

Solution Synthesis of Human Neuropeptide Y (hNPY)¹⁾

Seishi ONO,^a Kouki KITAGAWA,*^a Shiroh FUTAKI,^a Shinya KIYAMA,^a Yoshihiro OOI,^a Tadashi AKITA,^a Shunichi HIGASHIDE,^b Kazutomo INOUE,^b Syoichiro SUMI^b and Takayoshi TOBE^b

Faculty of Pharmaceutical Sciences, The University of Tokushima,^a Shō-machi 1–78, Tokushima 770, Japan and School of Medicine, Kyoto University,^b Sakyo-ku, Kyoto 606, Japan. Received December 21, 1988

Human neuropeptide Y (hNPY) was synthesized in a conventional manner by assembling seven peptide fragments followed by reduction of the Met(O) residue with phenylthiotrimethylsilane and subsequent deprotection with 1 M trimethylsilyl trifluoromethanesulfonate (TMSOTf)–thioanisole in trifluoroacetic acid (TFA). Alternatively, deprotection was performed in a two-step manner; first, treatment with 1 M trimethylsilyl bromide–thioanisole in TFA, and then with 1 M TMSOTf–thioanisole in TFA. After purification by gel-filtration on Sephadex G-25, followed by reversed-phase high-performance liquid chromatography, a highly purified sample of synthetic hNPY was obtained in both cases. When administered in dogs, synthetic hNPY was as active as porcine NPY in terms of the effects on systemic arterial blood pressure, pancreatic blood flow, and superior mesenteric artery (SMA) blood flow. Met(O)¹⁷-hNPY was found to be as active as the parent sample in these bioassays.

Keywords human neuropeptide Y (hNPY) synthesis; thioanisole-mediated deprotection; hard acid deprotection; trimethylsilyl trifluoromethanesulfonate deprotection; trimethylsilyl bromide deprotection; Met(O) reduction; phenylthiotrimethylsilane; β -cycloheptyl aspartate; hNPY activity *in vivo*

Neuropeptide Y (NPY) is one of the most abundant and widely distributed neuropeptides in the nervous system and is considered to be involved in the brain functions as a neurotransmitter or neuromodulator. Since the structure of porcine NPY was first elucidated by Tatemoto *et al.*^{2,3)} in 1982, NPYs from various mammalian species including human have been characterized by either the standard chemical method^{4–6)} or the cDNA cloning method.^{7–9)} Mammalian NPYs are highly conserved in structure and have a Met residue at position 17 in common, except for porcine NPY which has Leu at the same position. Following the syntheses of structurally related peptides, porcine NPY (pNPY)¹⁰⁾ and porcine peptide YY (PYY),¹¹⁾ we wish to report herein the solution-phase synthesis of human NPY, for which the newly established hard acid deprotecting procedure^{12–15)} was applied. The synthetic peptide and its Met-sulfoxide derivative, Met(O)¹⁷-hNPY,

were assayed in dogs.

Our synthetic scheme for hNPY is illustrated in Fig. 1. Seven peptide fragments were used to construct the peptide backbone of hNPY. Of these, fragments [5], [6], and [7] were employed for our previous synthesis of pNPY and four fragments [1], [2], [3] and [4] were newly prepared. Amino acid derivatives bearing protecting groups removable by 1 M trimethylsilyl trifluoromethanesulfonate (TMSOTf)–thioanisole/TFA were employed, *i.e.*, Lys(Z), Arg(Mts),¹⁶⁾ Glu(OBzl), Tyr(Cl₂Bzl)¹⁷⁾ and Asp(OChp).¹⁸⁾ Asp(OChp) was employed to suppress base-catalyzed succinimide formation¹⁹⁾ at the three Asp-X sequences. The Met residue at position 17 was reversibly protected as its sulfoxide²⁰⁾ to prevent air oxidation during peptide assembly and S-alkylation during N²-deprotection.

First, the C-terminal fragment [1], Z(OMe)–His–Tyr–Ile–Asn–Leu–Ile–Thr–Arg(Mts)–Gln–Arg(Mts)–Tyr(Cl₂Bzl)–NH₂–

