Solution-Phase Synthesis of Porcine Brain Natriuretic Peptide (pBNP) Using S-Trimethylacetamidomethylcysteine

Yoshiaki KISO,* Makoto YOSHIDA, Tooru KIMURA, Yoichi FUJIIWA, Masanori SHIMOKURA, and Kenichi AKAJI

Department of Medicinal Chemistry, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607, Japan. Received October 16, 1989

The hexadodecapetide 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. 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-rat atrial natriuretic peptide (α-rANP) (Fig. 1). This peptide exhibited similar biological activities to those of α-rANPs, i.e., regulation of the hemostatic balance of body fluid and blood pressure. Following our synthetic studies on α-rANPs, we have synthesized pBNP in order to obtain a sufficient amount to examine its biological relationship with α-rANPs. A part of this work has been reported preliminarily. Solid phase synthesis of this peptide has also been reported preliminarily by Yajima et al. 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 was employed as a cysteine S-protecting group to examine its usefulness in practical peptide synthesis. As described in the preceding paper, this Tacm group was stable under acidic conditions and removable with iodine. These properties are similar to those of the Acm group, but Cys(Tacm) was less susceptible to air-oxidation than Cys(Acm).

Alternatively, the synthesis of pBNP using S-2,4,6-trimethylbenzylcysteine [Cys(Tmb)] 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-protection, amino acid derivatives bearing protecting groups removable with HF were employed, i.e., Asp(OcHex), Ser(Bzl), Arg(Tos), and Tyr(BrZ), 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. 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 plus 1-hydroxybenzotriazole (HOBT) of these segments, segments were synthesized by the use of phenacyl (Pac) ester at the C-terminus. Prior to condensation of each segment, removal of the Pac group was conducted with Z-anthranilic acid in a mixture of DMF-pyridine.

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–

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

- pBNP:
- α-rANP:

Fig. 2. Synthetic Route to pBNP

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Tyr(BrZ)-OBzl, two Boc-Arg(Tos)-OH and Boc-Leu-OH were each prepared 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 6N 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 ester26) 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-NHH2, prepared by hydrazinolysis of Boc-Ser(Bzl)-Leu-OBzl, by the azide28) 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

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### Table 1: Amino Acid Ratios in 6N HCl Hydrolysates of Synthetic pBNP and Its Intermediates

<table>
<thead>
<tr>
<th>Protected intermediates</th>
<th>Synthetic pBNP</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>20–26</td>
</tr>
<tr>
<td>Asp</td>
<td>0.96</td>
</tr>
<tr>
<td>Ser</td>
<td>1.64</td>
</tr>
<tr>
<td>Gly</td>
<td>1.99</td>
</tr>
<tr>
<td>Val</td>
<td>1.01</td>
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<tr>
<td>Gly</td>
<td>0.25</td>
</tr>
<tr>
<td>Cys</td>
<td>1.04</td>
</tr>
<tr>
<td>Tyr</td>
<td>1.03</td>
</tr>
<tr>
<td>LeuF</td>
<td>1.00</td>
</tr>
<tr>
<td>Tyr</td>
<td>1.03</td>
</tr>
<tr>
<td>Arg</td>
<td>1.97</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>73</td>
</tr>
</tbody>
</table>

*a)* Synthesized using Cys(Tactm). *b)* Synthesized using Cys(Tmbm). *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)4–25]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 sulfide29 in an ice-bath for 60 min to remove all protecting groups except for two Tacm groups. The di-Tacm peptide was dissolved in H2O and the pH of the solution was adjusted to 8 with 5% NH4OH to reverse any possible N→O shift at three Ser residues30. 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—

![Image](protected_pBNP)

**Fig. 7.** Deprotection and Purification of pBNP

![Image](HPLC_Profile)

**Fig. 8.** HPLC Profile of Synthetic pBNP

(a) Gel-filtered sample. b) HPLC-purified sample.

![Image](FAB-MS_of_Synthetic_pBNP)

**Fig. 9.** FAB-MS of Synthetic pBNP

a) [Cys(Tacm)4–25]pBNP. b) Synthetic pBNP.
0.25 M AcONH₂, followed by fast protein liquid chromatography (FPLC) on a YMC-ODS-AQ 120 (SS50) 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⁺) + 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 SC18) (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 2686.4 (theoretical value: 2686.447) and a base peak of 2689.4 (theoretical value: 2689.449) 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 ANP, in reasonable agreement with literature values.21

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 H₂O and oxidized with K₂[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 SC18,5) 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%).

