Solution Synthesis of Human Neuropeptide Y (hNPY)[1)]

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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 mesentric 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(Cl_2Bzl)[17)] and Asp(OChp).[18)] Asp(OChp) was employed to suppress base-catalyzed succinimide formation[19)] at the three Asp-X sequences. The Met residue at position 17 was reversibly protected as its sulfoxide[20)] to prevent air oxidation during peptide assembly and S-alkylation during N^{α}-deprotection.

First, the C-terminal fragment [1], Z(OMe)–His–Tyr–Ile–Asn–Leu–Ile–Thr–Arg(Mts)–Gln–Arg(Mts)–Tyr(Cl_2Bzl)–

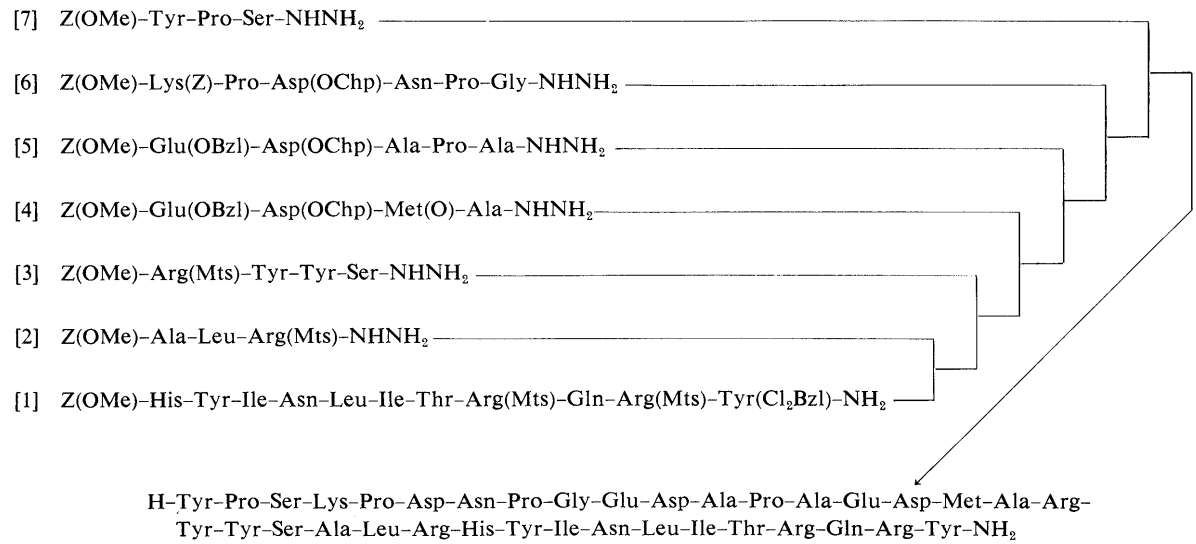

Fig. 1. Synthetic Route to Human Neuropeptide Y (hNPY)

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

NH_2, was prepared in essentially the same manner as described previously. The C-terminal tetrapeptide amide, Z(OMe)–Arg(Mts)–Gln–Arg(Mts)–Tyr(Cl_2Bzl)–NH_2,[11] was elongated to the octapeptide amide by successive azide condensations[21] with Z(OMe)–Ile–Thr–$NHNH_2$[10] and then Z(OMe)–Asn–Leu–$NHNH_2$.[10] Onto this octapeptide amide, Boc–Ile–OH was introduced by the Su active ester method,[22] then Z(OMe)–His–Tyr–$NHNH_2$[11] 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)–$NHNH_2$, 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–$NHNH_2$, was prepared by the azide condensation of Z(OMe)–Arg(Mts)–Tyr–$NHNH_2$[10] 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–$NHNH_2$, was prepared with the aid of Troc–$NHNH_2$[23] 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 tetrapeptide derivative by treatment with Cd–AcOH[27] to give [4].

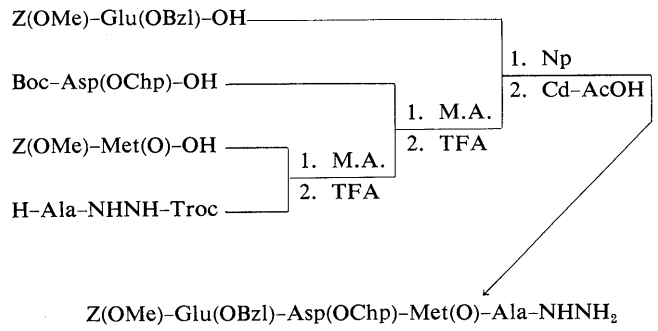

Fig. 2. Synthetic Scheme for the Protected Tetrapeptide Hydrazide [4]

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)–NH_2 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 phenylthiotrimethylsilane[28] 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 NH_4F 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 7C_{18} 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 6 N 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).