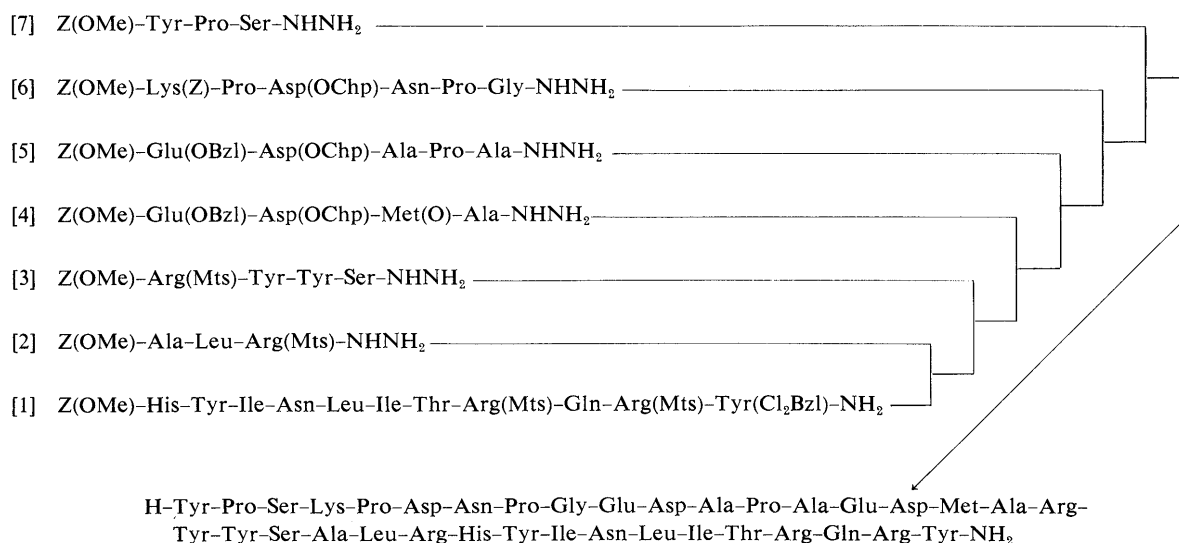


Fig. 1. Synthetic Route to Human Neuropeptide Y (hNPY)

This paper is dedicated to Professor Haruaki Yajima on this occasion of his retirement from Kyoto University in March 1989.

NH₂, was prepared in essentially the same manner as described previously. The C-terminal tetrapeptide amide, Z(OMe)-Arg(Mts)-Gln-Arg(Mts)-Tyr(Cl₂Bzl)-NH₂,¹¹⁾ was elongated to the octapeptide amide by successive azide condensations²¹⁾ with Z(OMe)-Ile-Thr-NHNH₂¹⁰⁾ and then Z(OMe)-Asn-Leu-NHNH₂.¹⁰⁾ Onto this octapeptide amide, Boc-Ile-OH was introduced by the Su active ester method,²²⁾ then Z(OMe)-His-Tyr-NHNH₂¹¹⁾ by the azide method to give fragment [1]. The purity of fragment [1] was ascertained by thin layer chromatography (TLC), elemental analysis and amino acid analysis after acid hydrolysis, as was done with other fragments.

Fragment [2], Z(OMe)-Ala-Leu-Arg(Mts)-NHNH₂, was prepared by the Su condensation of Z(OMe)-Ala-OH with a TFA-treated sample of Z(OMe)-Leu-Arg(Mts)-OMe,¹¹⁾ followed by the usual hydrazine treatment of the resulting protected tripeptide ester. Fragment [3], Z(OMe)-Arg(Mts)-Tyr-Tyr-Ser-NHNH₂, was prepared by the azide condensation of Z(OMe)-Arg(Mts)-Tyr-NHNH₂¹⁰⁾ with a TFA-treated sample of Z(OMe)-Tyr-Ser-OMe, followed by the usual hydrazine treatment of the resulting tetrapeptide ester.

Fragment [4], Z(OMe)-Glu(OBzl)-Asp(OChp)-Met(O)-Ala-NHNH₂, was prepared with the aid of Troc-NHNH₂²³⁾ as shown in Fig. 2. Onto a TFA-treated sample of Z(OMe)-Ala-NHNH-Troc,²⁴⁾ the Met(O) and the Asp(OChp) residues were successively introduced by the MA method,²⁵⁾ then the Glu(OBzl) residue by the Np method.²⁶⁾ The Troc group was removed from the resulting tetrapep-

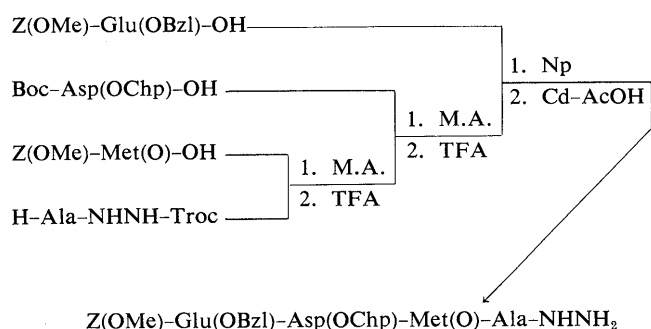


Fig. 2. Synthetic Scheme for the Protected Tetrapeptide Hydrazide [4]

ptide derivative by treatment with Cd-AcOH²⁷⁾ to give [4].

The seven fragments thus obtained were assembled successively by the azide procedure as shown in Fig. 1. Every reaction was performed in DMF and the amount of acyl component was increased from 1.5 to 3 eq as the chain elongation progressed. Each product was purified by either precipitation from DMF with AcOEt, or by column chromatography on silica gel, or by gel-filtration on Sephadex LH-60 using DMF as an eluant. In the purification of the protected-(4-36)-NH₂ and protected hNPY, gel-filtration on Sephadex LH-60 was effective to remove the possible over-reacted products at the unprotected Tyr residues. Throughout this synthesis, Leu was selected as a diagnostic amino acid in acid hydrolysis (Table I). By comparison of the recovery of Leu with those of newly incorporated amino acids after each condensation reaction, satisfactory incorporation of each fragment and purification of products were ascertained.

