in an ice-bath. The mixture, after being stirred overnight, was concentrated. The product was purified by procedure A and recrystallized from THF with ether: yield 4.78 g (89%), mp 120—123 °C, $[\alpha]_D^{19}$ −14.3° (c=0.5, DMF), Rf_1 0.78. Amino acid ratios in a 6 N HCl hydrolysate are listed in Table I. *Anal.* Calcd for $C_{79}H_{108}BrN_{15}O_{19}S_3$: C, 54.28; H, 6.23; N, 12.20. Found: C, 54.07; H, 6.08; N, 11.99.

Boc–Leu–Gly–OPac A solution of Boc–Leu–OSu (6.57 g, 20.0 mmol) and Et_3N (2.78 ml, 24.9 mmol) in DMF (20 ml) was added to a solution of H–Gly–OH (4.44 g, 60.0 mmol) in H_2O (5 ml) containing Et_3N (8.34 ml, 60.0 mmol) and the mixture was stirred overnight. The solvent was removed by evaporation and the residue was dissolved in AcOEt (50 ml). The organic phase was washed with 5% citric acid and NaCl–H_2O, dried over Na_2SO_4, and concentrated to give an oily product. This product and Cs_2CO_3 (3.26 g, 10.0 mmol) were dissolved in MeOH–H_2O (9:1, 20 ml) and concentrated *in vacuo*. The residue was dissolved in DMF (20 ml) and evaporation was repeated three times. The resulting Cs salt was dissolved in DMF (30 ml) and phenacyl bromide (4.38 g, 22.0 mmol) was added. After being stirred for 30 min, the mixture was filtered and the filtrate was evaporated. The product was purified by procedure A, followed by recrystallization from MeOH with ether: yield 5.93 g (73%), mp 106—108 °C, $[\alpha]_D^{23}$ −25.0° (c=0.5, DMF), Rf_2 0.67. *Anal.* Calcd for $C_{21}H_{30}N_2O_6$: C, 62.05; H, 7.44; N, 6.89. Found: C, 61.85; H, 7.46; N, 6.89.

Boc–Ser(Bzl)–Gly–OH·CHA A solution of Boc–Ser(Bzl)–OSu (6.63 g, 16.9 mmol) and Et_3N (2.35 ml, 16.9 mmol) in DMF (30 ml) was added to a solution of H–Gly–OH (3.81 g, 50.7 mmol) in H_2O (10 ml) containing Et_3N (7.05 ml, 50.7 mmol). After being stirred overnight, the solvent was removed by evaporation and the oily residue was dissolved in AcOEt (70 ml). The organic phase was washed with 5% citric acid and NaCl–H_2O, dried over Na_2SO_4 and concentrated *in vacuo* to give an oily product. This product was converted to the CHA salt as usual and recrystallized from MeOH with ether: yield 6.70 g (82%), mp 144—146 °C, $[\alpha]_D^{19}$ −2.0° (c=0.5, DMF), Rf_1 0.62. *Anal.* Calcd for $C_{17}H_{23}N_2O_6 \cdot C_6H_{13}N$: C, 61.17; H, 8.26; N, 9.31. Found: C, 61.40; H, 8.37; N, 9.22.

Boc–Ser(Bzl)–Gly–Leu–Gly–OPac A solution of Boc–Ser(Bzl)–Gly–ONB [prepared from Boc–Ser(Bzl)–Gly–OH·CHA (3.00 g, 6.64 mmol) HONB (2.65 g, 7.31 mmol) and WSC·HCl (1.53 g, 7.97 mmol)] in THF (50 ml) was added to a TFA-treated sample of the Boc–Leu–Gly–OPac (2.25 g, 5.54 mmol) in DMF (20 ml) together with Et_3N (1.11 ml, 7.97 mmol). The mixture was stirred overnight. After removal of the solvent by evaporation, the product was purified by procedure A and recrystallized from THF with ether: yield 2.44 g (58%), mp 75—77 °C, $[\alpha]_D^{23}$ −10.7° (c=0.6, DMF), Rf_2 0.65. *Anal.* Calcd for $C_{33}H_{44}N_4O_9$: C, 61.86; H, 6.92; N, 8.57. Found: C, 61.67: H, 6.96; N, 8.69.