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 ANPα and α-rat ANP.3

Prior to the coupling reaction, the Nα-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% NaHCO₃ and H₂O–NaCl, then dried over Na₂SO₃ 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% NaHCO₃ and H₂O, and recrystallized or reprecipitated from appropriate solvents.

TLC was performed on silica gel (Kiesel-gel 60F₂₅₄, Merck). Rf values refer to the following v/v solvent systems: Rf₁ CHCl₃–MeOH–H₂O (8:3:1; lower phase), Rf₂ CHCl₃–MeOH (10:5:5), Rf₃ CHCl₃–MeOH (9:1), Rf₄ n-BuOH–AcOH–pyridine–H₂O (4:1:1:2), Rf₅ n-BuOH–AcOH–pyridine–H₂O (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-HX110 double-focusing 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, 8.90 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 0.94, [α]D₂⁰ = 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 Et₃N (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 CHCl₃–MeOH (20:0.5) as an eluant. The product was triturated with n-hexane to give a powder: yield 4.65 g (69%), Rf 0.33, mp 69–70°C, [α]D₂⁰ = 11.9° (c = 0.5, DMF). Anal. Calcd for C₃₂H₅₄BrN₂O₅·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 Et₃N (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 CHCl₃-MeOH (30:0.5) and trituration with n-hexane: yield 1.26 g (50%), RFₖ, 0.51, mp 95–96°C, [α]=center 16.1° (c=0.4, DMF). Anal. Calcd for C₁₅H₁₈Br₂N₂O₅S₂: 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(Bz)-OBzl A mixed anhydride [prepared from 0.29 g (1.14 mmol) of Boc-LeuOH in DMF (5 ml) was added to an ice-cooled suspension of Boc-Arg(Tos)-Bzl (0.28 g, 1.11 mmol) of the above tripeptide ester (1.15 g, 0.95 mmol) in DMF (10 ml) containing Et₃N (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%), RFₖ, 0.56, mp 98–99°C, [α]=center 25.6° (c=0.5, DMF). Anal. Calcd for C₁₅H₁₈Br₂N₂O₅S₂: C, 54.82; H, 5.89; N, 5.89; 10.63. Found: C, 54.91; H, 5.96; N, 10.48.

Boc-Leu-Arg(Tos)-Arg(Tos)-Tyr(Bz)-OBzl A mixture of Boc-LeuOSu (1.48 g, 4.72 mmol), Et₃N (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, [α]=center 18.6° (c=0.5, DMF). RFₖ, 0.29, RFₖ, 0.57. Anal. Calcd for C₁₅H₁₈Br₂N₂O₅S₂: C, 55.92; H, 6.12; N, 10.87. Found: C, 55.63; H, 6.08; N, 11.01.

Arg-Val-Leu-Arg(Tos)-Arg(Tos)-Tyr(Bz)-OBzl A mixture of Arg-Val-Asn-OPn (1.49 g, 4.72 mmol), Et₃N (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, [α]=center 14.6° (c=0.4, DMF), RFₖ, 0.90, RFₖ, 0.56. Anal. Calcd for C₁₉H₂₁Br₂N₄O₅S₂: C, 54.89; H, 6.05; N, 11.89. Found: C, 54.67; H, 6.05; N, 11.82.

Cys(Tzm)-Asn-Val-Leu-Arg(Tos)-Arg(Tos)-Tyr(Bz)-OBzl [Boc-Cys(Tzm)-AcOH] prepared from 1.60 g (3.68 mmol) of the CH₃ salt, HOBT (0.62 g, 4.05 mmol) andWSC·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 Et₃N (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, recrystallized from THF with ether: yield 4.78 g (89%), mp 120–123°C, [α]=center 14.3° (c=0.5, DMF), RFₖ, 0.78. Alanine acid ratios in a 6N HCl hydrolysates are listed in Table 1. Anal. Calcd for C₁₉H₂₁Br₂N₄O₅S₂: C, 54.26; H, 6.23; N, 12.20. Found: C, 54.07; H, 6.08; N, 11.99.