In the final step, we conducted deprotection in two alternative ways. First, the fully protected hNPY was treated with phenylthiotrimethylsilane²⁸⁾ in DMF to reduce the Met(O) residue to Met prior to deprotection. The progress of the reduction was monitored on TLC. Next, all protecting groups were removed from the reduced peptide by treatment with 1 M TMSOTf-thioanisole/TFA in the presence of *m*-cresol in an ice-bath for 3 h. The deprotected peptide was then treated with dilute ammonia containing NH₄F at pH 8.0 to hydrolyze the attached trimethylsilyl groups and to reverse the possible N→O shift.²⁹⁾ The deprotected product was purified by gel-filtration on Sephadex G-25, followed by high-performance liquid chromatography (HPLC) on a Chemcopak 7C₁₈ column (Fig. 3-A). The purity of synthetic hNPY was ascertained by TLC, analytical HPLC (Fig. 3-C), and amino acid analyses after acid hydrolysis and enzymic digestion.

As an alternative deprotection, we applied the two-step hard-acid treatments. It is known that 1 M trimethylsilyl bromide (TMSBr)-thioanisole/TFA has an ability to reduce Met(O) effectively and at the same time to cleave the benzyl-based protecting groups, while 1 M TMSOTf-thioanisole/TFA cleaves various protecting groups more readily than the above reagent, but reduces Met(O) par-

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

	Protected human NPY							Syn. hNPY	Residue
	26-36	23-36	19-36	15-36	10-36	4-36	1-36		
Asp	0.98	1.00	1.11	2.20	3.38	5.16	5.43	5.22	5
Thr	0.94	1.00	1.07	1.04	0.97	0.99	0.98	1.06	1
Ser			1.22	1.07	1.15	0.94	2.37	2.23	2
Glu	0.97	1.03	1.11	2.22	3.52	3.06	3.52	3.38	3
Pro					1.16	2.89	4.22	4.49	4
Gly						1.07	1.15	1.29	1
Ala		1.03	0.96	2.11	4.26	4.15	4.33	4.48	4
Met ^{a)}				0.96	1.04	0.79	0.92	1.12	1
Ile	1.71	1.87	1.94	1.91	1.74	1.93	1.69	1.71	2
Leu	1.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2
Tyr	1.77	1.77	4.40	3.95	4.06	3.91	4.98	5.11	5
Lys						1.06	1.15	1.06	1
His	0.87	0.96	0.95	0.93	0.94	0.96	0.87	0.96	1
Arg	2.10	2.66	4.45	4.21	4.18	4.16	4.00	4.15	4
Recovery (%)	87	90	79	77	97	93	88	80	

a) Met + Met(O).

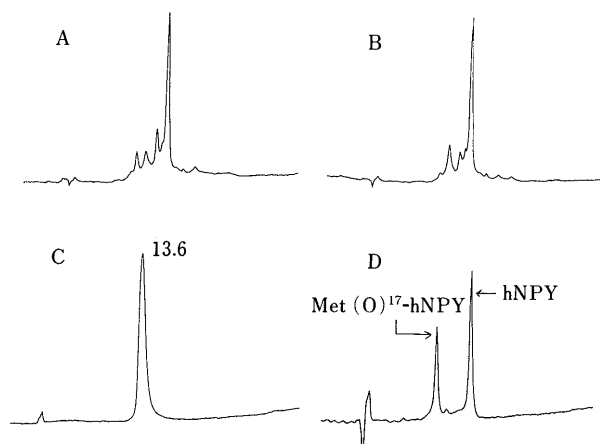


Fig. 3. HPLC Profiles of Deprotected Products and Purified hNPY

A. Product from phenylthio(trimethyl)silane reduction followed by 1 M TMSOTf-thioanisole/TFA treatment. B. Product from 1 M TMSBr-thioanisole/TFA followed by 1 M TMSOTf-thioanisole/TFA treatment. C. Finally purified hNPY from A. D. Co-chromatography of purified hNPY and purified Met(O)¹⁷-hNPY. HPLC was performed on a Chemcopak 7C₁₈ column (10 × 250 mm) for A, B, D and on a Chemcopak 7C₁₈ column (4.6 × 250 mm) for C. Isocratic elution with (A) (3 min) was followed by linear gradient elution from (A) to (B) (27 min) at a flow rate 3 ml/min (for A, B, D) and 1 ml/min (for C). (A): 32% CH₃CN (0.1% TFA). (B): 59% CH₃CN (0.1% TFA).

tially. Thus we first treated the protected hNPY with 1 M TMSBr-thioanisole/TFA at 0°C for 3 h to remove the benzyl-based protecting groups and to reduce the Met(O) residue, then with 1 M TMSOTf-thioanisole/TFA at 0°C for 2 h to remove the remaining protecting groups, such as the Chp group from the Asp and the Mts group from the Arg residues. The deprotected product was purified in the same way as above, i.e., gel-filtration on Sephadex G-25, followed by HPLC (Fig. 3-B). In this approach, the Met(O) was completely reduced, as expected, and the isolation yield (34%) was better than that of the former method (22%). This two-step hard acid deprotection can be performed in TFA, a good solvent for all protected peptides. Thus, this procedure seems to be suitable for deprotection of Met(O)-containing peptides with poor solubility in DMF.