Boc–Ser(Bzl)–Leu–NHNH$_2$ A mixed anhydride [prepared from 24.4 g, (82.7 mmol) of Boc–Ser(Bzl)–OH] in DMF (30 ml) was added to a solution of H–Leu–OBzl [prepared from 18.0 g (99.2 mmol) of the tosylate] in DMF (30 ml) together with Et_3N (13.8 ml, 99.2 mmol) at an ice-bath temperature. The mixture, after being stirred for 3 h, was concentrated *in vacuo*. The residue was purified by procedure A to give an oily product. This dipeptide ester was dissolved in MeOH (100 ml) and treated with hydrazine monohydrate (24.8 ml, 0.41 mol) for 24 h at 25 °C. The solvent was removed by evaporation, then the residue was precipitated with H_2O. The resulting powder was recrystallized from MeOH with ether: yield 12.9 g (37%), mp 120—121 °C, $[\alpha]_D^{23}$ −13.2° (c=0.5, DMF), Rf_1 0.76. *Anal.* Calcd for $C_{21}H_{34}N_4O_5 \cdot 1/2H_2O$: C, 58.44; H, 8.17; N, 12.98. Found: C, 58.17; H, 8.04; N, 13.46.

Boc–Ser(Bzl)–Leu–Ser(Bzl)–Gly–Leu–Gly–OPac The azide [prepared from 2.51 g (5.93 mmol) of Boc–Ser(Bzl)–Leu–NHNH$_2$] in DMF (10 ml) was added to a TFA-treated sample of the above tetrapeptide derivative (3.45 g, 5.39 mmol) in DMF (15 ml) together with Et_3N (1.12 ml, 5.39 mmol) at an ice-bath temperature. After being stirred overnight, the solvent was removed by evaporation. The product was purified by procedure B and reprecipitated from DMF with 2-propanol: yield 3.81 g (76%), mp 175—176 °C, $[\alpha]_D^{23}$ −7.8° (c=0.5, DMF), Rf_2 0.37. *Anal.* Calcd for $C_{49}H_{66}N_6O_{12} \cdot H_2O$: C, 62.01; H, 7.22; N, 8.66. Found: C, 61.94; H, 7.22; N, 8.82.

Boc–Ser(Bzl)–Leu–Ser(Bzl)–Gly–Leu–Gly–OH, Boc–(pBNP 14—19)–OH [2] The above hexapeptide derivative (3.52 g, 3.78 mmol) in DMF–pyridine (5:1, 30 ml) was treated with Zn powder (2.47 g, 37.8 mmol) in the presence of anthranilic acid (5.18 g, 37.8 mmol) at 50 °C for 2 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 3.00 g (97%), mp 180 °C (dec.), $[\alpha]_D^{23}$ −8.2° (c=0.5, DMF), Rf_1 0.41. *Anal.* Calcd for $C_{41}H_{60}N_6O_{11} \cdot 4H_2O$: C, 55.64; H, 7.74; N, 9.50. Found: C, 55.92; H, 7.95; N, 9.13. Amino acid ratios in a 6 N HCl hydrolysate: Ser 1.68, Gly 2.00, Leu 1.96 (recovery of Gly 64%).

Boc–Ile–Gly–OPac A solution of Boc–Ile–OSu (10.0 g, 30.4 mmol) and Et_3N (5.08 ml, 36.4 mmol) in DMF (25 ml) was added to a solution of H–Gly–OH (6.84 g, 91.2 mmol) in H_2O (10 ml) containing Et_3N (12.7 ml 91.2 mmol). The mixture, after being stirred overnight, was concentrated and the residue was dissolved in AcOEt (100 ml). The organic phase was washed with 5% citric acid and NaCl–H_2O, dried over Na_2SO_4 and concentrated. The oily product and Cs_2CO_3 (4.94 g, 15.2 mmol) were dissolved in MeOH–H_2O (9:1, 40 ml) and the solvent was removed by evaporation. The residue was dissolved in DMF (25 ml) and evaporation was repeated three times. The resulting Cs salt was redissolved in DMF (30 ml) and phenacyl bromide (6.66 g, 34.4 mmol) was added. The mixture, after being stirred for 30 min, was filtered, the filtrate was concentrated *in vacuo*. The product was purified by procedure A, and recrystallized from MeOH with ether: yield 9.12 g (74%), mp 130—132 °C, $[\alpha]_D^{23}$ −10.9° (c=1.1, DMF), Rf_2 0.74. *Anal.* Calcd for $C_{21}H_{30}N_2O_6$: C, 62.05; H, 7.44; N, 6.89. Found: C, 61.79; H, 7.51; N, 6.90.