Boc-Leu-Gly-OPac A solution of Boc-LeuOSu (6.57 g, 20.0 mmol) and Et₃N (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 H₂O (5 ml) containing Et₃N (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 NaCI 0.05M, dried over Na₂SO₄ and concentrated to give an oil product. This product was purified by procedure A and recrystallized from CH₂Cl₂-CH₂Cl₂ (30 ml) were added to a solution of H-Gly-OH (3.81 g, 50.7 ml) in H₂O (10 ml) containing Et₃N (1.20 ml, 4.00 mmol) and the residue was dissolved in AcOEt (100 ml). The organic phase was washed with 5% citric acid and NaCI 0.05M, dried over Na₂SO₄ and concentrated. The oil product was purified by procedure A, recrystallized from MeOH with ether: yield 1.6 g (94%), mp 32.0°C (5%, DMF), RFₖ, 0.75. Anal. Calcd for C₂₁H₂₆N₄O₅S₂: C, 56.07; H, 6.74; N, 5.74. Found: C, 55.92; H, 6.70; N, 5.93. Alanine acid ratios in a 6N HCl hydrolysates: Ser 1.68, Gly 2.00, Leu 1.96 (recovery of Gly 64%).

Boc-Val-Leu-Gly-OPac A solution of Boc-Val-OH (10.0 g, 30.4 mmol) and Et₃N (5.08 ml, 36.4 mmol) in DMF (25 ml) was added to a solution of H-Gly-OH (6.84 g, 72.2 mmol) in H₂O (10 ml) containing Et₃N (12.7 ml, 99.2 mmol). The mixture, after being stirred overnight, was concentrated and the residue was dissolved in AcOEt (100 ml). The reaction phase was washed with 5% citric acid and NaCI 0.05M, dried over Na₂SO₄ and concentrated. The oil product was purified by procedure A and recrystallized from MeOH with ether: yield 9.12 g (74%), mp 130–132°C, [α]=center 10.9° (c=1.1, DMF), RFₖ, 0.74. Anal. Calcd for C₁₉H₂₆N₄O₅S₂: C, 56.05; H, 6.74; N, 5.69. Found: C, 56.19; 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, 20.9 mmol) in DMF (25 ml) containing Et₃N (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 CHCl₃-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, [α]=center 8.7° (c=0.6, DMF), RFₖ, 0.25. Anal. Calcd for C₂₅H₂₂N₄O₅S₂: C, 55.57; H, 6.90; N, 11.64. Found: C, 55.59; H, 6.80; N, 11.59.

Boc-Arg(OicHex)-Arg(Tos)-Ile-Gly-OPac A solution of Boc-Arg(OicHex)-ONp [prepared from Boc-Arg(OicHex)-OH (3.69 g, 11.7 mmol)] was added to a TFA-treated sample of the above tripeptide ester (7.0 g, 9.76 mmol) in DMF (25 ml) together with Et₃N (1.63 ml, 11.7 mmol). The mixture, after being stirred overnight, was evaporated. The product was purified by procedure B and recrystallized from THF with ether: yield 7.2g (67%), mp 113–115°C, [α]=center 10.7° (c=0.5, DMF), RFₖ, 0.73. Anal. Calcd for C₂₅H₂₆N₄O₅S₂: C, 57.81; H, 6.95; N, 10.73. Found: C, 57.98; H, 6.95; N, 10.73.

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Boc-Leu-Asp(Och-Hex)-Arg(Tos)-Ile-Gly-OAc
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$_3$N (1.39 ml, 9.58 mmol) and for $C_{4}$H$_{8}$O$_{3}$N$_{2}$S$_{2}$ of 0.81. The mixture was stirred overnight and concentrated. The product was purified by procedure B and recrystallized from THF with ether: yield 6.13 g (92%), mp 119–123°C, $[\alpha]_D^{20}$ –17.0° (c=0.5, DMF), RF$_f$ 0.83. Anal. Calcd for $C_{14}H_{18}N_4O_7S_2$: H 7.35; C 57.45; H 7.33; N 10.72. Found: C 57,78; H 7.35; N 11.07.