Next, in order to evaluate the biological properties of the oxidized hNPY, Met(O)¹⁷-hNPY was prepared as follows. The fully protected hNPY was treated with 1 M TMSOTf-thioanisole/TFA and the deprotected product was treated with dilute ammonia containing NH₄F as stated above. The deprotected sample was oxidized by treatment with dilute H₂O₂ solution and purified by gel-filtration, then by HPLC to afford Met(O)¹⁷-hNPY, which was apparently distinguishable from hNPY on TLC and analytical HPLC (Fig. 3-D).

Female mongrel dogs were used to determine the biological activities of synthetic hNPY and Met(O)¹⁷-hNPY, such as effects on systemic arterial blood pressure, pancreatic tissue blood flow, and blood flow in the superior mesenteric artery (SMA) and celiac artery (CA). The results were compared with those for synthetic pNPY. Injection of synthetic hNPY (over 5 μg/kg body weight) caused a slight increase in systemic mean blood pressure comparable to that in the case of pNPY.¹⁰ At the dose of 2 μg/kg body weight, hNPY, Met(O)¹⁷-hNPY, and pNPY decreased the pancreatic blood flow (83.7%, 77.5%, and 84.0% of the basal flow expressed as 100%, respectively; *n* = 2) and SMA blood flow (77.8%, 76.5%, and 92.5% of the basal flow,

respectively; *n* = 2). At the dose of 0.5 μg/kg body weight, hNPY and Met(O)¹⁷-hNPY decreased the CA blood flow (72.6 ± 7.6%, and 82.8 ± 5.7%, respectively; *n* = 4). From these results, it can be concluded that there is no significant difference in biological activities between hNPY and pNPY. It is interesting that Met(O)¹⁷-hNPY retains high activities, since many biologically active peptides containing Met lose their biological activities on oxidation.

Experimental

General experimental procedures employed here are essentially the same as described for the porcine NPY¹⁰ and PYY syntheses.¹¹ *R_f* values in TLC, performed on silica gel (precoated Silica gel 60 F₂₅₄, Merck), refer to the following solvent systems: *R_f1* CHCl₃-MeOH-H₂O (8:3:1), *R_f2* *n*-BuOH-AcOH-pyridine-H₂O (4:1:1:2), *R_f3* *n*-BuOH-AcOH-AcOEt-H₂O (1:1:1:1). The melting points are uncorrected. The optical rotation was determined with a Union PM-201 polarimeter. HPLC was conducted with a Hitachi L-6200 model equipped with a Chemcopak (Nucleosil 7C₁₈, 4.6 × 250 mm or 10 × 250 mm) column. Acid hydrolysis with 6 N HCl was carried out in a sealed tube, and amino acid analysis was performed on a Hitachi 835 model amino acid analyzer. Leucine-aminopeptidase (Lot No. 62F-8000) was purchased from Sigma Chemical Co.

Products were purified by one of the following procedures. Procedure A: For purification of a product soluble in AcOEt, the extract was washed with 5% citric acid, 5% Na₂CO₃ and brine, then dried over Na₂SO₄ and concentrated. The residue was recrystallized or precipitated from appropriate solvents. Procedure B: For purification of a peptide 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% NaHCO₃ and H₂O, and recrystallized or precipitated from appropriate solvents.

Z(OMe)-Ile-Thr-Arg(Mts)-Gln-Arg(Mts)-Tyr(Cl₂Bzl)-NH₂ (1) The azide [prepared from 6.28 g (15.3 mmol) of Z(OMe)-Ile-Thr-NHNH₂¹⁰] in DMF (60 ml) and Et₃N (2.14 ml, 15.3 mmol) were added to an ice-chilled solution of a TFA-treated sample of Z(OMe)-Arg(Mts)-Gln-Arg(Mts)-Tyr(Cl₂Bzl)-NH₂¹¹ (13.4 g, 10.2 mmol) in DMF (100 ml) containing Et₃N (1.14 ml, 10.2 mmol) and the mixture was stirred for 24 h. The product was purified by procedure B, followed by reprecipitation from DMF with AcOEt; yield 14.8 g (95%), mp 207–208°C, [α]_D²⁵ –10.5° (*c* = 0.5, DMF), *R_f1* 0.43. *Anal.* Calcd for C₇₀H₉₄Cl₂N₁₄O₁₆S₂·H₂O: C, 54.56; H, 6.28; N, 12.73. Found: C, 54.27; H, 6.14; N, 12.47.

Z(OMe)-Asn-Leu-Ile-Thr-Arg(Mts)-Gln-Arg(Mts)-Tyr(Cl₂Bzl)-NH₂ (2) The azide [prepared from 3.49 g (8.24 mmol) of Z(OMe)-Asn-Leu-NHNH₂¹⁰] in DMF (35 ml) and Et₃N (1.15 ml, 8.24 mmol) were added to an ice-chilled solution of a TFA-treated sample of 1 (9.65 g, 6.34 mmol) in DMF (100 ml) containing Et₃N (0.88 ml, 6.34 mmol) and the mixture was stirred for 24 h. The product was purified by procedure B, followed by reprecipitation from DMF with AcOEt; yield 10.4 g (94%), mp 250–252°C, [α]_D²⁵ –11.0° (*c* = 0.5, DMF), *R_f1* 0.50. *Anal.* Calcd for C₈₀H₁₁₁Cl₂N₁₇O₁₉S₂·2H₂O: C, 53.79; H, 6.49; N, 13.33. Found: C, 53.77; H, 6.38; N, 12.98.