Boc–Arg(Tos)–Ile–Gly–OPac A mixed anhydride [prepared from 9.85 g (23.0 mmol) of Boc–Arg(Tos)–OH] in DMF (25 ml) was added to an ice-chilled solution of a TFA-treated sample of the above dipeptide ester (8.50 g, 20.9 mmol) in DMF (25 ml) containing Et_3N (2.91 ml, 20.9 mmol). The mixture was stirred for 3 h and concentrated. The product was purified by procedure A, followed by column chromatography on silica using $CHCl_3$–MeOH (20:0.5) as an eluant. The product was triturated with ether to give a powder: yield 11.1 g (74%), mp 91—94 °C, $[\alpha]_D^{23}$ −8.7° (c=0.6, DMF), Rf_2 0.25. *Anal.* Calcd for $C_{34}H_{48}N_6O_9S \cdot H_2O$: C, 55.57; H, 6.80; N, 11.44. Found: C, 55.69; H, 6.80; N, 11.59.

Boc–Asp(OcHex)–Arg(Tos)–Ile–Gly–OPac A solution of Boc–Asp(OcHex)–ONp [prepared from Boc–Asp(OcHex)–OH (3.69 g, 11.7 mmol), HONp (1.63 g) and DCC (2.90 g, 14.0 mmol)] in THF (30 ml) and NMM (1.54 ml, 14.0 mmol) was added to an ice-chilled solution of a TFA-treated sample of the above tripeptide ester (7.0 g, 9.76 mmol) in DMF (25 ml) together with Et_3N (1.63 ml, 11.7 mmol). The mixture, after being stirred overnight, was evaporated. The product was purified by procedure B and reprecipitated from THF with ether: yield 7.2 g (67%), mp 113—115 °C, $[\alpha]_D^{23}$ −10.1° (c=0.5, DMF), Rf_1 0.73. *Anal.* Calcd for $C_{44}H_{63}N_7O_{12}S_2$: C, 57.81; H, 6.95; N, 10.73. Found C, 57.98; H, 6.95; N,

10.49.

Boc–Leu–Asp(OcHex)–Arg(Tos)–Ile–Gly–OPac A mixed anhydride [prepared from 1.82 g (7.32 mmol) of Boc–Leu–OH] in DMF (10 ml) was added to an ice-chilled solution of a TFA-treated sample of the above tetrapeptide ester (6.50 g, 6.65 mmol) in DMF (20 ml) containing Et_3N (1.39 ml, 9.98 mmol). The mixture was stirred for 3 h and concentrated. The product was purified by procedure B and reprecipitated from THF with ether: yield 6.13 g (90%), mp 119—123 °C, $[\alpha]_D^{23}$ −17.0° (c=0.5, DMF), Rf_1 0.83. *Anal.* Calcd for $C_{50}H_{74}N_8O_{13}S \cdot H_2O$: C, 57.45; H, 7.33; N, 10.72. Found: C, 57.88; H, 7.35; N, 11.07.

Boc–Arg(Tos)–Leu–Asp(OcHex)–Arg(Tos)–Ile–Gly–OPac A mixed anhydride [prepared from 2.76 g (6.43 mmol) of Boc–Arg(Tos)–OH] in DMF (15 ml) was added to an ice-chilled solution of a TFA-treated sample of the above pentapeptide ester (6.0 g, 5.84 mmol) in DMF (25 ml) containing Et_3N (1.22 ml, 5.84 mmol). The mixture was stirred for 3 h and concentrated. The product was purified by procedure A, followed by column chromatography on silica using $CHCl_3$–MeOH (20:0.5) and trituration with ether: yield 6.59 g (82%), mp 121—123 °C, $[\alpha]_D^{23}$ −14.3° (c=0.5, DMF), Rf_1 0.62. *Anal.* Calcd for $C_{63}H_{92}N_{12}O_{16}S_2 \cdot H_2O$: C, 55.82; H, 6.98; N, 12.40. Found: C, 55.72; H, 6.96; N, 12.46.

Boc–Arg(Tos)–Arg(Tos)–Leu–Asp(OcHex)–Arg(Tos)–Ile–Gly–OPac A mixed anhydride [prepared from 2.15 g (5.02 mmol) of Boc–Arg(Tos)–OH] in DMF (15 ml) was added to an ice-chilled solution of a TFA-treated sample of the above hexapeptide ester (6.30 g, 4.57 mmol) containing Et_3N (0.95 ml, 4.57 mmol). The mixture was stirred for 3 h and concentrated. The product was purified by procedure A, followed by column chromatography on silica using $CHCl_3$–MeOH (20:0.5) as described above: yield 6.01 g (80%), mp 122—124 °C, $[\alpha]_D^{23}$ −20.6° (c=0.5, DMF), Rf_1 0.66. *Anal.* Calcd for $C_{76}H_{110}N_{16}O_{19}S_3 \cdot 2H_2O$: C, 54.21; H, 6.82; N, 13.31. Found: C, 54.22; H, 6.60; N, 13.41.