Boc-(Arg-Tos)-Leu-Asp(Och-Hex)-Arg(Tos)-Ile-Gly-OAc
A mixed anhydride [prepared from 2.76 g (6.65 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$_3$N (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 (20:5) and trituration with ether: yield 6.59 g (82%), mp 121–123°C, $[\alpha]_D^{20}$ +14.3° (c=0.5, DMF), RF$_f$ 0.62. Anal. Calcd for $C_{14}H_{18}N_4O_7S_2$: H 6.99; C 55.82; N 12.40. Found: C 55.72; H 6.96; N 12.46.

Boc-(Arg-Tos)-Arg-(Tos)-Leu-Asp(Och-Hex)-Arg(Tos)-Ile-Gly-OAc
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 (6.0 g, 4.57 mmol) containing Et$_3$N (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 (20:5) as described above: yield 6.91 g (80%), mp 122–124°C, $[\alpha]_D^{20}$ +20.6° (c=0.5, DMF), RF$_f$ 0.66. Anal. Calcd for $C_{15}H_{18}N_5O_8S_2$: H 5.28; C 54.21; H 6.85; N 13.34. Found: C 54.22; H 6.66; N 13.41.

Boc-(Arg-Tos)-Arg-(Tos)-Leu-Asp(Och-Hex)-Arg(Tos)-Ile-Gly-OH
(b-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 anhydrous acetic acid (4.31 g, 34.14 mmol) at 50°C for 2 h. The reaction was filtered, the filtrate was concentrated in vacuo and the residue was triturated with 2% EDTA. The resulting powder was washed with H$_2$O, followed by recrystallization from DMF with ether: yield 1.90 g (86%), mp 102–105°C, $[\alpha]_D^{20}$ –12.5° (c=0.6, DMF), RF$_f$ 0.69. Acid amino acids in a 6 N HCl hydrolysate are listed in Table 1. Anal. Calcd for $C_{15}H_{18}Br$_{2}N$_{2}$O$_{3}$S$_{2}$: C 58.19; H 6.96; N 10.11. Found: C 57.96; H 6.87; N 9.88.

Boc-(Ser(Bzl)-Gly-Cys(Tacm)-Phy-Gly-OAc
A solution of Boc-Ser(Bzl)-Gly-Cys(Tacm)-Phy-Gly-OAc [prepared from Boc-Ser(Bzl)-Gly-Cys(OH)-CHO] 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$_3$N (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: yield 4.60 g (79%), mp 125–127°C, $[\alpha]_D^{20}$ +19.4° (c=0.5, DMF), RF$_f$ 0.59. Anal. Calcd for $C_{14}H_{18}N_4O_7S_2$: H 7.39; C 55.3; H 7.39; C 55.3; H 7.35; C 55.3; H 7.39. The product was stirred overnight and concentrated. The product was purified by procedure A and recrystallized from MeOH with ether: yield 3.43 g (60%), mp 98–99°C, $[\alpha]_D^{20}$ –37.0° (c=0.5, DMF), RF$_f$ 0.52. Anal. Calcd for $C_{14}H_{18}N_4O_7S_2$: C 60.65; H 6.56; N 9.43. Found: C 61.02; H 6.86; N 9.48.

Boc-(Asp(Och-Hex)-Ser(Bzl)-Gly-Cys(Tacm)-Phy-Gly-OAc
A solution of Boc-(Asp(Och-Hex)-ONp [prepared from 1.32 g (4.18 mmol) of Boc-(Asp(Och-Hex)-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 tetrapeptide ester (4.30 g, 4.18 mmol) in DMF (25 ml) containing Et$_3$N (0.58 mmol, 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, $[\alpha]_D^{20}$ –12.6° (c=0.6, DMF), RF$_f$ 0.56. Anal. Calcd for $C_{14}H_{18}N_4O_7S_2$: C 60.66; H 6.70; N 9.92. Found: C 60.66; H 6.70; N 9.92.

Boc-(Asp(Och-Hex)-Ser(Bzl)-Gly-Cys(Tacm)-Phy-Gly-OH, Boc-(pBNP 1–6–OH)
A solution of the above dipeptide 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 anhydrous acetic acid (3.15 g, 33.0 mmol) at 50°C for 2 h. The solution was filtered, the filtrate was concentrated in vacuo and the residue was triturated with ether–2% EDTA. The resulting powder was washed with H$_2$O, followed by recrystallization from DMF with ether: yield 1.90 g (86%), mp 102–105°C, $[\alpha]_D^{20}$ –12.5° (c=0.6, DMF), RF$_f$ 0.69. Acid amino acids in a 6 N HCl hydrolysate are listed in Table 1. Anal. Calcd for $C_{14}H_{18}Br$_{2}N$_{2}$O$_{3}$S$_{2}$: C 58.19; H 6.96; N 10.11. Found: C 57.96; H 6.87; N 9.88.