Boc-Ile-Asn-Leu-Ile-Thr-Arg(Mts)-Gln-Arg(Mts)-Tyr(Cl₂Bzl)-NH₂ (3) A mixture of Boc-Ile-OSu (2.28 g, 6.94 mmol), Et₃N (1.40 ml, 12.7 mmol), and a TFA-treated sample of 2 (10.1 g, 5.78 mmol) in DMF (100 ml) was stirred for 72 h. The product was purified by procedure B, followed by reprecipitation from DMF with AcOEt; yield 6.82 g (66%), mp 255–256°C, [α]_D²⁵ –20.3° (*c* = 0.5, DMF), *R_f1* 0.57. *Anal.* Calcd for C₈₂H₁₂₂Cl₂N₁₈O₁₉S₂·H₂O: C, 54.20; H, 6.88; N, 13.87. Found: C, 54.06; H, 6.90; N, 13.79.

Z(OMe)-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg(Mts)-Gln-Arg(Mts)-Tyr(Cl₂Bzl)-NH₂ [1] The azide [prepared from 2.13 g (4.27 mmol) of Z(OMe)-His-Tyr-NHNH₂¹¹] in DMF (20 ml) and Et₃N (0.60 ml, 4.27 mmol) were added to an ice-chilled solution of a TFA-treated sample of 3 (6.40 g, 3.56 mmol) in DMF (60 ml) containing Et₃N (0.49 ml, 3.56 mmol) and the mixture was stirred for 24 h. The product was purified by procedure B, followed by reprecipitation from DMF with AcOEt; yield 5.55 g (86%), mp 251–253°C, [α]_D²⁵ –2.8° (*c* = 0.5, DMF), *R_f1* 0.42. *Anal.* Calcd for C₁₀₁H₁₃₈Cl₂N₂₂O₂₃S₂·3H₂O: C, 54.70; H, 6.55; N, 13.90. Found: C, 54.62; H, 6.45; N, 13.89.

Z(OMe)-Ala-Leu-Arg(Mts)-OMe (4) A mixture of Z(OMe)-Ala-OSu (4.55 g, 13.0 mmol), Et₃N (3.36 ml, 24.0 mmol), and a TFA-treated sample of Z(OMe)-Leu-Arg(Mts)-OMe¹¹ (7.64 g, 11.8 mmol) in DMF (50 ml) was stirred for 24 h. The product was purified by procedure A, followed by column chromatography on silica gel using CHCl₃-MeOH

(20:1) as an eluant. The product was triturated with isopropylether to give a powder; yield 4.50 g (53%), mp 71°C, $[\alpha]_D^{25} = -24.5^\circ$ ($c=0.5$, MeOH), R_f 0.67. *Anal.* Calcd for $C_{34}H_{50}N_6O_9S$: C, 56.80; H, 7.01; N, 11.69. Found: C, 56.88; H, 7.28; N, 11.37.

Z(OMe)-Ala-Leu-Arg(Mts)-NHNH₂ [2] The above tripeptide methyl ester **4** (4.00 g, 5.56 mmol) in MeOH (40 ml) was treated with hydrazine hydrate (3.40 ml, 10 eq) at 37°C for 24 h. The solvent was evaporated off *in vacuo* and the residue was triturated with AcOEt. The resulting solid was recrystallized from MeOH with AcOEt; yield 3.80 g (95%), mp 129–130°C, $[\alpha]_D^{25} = -35.4^\circ$ ($c=0.5$, MeOH), R_f 0.57. *Anal.* Calcd for $C_{33}H_{50}N_8O_8S \cdot 1/2H_2O$: C, 54.45; H, 7.06; N, 15.39. Found: C, 54.24; H, 7.22; N, 15.05.

Z(OMe)-Tyr-Ser-OMe (5) The title compound was prepared by the azide method and the product was purified by procedure A (*n*-BuOH was used instead of AcOEt), followed by recrystallization from MeOH with AcOEt; yield 89%, mp 137–139°C, $[\alpha]_D^{25} = -17.8^\circ$ ($c=0.5$, MeOH), R_f 0.58. *Anal.* Calcd for $C_{22}H_{26}N_2O_8$: C, 59.18; H, 5.87; N, 6.28. Found: C, 59.17; H, 5.97; N, 6.25.