Boc–Arg(Tos)–Arg(Tos)–Leu–Asp(OcHex)–Arg(Tos)–Ile–Gly–OH, Boc-(pBNP 10—16)–OH [3] The above heptapeptide derivative (6.0 g, 3.64 mmol) in DMF–pyridine (5:1, 30 ml) was treated with Zn powder (4.70 g, 72.8 mmol) in the presence of anthranilic acid (4.31 g, 31.4 mmol) at 50 °C for 2 h. The solution was filtered, the filtrate was concentrated *in vacuo* and the residue was triturated with 2% EDTA. The resulting powder was washed with H_2O and reprecipitated from DMF with AcOEt: yield 5.09 g (92%), mp 140—146 °C, $[\alpha]_D^{23}$ −12.6° (c=0.5, DMF), Rf_1 0.46. *Anal.* Calcd for $C_{68}H_{104}N_{16}O_{18}S_3 \cdot 3/2H_2O$: C, 52.45; H, 6.92; N, 14.39. Found: C, 52.72; H, 7.06; N, 13.83. Amino acid ratios in a 6 N HCl hydrolysate: Asp 0.89, Gly 1.00, Ile 0.97, Leu 0.98, Arg 2.79 (recovery of Gly 71%).

Boc–Phe–Gly–OPac A solution of Boc–Phe–OSu (6.81 g, 18.8 mmol) and Et_3N (2.88 ml, 20.7 mmol) in DMF (25 ml) was added to a solution of H–Gly–OH (4.25 g, 56.5 mmol) in H_2O (10 ml) containing Et_3N (7.86 ml, 56.5 mmol) and the mixture was stirred overnight. The solvent was removed by evaporation and the residue was dissolved in AcOEt (100 ml). The organic phase was washed with 5% citric acid and NaCl–H_2O, dried over Na_2SO_4 and concentrated. The oily product and Cs_2CO_3 (3.08 g, 9.40 mmol) were dissolved in MeOH–H_2O (9:1, 20 ml) and concentrated. The residue was dissolved in DMF (20 ml) and evaporation was repeated three times. The resulting Cs salt was redissolved in DMF (25 ml) and phenacyl bromide (4.13 g, 20.7 mmol) was added. After being stirred for 30 min, the product was purified by procedure A and recrystallized from THF with ether: yield 4.21 g (51%), mp 137—139 °C, $[\alpha]_D^{22}$ −7.9° (c=0.5, DMF), Rf_2 0.80. *Anal.* Calcd for $C_{24}H_{28}N_2O_6$: C, 65.44; H, 6.40; N, 6.36. Found: C, 65.21; H, 6.37; N, 6.39.

Boc–Cys(Tacm)–Phe–Gly–OPac A mixed anhydride [prepared from 3.78 g (8.72 mmol) of Boc–Cys(Tacm)–OH·CHA] in DMF (20 ml) was added to an ice-chilled solution of a TFA-treated sample of the above dipeptide ester (3.50 g, 7.93 mmol) in DMF (20 ml) containing Et_3N (1.45 ml, 10.5 mmol). The mixture was stirred for 3 h and concentrated. The product was purified by procedure A and recrystallized from MeOH with ether–*n*-hexane (1:1): yield 4.65 g (89%), mp 125—127 °C, $[\alpha]_D^{23}$ −19.4° (c=0.5, DMF), Rf_2 0.59. *Anal.* Calcd for $C_{33}H_{44}N_4O_8S \cdot 1/2H_2O$: C, 59.53; H, 6.81; N, 8.41. Found: C, 59.68; H, 7.08; N, 8.01.

Boc–Ser(Bzl)–Gly–Cys(Tacm)–Phe–Gly–OPac A solution of Boc–Ser(Bzl)–Gly–ONB [prepared from Boc–Ser(Bzl)–Gly–OH·CHA (3.15 g, 8.38 mmol), HONB (1.38 g, 7.68 mmol) and DCC (1.73 g, 8.38 mmol)] in THF (30 ml) was added to an ice-chilled solution of a TFA-treated sample of the above tripeptide ester (4.0 g, 7.93 mmol) in DMF (15 ml) containing Et_3N (1.32 ml, 7.93 mmol). The mixture was stirred overnight and concentrated. The product was purified by procedure A and recrystallized from MeOH with ether: yield 3.23 g (60%), mp 98—99 °C, $[\alpha]_D^{23}$ −37.0° (c=0.5, DMF), Rf_2 0.52. *Anal.* Calcd for $C_{45}H_{58}N_6O_{11}S$: C, 60.65; H, 6.56; N, 9.43. Found: C, 61.02; H, 6.86; N, 9.48.