Boc-(Ser(Bzl)-Gly-Cys(Tacm)-Phy-Gly-OAc
A solution of Boc-Ser(Bzl)-Gly-Cys(Tacm)-Phy-Gly-OAc [prepared from Boc-Ser(Bzl)-Gly-Cys(OH)-CHO] in DMF (20 ml) was added to an ice-chilled solution of a TFA-treated sample of the above dipeptide ester (4.0 g, 7.93 mmol) in DMF (25 ml) containing Et$_3$N (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 3.43 g (60%), mp 98–99°C, $[\alpha]_D^{20}$ –37.0° (c=0.5, DMF), RF$_f$ 0.52. Anal. Calcd for $C_{14}H_{18}N_4O_7S_2$: C 60.65; H 6.56; N 9.43. Found: C 61.02; H 6.86; N 9.48.
### Table II. Physical Constants and Analytical Data for Protected pBNP and Its Intermediates Using Cys(Tacm)

<table>
<thead>
<tr>
<th>Boc-protected peptide (Positions)</th>
<th>Coupling yield (%)</th>
<th>mp (°C)</th>
<th>[α]² (°) (DMF)</th>
<th>Analysis (%) Cacl (Found)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>7-Residue (20–26)</td>
<td>87</td>
<td>161–163</td>
<td>-13.6 (c=0.6)</td>
<td></td>
</tr>
<tr>
<td>13-Residue (14–26)</td>
<td>89</td>
<td>218 (dec.)</td>
<td>-6.8 (c=0.4)</td>
<td></td>
</tr>
<tr>
<td>Residue (7–26)</td>
<td>84</td>
<td>222 (dec.)</td>
<td>-17.0 (c=0.5)</td>
<td></td>
</tr>
<tr>
<td>26-Residue (1–26)</td>
<td>84</td>
<td>-18.2 (c=0.4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\[ \text{C_{6}H_{10}Br_{2}N_{2}O_{2}S_{2}H_{2}O} \]

- 55.84 6.27 10.98
- 56.67 6.24 11.08
- 56.81 6.61 11.13
- 56.74 6.78 10.98
- 55.80 7.1 (c=0.5, DMF), Rf 0.87. Anal. Calc'd for C_{6}H_{10}Br_{2}N_{2}O_{2}S_{2} [1/2H_{2}O]. C: 63.47, H: 6.77, N: 7.33. Found C: 63.32, H: 6.76, N: 7.72.

Boc-Asp(OeHex)-Ser(Bzl)-Gly-Cys(Tacm)-Phe-Gly-OPac | The title compound was prepared by the same procedure as employed for the preparation of the corresponding Cys(Tacm) derivative: yield 70% mp 169–171 °C, [α]² = -16.0° (c=0.5, DMF), Rf 0.87. Anal. Calc'd for C_{6}H_{10}Br_{2}N_{2}O_{2}S_{2}H_{2}O, C: 66.45, H: 6.77, N: 7.97. Found: C: 62.7; H: 6.65; N: 7.97.

Boc-Asp(OeHex)-Ser(Bzl)-Gly-Cys(Tacm)-Phe-Gly-OPac | The title compound was prepared by the same procedure as employed for the preparation of the corresponding Cys(Tacm) derivative: yield 76% mp 157–159 °C, [α]² = -21.1° (c=0.6, DMF), Rf 0.54. Ammonium acid ratio 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. Calc'd for C_{6}H_{10}N_{2}O_{2}S_{2}·2H_{2}O. C: 61.36, H: 7.01, N: 8.42. Found: C: 61.06, H: 6.98, N: 8.70.

Boc-Cys(Tacm)-Asn-Val-Leu-Arg(Tos)-Arg(Tos)-Trty(Bz)-OBzl, Boc-(pBNP 20–26)-OBzl | The title compound was prepared by the WSC plus HOBt coupling of Boc-Cys(Tacm)-OH and purified by procedure B, followed by recrystallization from THF with ether: yield 83%, Rf 0.76. Ammonium acid ratio in a 6 N HCl hydrolysate are listed in Table I. Physical constants and analytical data of each intermediate are listed in Table II.