Z(OMe)-Arg(Mts)-Tyr-Tyr-Ser-OMe (6) The azide [prepared from 13.2 g (18.8 mmol) of Z(OMe)-Arg(Mts)-Tyr-NHNH₂¹⁰] in DMF (100 ml) and Et₃N (2.63 ml, 18.8 mmol) were added to an ice-chilled solution of a TFA-treated sample of **5** (7.00 g, 15.7 mmol) in DMF (70 ml) containing Et₃N (2.17 ml, 15.7 mmol) and the mixture was stirred for 24 h. The product was purified by procedure A, followed by column chromatography on silica gel using CHCl₃-MeOH (20:1) as an eluant. The product was triturated with ether to give a powder; yield 7.89 g (53%), mp 123–124°C, $[\alpha]_D^{25} = -14.9^\circ$ ($c=0.5$, MeOH), R_f 0.52. *Anal.* Calcd for $C_{46}H_{57}N_7O_{13}S \cdot H_2O$: C, 57.19; H, 6.16; N, 10.15. Found: C, 57.09; H, 6.04; N, 10.02.

Z(OMe)-Arg(Mts)-Tyr-Tyr-Ser-NHNH₂ [3] The above tetrapeptide methyl ester **6** (7.50 g, 7.90 mmol) in DMF (50 ml) was treated with hydrazine hydrate (3.95 ml, 10 eq) at 37°C for 24 h. The solvent was evaporated off *in vacuo* and the residue was triturated with EtOH. The resulting powder was washed with cold EtOH; yield 5.85 g (78%), mp 203–204°C, $[\alpha]_D^{25} = -13.4^\circ$ ($c=0.5$, DMF), R_f 0.45. *Anal.* Calcd for $C_{45}H_{57}N_9O_{12}S \cdot H_2O$: C, 55.88; H, 6.15; N, 13.04. Found: C, 56.09; H, 6.07; N, 13.02.

Z(OMe)-Met(O)-Ala-NHNH-Troc (7) The title compound was prepared by the MA method and the product was purified by procedure A, followed by recrystallization from MeOH with *n*-hexane; yield 5.31 g (85%), mp 185–186°C, $[\alpha]_D^{25} = -20.2^\circ$ ($c=0.5$, MeOH), R_f 0.63. *Anal.* Calcd for $C_{20}H_{27}Cl_3N_4O_8S$: C, 40.72; H, 4.61; N, 9.50. Found: C, 40.45; H, 4.55; N, 9.37.

Boc-Asp(OChp)-Met(O)-Ala-NHNH-Troc (8) The MA [prepared from 3.48 g (10.6 mmol) of Boc-Asp(OChp)-OH] in DMF (30 ml) was added to an ice-chilled solution of a TFA-treated sample of **7** (5.20 g, 8.80 mmol) in DMF (40 ml) containing Et₃N (1.21 ml, 8.80 mmol) and the mixture was stirred for 5 h. The product was purified by procedure A, followed by recrystallization from AcOEt with ether; yield 4.90 g (71%), mp 131–132°C, $[\alpha]_D^{25} = -23.6^\circ$ ($c=0.5$, MeOH), R_f 0.59. *Anal.* Calcd for $C_{27}H_{44}Cl_3N_5O_{10}S$: C, 43.99; H, 6.02; N, 9.50. Found: C, 44.25; H, 6.23; N, 9.53.

Z(OMe)-Glu(OBzl)-Asp(OChp)-Met(O)-Ala-NHNH-Troc (9) A mixture of Z(OMe)-Glu(OBzl)-ONp (3.60 g, 6.90 mmol), NMM (1.45 ml,

13.2 mmol) and a TFA-treated sample of **8** (4.63 g, 6.30 mmol) in DMF (80 ml) was stirred for 24 h. The product was purified by procedure A, followed by recrystallization from MeOH with ether; yield 5.03 g (79%), mp 125–126°C, $[\alpha]_D^{25} = -76.8^\circ$ ($c=0.5$, MeOH), R_f 0.70. *Anal.* Calcd for $C_{43}H_{57}Cl_3N_6O_{14}S$: C, 50.61; H, 5.63; N, 8.24. Found: C, 50.40; H, 5.71; N, 8.17.

Z(OMe)-Glu(OBzl)-Asp(OChp)-Met(O)-Ala-NHNH₂ [4] The above protected tetrapeptide derivative **9** (4.80 g, 4.70 mmol) in DMF-AcOH (40 ml–10 ml) was treated with Cd powder (4.00 g) for 4 h. The solution was filtered, and the filtrate was concentrated *in vacuo*. The residue was treated with 10% EDTA to form a powder, which was washed with 10% EDTA and H₂O in a batchwise manner, then recrystallized from DMF with AcOEt; yield 2.92 g (74%), mp 149–150°C, $[\alpha]_D^{25} = -15.5^\circ$ ($c=0.5$, DMF), R_f 0.60. *Anal.* Calcd for $C_{40}H_{56}N_6O_{12}S \cdot H_2O$: C, 55.67; H, 6.77; N, 9.74. Found: C, 55.71; H, 6.64; N, 9.61.