Boc–Asp(OcHex)–Ser(Bzl)–Gly–Cys(Tacm)–Phe–Gly–OPac A solution of Boc–Asp(OcHex)–ONp [prepared from 1.32 g (4.18 mmol) of Boc–Asp(OcHex)–OH, HONp (0.64 g, 4.60 mmol) and DCC (1.04 g, 5.02 mmol)] in THF (25 ml) was added to an ice-chilled solution of a TFA-treated sample of the above pentapeptide ester (3.10 g, 4.18 mmol) containing Et_3N (0.58 ml, 4.18 mmol). The mixture was stirred overnight and concentrated. The product was purified by procedure A and recrystallized from THF with ether: yield 2.89 g (77%), mp 128—129 °C, $[\alpha]_D^{23}$ −12.6 (c=0.6, DMF), Rf_2 0.56. *Anal.* Calcd for $C_{55}H_{77}N_7O_{14}S$: C, 60.70; H, 6.76; N, 9.01. Found: C, 60.64; H, 6.70; N, 8.92.

Boc–Asp(OcHex)–Ser(Bzl)–Gly–Cys(Tacm)–Phe–Gly–OH, Boc–(pBNP 1—6)–OH [4] The above hexapeptide ester (2.50 g, 2.30 mmol) in DMF–pyridine (5:1, 30 ml) was treated with Zn powder (1.50 g, 23.0 mmol) in the presence of anthranilic acid (3.15 g, 23.0 mmol) at 50 °C for 2 h. The mixture was filtered, the filtrate was concentrated *in vacuo*, and the residue was triturated with ether–2% EDTA. The resulting powder was washed with H_2O, followed by reprecipitation from DMF with ether: yield 1.90 g (86%), mp 102—105 °C, $[\alpha]_D^{23}$ −12.5° (c=0.6, DMF), Rf_1 0.49. Amino acid ratios in a 6 N HCl hydrolysate: Asp 0.99, Ser 0.91, Gly 2.00, Cys 0.10, Phe 0.99 (recovery of Gly 97%). *Anal.* Calcd for $C_{47}H_{67}N_7O_{13}S$: C, 58.19; H, 6.96; N, 10.11. Found: C, 57.96; H, 6.87; N, 9.88.

Boc–Ser(Bzl)–Leu–Ser(Bzl)–Gly–Leu–Gly–Cys(Tacm)–Asn–Val–Leu–Arg(Tos)–Arg(Tos)–Tyr(BrZ)–OBzl, Boc–(pBNP 14—26)–OBzl Segment [2] (0.45 g, 0.52 mmol), HOBt (77 mg, 0.57 mmol) and WSC·HCl (138 mg, 0.72 mmol) were successively added to an ice-chilled solution of a TFA-treated sample of segment [1] (0.85 g, 0.43 mmol) in DMF (15 ml) together with Et_3N (60 μl, 0.43 mmol). The mixture was stirred overnight and concentrated. The product was purified by procedure B and reprecipitated from DMF with EtOH: yield 0.94 g (82%), mp 214—218 °C, $[\alpha]_D^{18}$ −33.1° (c=0.7, DMF), Rf_1 0.44. Amino acid ratios in a 6 N HCl acid hydrolysate are listed in Table I. *Anal.* Calcd for $C_{115}H_{158}BrN_{21}O_{27}S_3$: C, 56.54; H, 6.52; N, 12.04. Found: C, 56.28; H, 6.39; N, 11.86.

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, Boc–(pBNP 7—26)–OBzl Segment [3] (549 mg, 0.36 mmol), HOBt (58 mg, 0.43 mmol) and WSC·HCl (97 mg, 0.50 mmol) were successively added to an ice-chilled solution of a TFA-treated sample of the above Boc–(pBNP 14—26)–OBzl (0.82 g, 0.30 mmol) together with Et_3N (42 μl, 0.30 mmol). The mixture was stirred overnight and concentrated. The product was purified by procedure B and reprecipitated from DMF with EtOH: yield 1.16 g (95%), mp 238 °C (dec.), $[\alpha]_D^{18}$ −35.0° (c=0.6, DMF), Rf_1 0.68. Amino acid ratios in a 6 N HCl hydrolysate are listed in Table I. *Anal.* Calcd for $C_{178}H_{252}BrN_{37}O_{42}S_6 \cdot 2H_2O$: C, 54.95; H, 6.63; N, 13.32. Found: C, 54.84; H, 6.62; N, 13.43.