Boc-Asp(OeHex)-Ser(Bzl)-Gly-Cys(Tacm)-Phe-Gly-Arg(Tos)-Arg(Tos)-Leu-Asp(OeHex)-Arg(Tos)-Ile-Gly-Ser(Bzl)-Leu-Ser(Bzl)-Gly-Cys(Tacm)-Asn-Val-Leu-Arg(Tos)-Arg(Tos)-Trty(Bz)-OBzl, Boc-(Cys(Tacm))²°-pBNP(26–26)-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 from Cys(Tacm)²°-pBNP by Use of 1: The purified [Cys(Tacm)]²°-pBNP (28 mg) was dissolved in 90% AcOH (200 ml) and 20% L/1EtOH (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 x 10 cm), which was eluted with 1% AcOH. The UV absorption at 280 nm was determined in each fraction (6.5 ml). The fractions containing the main peak (tube Nos. 75–90) were combined and lyophilized to give a fluffy white powder (24.6 mg). The column elution with 0.3% aqueous TFA containing 20% MeCN and applied to a YM-3 gel column was eluted with a linear gradient of MeCN (20–40%), in 0.3% aqueous TFA for 100 min (flow rate 40 ml/min). The eluate corresponding to the main peak was collected and lyophilized to give a fluffy white powder (14% yield 52% calculated from di-Tacm-pBNP, overall yield 12%, calculated from the fully protected pBNP), [α]² = -50° (c=0.1, 1% AcOH), Rf 0.35, Rf 0.69, HPLC on a Cosmosil SC-18 column (4.6 x 150 mm) [retention time: 18.5 min, gradient elution with MeCN (10–60%) 30 min 0.1% aqueous TFA, 0.7 ml/min] and a TSK gel G-2000 SW column (7.5 x 600 mm) [eluted with 0.1% MeCN, pH 4.0, 0.5 ml/min; the retention time (46.2 min) was between those of α-ANP (45.7 min, MW 3063) and adrenorphin (47.8 min, MW 9849). FAB-MS (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 calculated for C_{6}H_{10}N_{2}O_{2}S_{2} m/z 2868.447 [MH⁺] and 2869.449 (base peak in the molecular ion region). Ammonium acid ratios in a 6 N HCl hydrolysate are listed in Table I.

Boc-Cys(Tacm)-Phe-Gly-OPac | Boc-Cys(Tacm)-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, [α]² =-17.2° (c=0.6, DMF), Rf 0.55. Anal. Calc'd for C_{6}H_{10}N_{2}O_{2}S·2H_{2}O. C: 65.76, H: 6.71, N: 6.22. Found: C: 65.65, H: 6.83, N: 6.28.

Boc-Ser(Bzl)-Gly-Cys(Tacm)-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, [α]² =-13.0° (c=0.6, DMF), Rf 0.87. Anal. Calc'd for C_{6}H_{10}N_{2}O_{2}S·2H_{2}O, C: 62.80, H: 6.67, N: 7.47. Found: C: 62.77; H: 6.65; N: 7.97.

References and Notes
1) Ammonium and peptide derivatives mentioned in this paper are of the α-configuration. The following abbreviations are used: Boc = tert-butoxycarbonyl, Bzl = benzyl, Brz = 2-bromobenzyloxycarbonyl, cHex = cyclohexyl, cM = carbomethoxy, cVal = cyclohexylamino, DCHA = dicyclohexyl amine, DMF = N,N-dimethylformamide, DMSC = dimethyl sulfoxide, EDTA = ethylenediaminetetraacetic acid, HOBt = 1-hydroxybenzotriazole, MeCN = acetonitrile, Np = Na-hydroxy-2-norbornen-2,3-dicarboximidoyl.
phenyl, Pae = phenacetyl, NMM = N-methylmorpholine, Su = N-hydroxy succinimidy1, Tcm = trimethylacetimidomethyl, TFA = trithioacetic acid, THF = tetrahydrofuran, Tmb = 2,4,6-trimethylbenzyl, Tos = p-toluenesulfonyl, DCC = dicyclohexylcarbodiimide, Acn = acetimidomethyl.