Synthesis of Protected hNPY Successive azide condensations of the seven fragments were carried out according to the indicated route (Fig. 1). Prior to condensation, the Z(OMe) group was removed from the respective amino component by treatment with TFA (*ca.* 0.5 ml per 0.1 g of the peptide) in the presence of anisole (*ca.* 10 eq) in an ice-bath for 60 min. The TFA-treated sample was precipitated with dry ether, dried over KOH pellets *in vacuo* for 2 h and dissolved in DMF containing Et₃N (1 eq). The corresponding azide (the amount was increased from 1.5 to 3 eq as the chain elongation progressed) in DMF and Et₃N (1 eq) were added to the above ice-chilled solution and the mixture was stirred at –4°C until the solution became negative to the ninhydrin test. The DMF was evaporated off *in vacuo* and the residue was triturated with ether to afford a solid, which was purified by procedure B, followed by reprecipitation from DMF with AcOEt or ether. For the purification of Z(OMe)-(15–36)-NH₂, column chromatography on silica gel was employed using CHCl₃-MeOH-H₂O (45:10:1) as an eluant (procedure C). For the purification of Z(OMe)-(4–36)-NH₂ and Z(OMe)-(1–36)-NH₂, gel-filtration on Sephadex LH-60 (3.5 × 110 cm) was employed using DMF as an eluant. In this case, eluates (10 g each) were examined by measuring the ultraviolet (UV) absorption at 280 nm and the fractions corresponding to the front main peak were combined. The solvent was removed by evaporation *in vacuo* and the residue was treated with ether to afford a powder (procedure D). Purification procedures, yields, physical constants and analytical data of protected hNPY and its protected intermediates are listed in Table II.

H-Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Met-Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-NH₂, hNPY a) Phenylthiotrimethylsilane reduction procedure: The fully protected 36-peptide amide (118.9 mg, 20 μmol) was dissolved in distilled DMF (10 ml) and treated with phenylthiotrimethylsilane (200 μl, 50 eq) for 1 h at 40°C. The solvent was removed by evaporation *in vacuo* and the residue was triturated with dry ether to afford a powder, which was collected by centrifugation and dried over KOH pellets *in vacuo*; yield 102.5 mg (86%), R_f 0.40. The dried powder (50 mg, 8.44 μmol) was treated with 1 M TMSOTf-thioanisole/TFA (2.25 ml) in the presence of *m*-cresol (110 μl) in an ice-bath for 3 h, then dry ether was added. The resulting powder was collected by centrifugation, dried over KOH pellets *in vacuo* for 2 h and dissolved in H₂O (20 ml). The pH of the solution was adjusted to 8.0 with 5% NH₄OH and 5 M NH₄F (50 μl) was added. After 30 min, the pH of the ice-chilled

TABLE II. Characterization of Protected hNPY and Its Intermediates

	Puri.	Yield (%)	mp (°C)	$[\alpha]_D^{25}$ (DMF)	R_f	Formula	Analysis (%) Calcd (Found)		
							C	H	N
Z(OMe)-(23–36)-NH ₂	B	80	218–221	+4.3	0.30	C ₁₂₅ H ₁₇₆ Cl ₂ N ₂₈ O ₂₈ S ₃ ·5H ₂ O	54.08 (53.99)	6.75 (6.68)	14.13 (13.86)
Z(OMe)-(19–36)-NH ₂	B	53	172–173	+7.2	0.28	C ₁₆₁ H ₂₂₁ Cl ₂ N ₃₅ O ₃₇ S ₄ ·2H ₂ O	55.66 (55.81)	6.53 (6.75)	14.11 (13.70)
Z(OMe)-(15–36)-NH ₂	C	55	153–155	+11.1	0.32	C ₁₉₂ H ₂₆₅ Cl ₂ N ₃₉ O ₄₆ S ₅ ·3H ₂ O	55.69 (55.67)	6.60 (6.81)	13.19 (12.90)
Z(OMe)-(10–36)-NH ₂	B	95	158–160	+13.1	0.41	C ₂₂₆ H ₃₁₂ Cl ₂ N ₄₄ O ₅₅ S ₅ ·6H ₂ O	55.80 (55.85)	6.71 (6.60)	12.67 (12.20)
Z(OMe)-(4–36)-NH ₂	D	85	156–158	+11.5	0.45	C ₂₆₇ H ₃₇₀ Cl ₂ N ₅₂ O ₆₆ S ₅ ·6H ₂ O	56.22 (56.17)	6.75 (7.00)	12.77 (12.70)
Z(OMe)-(1–36)-NH ₂	D	74	162–164	+1.5	0.32	C ₂₈₄ H ₃₉₁ Cl ₂ N ₅₅ O ₇₁ S ₅ ·8H ₂ O	56.03 (55.79)	6.74 (6.65)	12.65 (12.44)

solution was adjusted to 5.5 with 1N AcOH and the solution was lyophilized. This sample was dissolved in 5% AcOH (3 ml) and applied to a column of Sephadex G-25 (2.5 × 110 cm), which was eluted with 5% AcOH. The fractions (5 g each) corresponding to the main peak (tube Nos. 49–61, monitored by UV measurement at 275 nm) were combined and the solvent was removed by lyophilization to give a fluffy powder; yield 21.8 mg (61%).