Boc–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 Segment [4] (328 mg, 0.34 mmol), HOBt (55 mg, 0.41 mmol) and WSC·HCl (91 mg, 0.47 mmol) were successively added to an ice-chilled solution of a TFA-treated sample of the above Boc–(pBNP 7—26)–OBzl (1.16 g, 0.28 mmol) together with Et_3N (40 μl, 0.28 mmol). The mixture was stirred overnight and concentrated. The product was purified by procedure B and reprecipitated from DMF with EtOH: yield 1.12 g (82%), mp 244 °C (dec.), $[\alpha]_D^{18}$ −31.7° (c=0.7, DMSO), Rf_1 0.70. Amino acid ratios in a 6 N HCl hydrolysate are listed in Table I. *Anal.* Calcd for $C_{220}H_{309}BrN_{44}O_{52}S_7 \cdot 2H_2O$: C, 55.11; H, 6.65; N, 13.00. Found: 55.42; H, 6.58; N, 13.05.

H–Asp–Ser–Gly–Cys(Tacm)–Phe–Gly–Arg–Arg–Leu–Asp–Arg–Ile–Gly–Ser–Leu–Ser–Gly–Leu–Gly–Cys(Tacm)–Asn–Val–Leu–Arg–Arg–Tyr–OH, [Cys(Tacm)^{4,20}]–pBNP The fully protected pBNP (200 mg) was treated with HF (5 ml) in the presence of *m*-cresol (250 μl) and dimethyl sulfide (250 μl) at an ice-bath temperature for 1 h. After evaporation of 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.* 5 ml). After being adjusted to pH 8 with 5% NH_4OH, the solution was stirred for 30 min. The solution was applied to a column of Sephadex G-25 (2.1 × 101 cm), which was eluted with 1 N AcOH. The ultraviolet (UV) absorption at 280 nm was determined in each fraction (10 ml). The fractions corresponding to the main peak (tube Nos. 28—48) were combined and lyophilized to give a fluffy powder (118 mg, 95%).

The above crude product was dissolved in 0.01 M $AcONH_4$ buffer and the solution was applied to a column of CM-cellulose (2.1 × 10 cm), which was eluted first with 0.01 M $AcONH_4$ (200 ml) and then with a linear

TABLE II. Physical Constants and Analytical Data for Protected pBNP and Its Intermediates Using Cys(Tmb)

Boc-protected peptide (Positions)	Coupling yield (%)	mp (°C)	$[\alpha]_D^{22}$ (°) (DMF)	Formula	Analysis (%) Calcd (Found)		
					C	H	N
7-Residue (20—26)	—	161—163	−13.6 (c=0.6)	$C_{83}H_{109}BrN_{14}O_{18}S_3 \cdot H_2O$	55.84 (55.67)	6.27 6.24	10.98 11.08)
13-Residue (14—26)	87	218 (dec.)	−6.8 (c=0.4)	$C_{119}H_{159}BrN_{20}O_{26}S_3 \cdot 3H_2O$	56.81 (56.74)	6.61 6.78	11.13 10.98)
20-Residue (7—26)	89	222 (dec.)	−17.0[a] (c=0.5)	$C_{182}H_{253}BrN_{36}O_{42}S_6 \cdot 3H_2O$	55.65 (55.60)	6.65 6.61	12.84 13.10)
26-Residue (1—26)	84	226 (dec.)	−18.2[a] (c=0.4)	$C_{228}H_{311}BrN_{42}O_{50}S_7 \cdot 7H_2O$	56.29 (56.31	6.73 6.74	12.09 12.47)

a) In DMSO.

gradient formed from 0.25 M $AcONH_4$ buffer (250 ml) through a mixing flask containing the starting buffer (250 ml). The UV absorption at 280 nm of each fraction (6.5 ml) was measured and fractions corresponding to the main peak (tube Nos. 42—50) were combined and lyophilized. Further purification was performed by preparative FPLC on a YMC-gel AQ-120 (s-50) column (1.6 × 50 cm). The CM-purified sample was dissolved in H_2O (*ca.* 5 ml) and applied to the above column, which was eluted with a linear gradient of 60% aqueous MeCN in 0.1% aqueous TFA for 200 min at a flow rate of 3.0 ml/min. The eluate corresponding to the main peak was collected and lyophilized to give a fluffy white powder: yield 28 mg (34%), $[\alpha]_D^{27}$ −54.5° (c=0.2, 1 N AcOH), Rf_4 0.37, HPLC on a YMC AM-302 column (4.6 × 150 mm) [retention time 19.6 min, gradient elution with MeCN (10—60%, 30 min) in 0.1% aqueous TFA], FAB-MS m/z (relative intensity): 3081.772 (32), 3082.776 (33), 3096.719 (MH^+, 74), 3097.715 (100), 3098.721 (91), 3099.709 (65), 3100.708 (39); theoretical mass value calcd for $C_{132}H_{233}N_{44}O_{38}S_2$ m/z 3096.631 $[MH]^+$ and 3097.634 (base peak in the molecular ion region). Amino acid ratios after AP-M digestion were Asp 1.95, Gly 5.06, Val 0.78, Ile 0.87, Leu 4.00, Tyr 1.23, Phe 1.18, Arg 5.26 [recovery of Leu 68%, Asn, Ser and Cys(Tacm) were not determined].

Synthetic pBNP from [Cys(Tacm)^{4,20}]-pBNP by Use of I_2 The purified [Cys(Tacm)^{4,20}]-pBNP (28 mg) was dissolved in 90% AcOH (200 ml) and 20% I_2/EtOH (115 μl, 10 eq) was added. The mixture was stirred at 25 °C for 60 min, then concentrated and the resulting solution (*ca.* 5 ml) was applied to a column of Sephadex G-25 (2.1 × 101 cm), which was eluted with 1 N AcOH. The UV absorption at 280 nm was determined in each fraction (6.5 ml). The fractions corresponding to the main peak (tube Nos. 20—29) were combined and lyophilized to give a fluffy powder (24.6 mg, 95%). The gel-filtered sample was dissolved in 0.3% aqueous TFA containing 20% MeCN and applied to a YMC-gel D-ODS-5 (2.15 × 25 cm) column, which was eluted with a linear gradient of MeCN (20—40%) in 0.3% aqueous TFA for 100 min (flow rate 4.0 ml/min). The eluate corresponding to the main peak was collected and lyophilized to give a fluffy white powder: 14 mg (52%, calculated from di-Tacm-pBNP; overall yield 12%, calculated from the fully protected pBNP), $[\alpha]_D^{26}$ −50.0° (c=0.1, 1 N AcOH), Rf_4 0.35, Rf_5 0.69, HPLC on a Cosmosil $5C_{18}$ ST column (4.6 × 150 mm) [retention time: 18.5 min, on gradient elution with MeCN (10—60%, 30 min) in 0.1% aqueous TFA, 0.7 ml/min] and a TSK-gel G-2000 SW column (7.5 × 600 mm) [eluted with 0.1 M $AcONH_4$ (pH 4.0), 0.5 ml/min; the retention time (46.2 min) was between those of α-rANP (45.7 min, MW. 3063) and adrenorphin (47.8 min, MW. 984)], FAB-MS m/z (relative intensity): 2853.414 (37), 2854.429 (38), 2855.456 (33), 2868.395 (MH^+, 76), 2869.397 (100), 2870.388 (95), 2871.401 (80), 2872.393 (51), 2873.384 (31); theoretical mass value calcd for $C_{120}H_{198}N_{42}O_{36}S_2$ m/z 2868.447 $[MH]^+$ and 2869.449 (base peak in the molecular ion region). Amino acid ratios in a 6 N HCl hydrolysate are listed in Table I.

Boc–Cys(Tmb)–Phe–Gly–OPac Boc–Cys(Tmb)–OH was coupled by the mixed anhydride method and the product was purified by procedure B, followed by recrystallization from THF with ether: yield 79%, mp 171—172 °C, $[\alpha]_D^{22}$ −17.2° (c=0.6, DMF), Rf_2 0.55. *Anal.* Calcd for $C_{37}H_{46}N_3O_7S$: C, 65.76; H, 6.71; N, 6.22. Found: C, 65.56; H, 6.83; N, 6.28.

Boc–Ser(Bzl)–Gly–Cys(Tmb)–Phe–Gly–OPac The title compound was prepared by the same procedure as used for the preparation of the corresponding Cys(Tacm) derivative: yield 90%, mp 132—134 °C, $[\alpha]_D^{22}$ −13.0° (c=0.6, DMF), Rf_1 0.87. *Anal.* Calcd for $C_{49}H_{59}N_5O_{10}S \cdot 3/2H_2O$: C, 62.80; H, 6.67; N, 7.47. Found: C, 62.77; H, 6.65; N, 7.97.

Boc–Asp(OcHex)–Ser(Bzl)–Gly–Cys(Tmb)–Phe–Gly–OPac The title compound was prepared by the same procedure as used for the preparation of the corresponding Cys(Tacm) derivative: yield 70%, mp 169—171 °C, $[\alpha]_D^{22}$ −16.0° (c=0.5, DMF), Rf_1 0.87. *Anal.* Calcd for $C_{59}H_{74}N_6O_{13}S \cdot 1/2H_2O$: C, 63.47; H, 6.77; N, 7.53. Found: C, 63.32; H, 6.76; N, 7.72.