Subsequent purification was performed by reversed-phase HPLC on a Chemcopak (Nucleosil 7C₁₈, 10 × 250 mm) column. A part of the above crude sample (ca. 2 mg each) was applied to a column, which was eluted with a linear gradient of acetonitrile (32–59% in 27 min) in 0.1% aqueous TFA at a flow rate of 3 ml/min. The eluate corresponding to the main peak (retention time 15.58 min, monitored by UV absorption measurement at 275 nm) was collected and the solvent was removed by lyophilization to give a white fluffy powder; yield 7.86 mg (22%). $[\alpha]_D^{25} = -52.1^\circ$ ($c = 0.1$, 1 M AcOH), R_f 0.28, R_f 0.33; retention time, 13.6 min in HPLC on an analytical Nucleosil 7C₁₈ column (4.6 × 250 mm) (Fig. 3-C). Amino acid ratios in a 6N HCl hydrolysate are shown in Table I. Amino acid ratios in a LAP digest (numbers in parentheses are theoretical): Asp 2.54 (3), Thr 1.25 (1), Ser 2.39 (2), Glu 2.04 (2), Pro 3.23 (4), Gly 1.13 (1), Ala 3.80 (4), Met 0.88 (1), Ile 2.14 (2), Leu 2.39 (2), Tyr 5.00 (5), Lys 1.11 (1), His 1.03 (1), Arg 3.87 (4), recovery of Tyr: 78%. As Asn (2) and Gln (1) were co-eluted with Thr and Ser, the ratios of both amino acids were slightly high.

b) Two-step hard acid deprotection procedure: The fully protected 36-peptide amide (50 mg, 8.44 μmol) was treated with 1 M TMSBr–thioanisole/TFA (2.25 ml) in the presence of *m*-cresol (110 μl) in an ice-bath for 3 h, then dry ether was added. The resulting precipitate was collected by centrifugation and dried over KOH pellets *in vacuo*. This dried sample was next treated with 1 M TMSOTf–thioanisole/TFA (2.25 ml) in the presence of *m*-cresol (110 μl) in an ice-bath for 2 h. The precipitate obtained by addition of dry ether was collected by centrifugation, then dissolved in H₂O (10 ml). The pH of the solution was adjusted to 8.5 with 5% NH₄OH containing 5 M NH₄F, then to 5.5 with 5% AcOH as described above. After lyophilization, this sample was applied to a Sephadex G-25 column and a fluffy powder was obtained from the main peak fraction after lyophilization; yield 30.2 mg (84%). This sample was similarly purified by HPLC to give a homogeneous product; yield 12.1 mg (34%).

Met(O)¹⁷-hNPY The fully protected 36-peptide amide (50 mg, 8.44 μmol) was treated with 1 M TMSOTf–thioanisole/TFA (2.25 ml) in the presence of *m*-cresol (110 μl) in an ice-bath for 3 h, then dry ether was added. The resulting powder was similarly treated with NH₄F in H₂O (pH 8.0) as described above, then lyophilized. This sample was dissolved in H₂O (5 ml) and treated with 3% H₂O₂ (200 μl) overnight at 4 °C, then the reaction mixture was subjected to gel-filtration on Sephadex G-25. The fluffy powder was obtained from the main peak fraction after lyophilization; yield 30.5 mg (85%). A part of this sample (15 mg) was purified by HPLC to give a homogeneous product; yield 5.90 mg. R_f 0.05, R_f 0.10; retention time, 12.8 min in HPLC on a Nucleosil 7C₁₈ column (10 × 250 mm) (Fig. 3-D). Amino acid ratios in a 6N HCl hydrolysate (numbers in parentheses are theoretical): Asp 5.25 (5), Thr 1.00 (1), Ser 1.97 (2), Glu 3.21 (3), Pro 4.46 (4), Gly 1.18 (1), Ala 4.00 (4), Met + Met(O) 0.74 (1), Ile 1.88 (2), Leu 2.00 (2), Tyr 4.97 (5), Lys 1.05 (1), His 1.03 (1), Arg 4.31 (4), recovery of Leu: 81%.

Acknowledgements We wish to express our gratitude to Professor Haruaki Yajima of Kyoto University for his encouragement during this investigation. Thanks are also due to Dr. Nobutaka Fujii and Dr. Susumu Funakoshi of Kyoto University for amino acid analysis.

References and Notes

- 1) Amino acids, peptides and their derivatives in this paper are of the L-configuration. Abbreviations used are those recommended by the I.U.P.A.C.-I.U.B. Commission on Biochemical Nomenclature, *J. Biol. Chem.*, **247**, 977 (1972). Z = benzyloxycarbonyl, Z(OMe) =

p-methoxybenzyloxycarbonyl, Bzl = benzyl, Mts = mesitylenesulfonyl, Chp = β -cycloheptyl, Cl₂Bzl = 2,6-dichlorobenzyl, Np = *p*-nitrophenyl, Su = *N*-hydroxysuccinimidyl, TFA = trifluoroacetic acid, TMSOTf = trimethylsilyl trifluoromethanesulfonate, TMSBr = trimethylsilyl bromide, Et₃N = triethylamine, DMF = dimethylformamide, MeOH = methanol, EtOH = ethanol, AcOEt = ethyl acetate, AcOH = acetic acid, cDNA = complementary deoxyribonucleic acid, EDTA = ethylenediamine tetraacetic acid.

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