Boc–Asp(OcHex)–Ser(Bzl)–Gly–Cys(Tmb)–Phe–Gly–OH [4′] The title compound was prepared by the same procedure as employed for the preparation of the corresponding Cys(Tacm) derivative: yield 66%, mp 157—159 °C, $[\alpha]_D^{22}$ −21.1° (c=0.6, DMF), Rf_1 0.54. Amino acid ratios in a 6 N HCl hydrolysate: Asp 0.99, Ser 0.87, Gly 1.00, Cys 0.30, Phe 1.01 (recovery of Gly 76%). *Anal.* Calcd for $C_{51}H_{68}N_6O_{12}S \cdot 1/2H_2O$: C, 61.36; H, 7.01; N, 8.42. Found: C, 61.06; H, 6.98; N, 8.70.

Boc–Cys(Tmb)–Asn–Val–Leu–Arg(Tos)–Arg(Tos)–Tyr(BrZ)–OBzl, Boc–(pBNP 20—26)–OBzl [1′] The title compound was prepared by the WSC plus HOBt coupling of Boc–Cys(Tmb)–OH and purified by procedure B, followed by recrystallization from THF with ether: yield 83%, Rf_1 0.76. Amino acid ratios in a 6 N HCl hydrolysate are listed in Table I. Physical constants and analytical data are listed in Table II.

Boc–Asp(OcHex)–Ser(Bzl)–Gly–Cys(Tmb)–Phe–Gly–Arg(Tos)–Arg(Tos)–Leu–Asp(OcHex)–Arg(Tos)–Ile–Gly–Ser(Bzl)–Leu–Ser(Bzl)–Gly–Leu–Gly–Cys(Tmb)–Asn–Val–Leu–Arg(Tos)–Arg(Tos)–Tyr(BrZ)–OBzl, Boc–[Cys(Tmb)^{4,20}–pBNP (1—26)]–OBzl Couplings of segment [1′] to [4′] were carried out by the same procedure as described for the corresponding Cys(Tacm) derivatives. The yield of each coupling, the physical constants and analytical data of each intermediate are listed in Table II.

Synthetic pBNP Prepared from Boc–[Cys(Tmb)^{4,20}-pBNP(1—26)]-OBzl The above protected pBNP containing Cys(Tmb) (200 mg) was deprotected with HF–*m*-cresol–dimethyl sulfide as described for the corresponding Cys(Tacm) derivative. The deprotected peptide was dissolved in H_2O (500 ml). To this solution, after it had been adjusted to pH 8 with 5% NH_4OH, a solution of $K_3[Fe(CN)_6]$ (138 mg, 10 eq) in H_2O (5 ml) was added slowly over 30 min, and the mixture was further stirred for 30 min at 25 °C. The oxidation reaction was stopped by adding AcOH and the mixture was treated with Dowex 1-X8 (Cl^- form). After removal of the Dowex resin by filtration, the filtrate was lyophilized. The product was purified in the same manner as employed for pBNP synthesis using Cys(Tacm): yield 6.8% (calculated from the protected peptide). The purified pBNP exhibited a single peak on an analytical HPLC column (Fig. 10b) and the retention time was identical with that of pBNP synthesized using Cys(Tacm). Amino acid ratios in a 6 N HCl hydrolysate are listed in Table I.

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References and Notes

1) Amino acids and peptide derivatives mentioned in this paper are of the L-configuration. The following abbreviations are used: Boc = *tert*-butoxycarbonyl, Bzl = benzyl, BrZ = 2-bromobenzyloxycarbonyl, cHex = cyclohexyl, CM = carboxymethyl, CHA = cyclohexylamine, DCHA = dicyclohexylamine, DMF = *N*,*N*-dimethylformamide, DMSO = dimethyl sulfoxide, EDTA = ethylenediaminetetraacetic acid, HOBt = 1-hydroxybenzotriazole, MeCN = acetonitrile, NB = *N*-hydroxy-5-norbornene-2,3-dicarboximidyl, Np = *p*-nitro-

phenyl, Pac=phenacyl, NMM=*N*-methylmorpholine, Su=*N*-hydroxysuccinimidyl, Tacm=trimethylacetamidomethyl, TFA=trifluoroacetic acid, THF=tetrahydrofuran, Tmb=2,4,6-trimethylbenzyl, Tos=*p*-toluenesulfonyl, DCC=dicyclohexylcarbodiimide, Acm=acetamidomethyl.

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