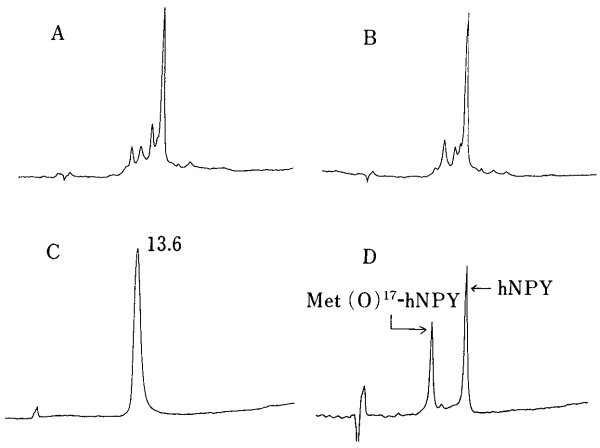

Fig. 3. HPLC Profiles of Deprotected Products and Purified hNPY

A. Product from phenylthiotrimethylsilane 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 $7C_{18}$ column (10 × 250 mm) for A, B, D and on a Chemcopak $7C_{18}$ 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% CH_3CN (0.1% TFA). (B): 59% CH_3CN (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 was 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 NH_4F as stated above. The deprotected sample was oxidized by treatment with dilute H_2O_2 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 NPY[10] and PYY syntheses.[11] *Rf* values in TLC, performed on silica gel (precoated Silica gel 60 F_{254}, Merck), refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH–H_2O (8:3:1), Rf_2 *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2), Rf_3 *n*-BuOH–AcOH–AcOEt–H_2O (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 $7C_{18}$, 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% Na_2CO_3 and brine, then dried over Na_2SO_4 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% $NaHCO_3$ and H_2O, and recrystallized or precipitated from appropriate solvents.

Z(OMe)–Ile–Thr–Arg(Mts)–Gln–Arg(Mts)–Tyr(Cl_2Bzl)–NH_2 (1) The azide [prepared from 6.28 g (15.3 mmol) of Z(OMe)–Ile–Thr–$NHNH_2$[10]] in DMF (60 ml) and Et_3N (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(Cl_2Bzl)–NH_2[11] (13.4 g, 10.2 mmol) in DMF (100 ml) containing Et_3N (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, $[\alpha]_D^{25}$ −10.5° (c = 0.5, DMF), Rf_1 0.43. *Anal.* Calcd for $C_{70}H_{94}Cl_2N_{14}O_{16}S_2 \cdot H_2O$: 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(Cl_2Bzl)–NH_2 (2) The azide [prepared from 3.49 g (8.24 mmol) of Z(OMe)–Asn–Leu–$NHNH_2$[10]] in DMF (35 ml) and Et_3N (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 Et_3N (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, $[\alpha]_D^{25}$ −11.0° (c=0.5, DMF), Rf_1 0.50. *Anal.* Calcd for $C_{80}H_{111}Cl_2N_{17}O_{19}S_2 \cdot 2H_2O$: 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(Cl_2Bzl)–NH_2 (3) A mixture of Boc–Ile–OSu (2.28 g, 6.94 mmol), Et_3N (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, $[\alpha]_D^{25}$ −20.3° (c=0.5, DMF), Rf_1 0.57. *Anal.* Calcd for $C_{82}H_{122}Cl_2N_{18}O_{19}S_2 \cdot H_2O$: 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(Cl_2Bzl)–NH_2 [1] The azide [prepared from 2.13 g (4.27 mmol) of Z(OMe)–His–Tyr–$NHNH_2$[11]] in DMF (20 ml) and Et_3N (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 Et_3N (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, $[\alpha]_D^{25}$ −2.8° (c=0.5, DMF), Rf_1 0.42. *Anal.* Calcd for $C_{101}H_{138}Cl_2N_{22}O_{23}S_2 \cdot 3H_2O$: 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), Et_3N (3.36 ml, 24.0 mmol), and a TFA-treated sample of Z(OMe)–Leu–Arg(Mts)–OMe[11] (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 $CHCl_3$–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° (c=0.5, MeOH), Rf_1 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)–NHNH$_2$ [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° (c=0.5, MeOH), Rf_1 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° (c=0.5, MeOH), Rf_1 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–NHNH$_2$[10]] in DMF (100 ml) and Et_3N (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 Et_3N (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 $CHCl_3$–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° (c=0.5, MeOH), Rf_1 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–NHNH$_2$ [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° (c=0.5, DMF), Rf_1 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° (c=0.5, MeOH), Rf_1 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 Et_3N (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° (c=0.5, MeOH), Rf_1 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° (c=0.5, MeOH), Rf_1 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–NHNH$_2$ [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 H_2O in a batchwise manner, then recrystallized from DMF with AcOEt; yield 2.92 g (74%), mp 149—150 °C, $[\alpha]_D^{25}$ −15.5° (c=0.5, DMF), Rf_1 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 Et_3N (1 eq). The corresponding azide (the amount was increased from 1.5 to 3 eq as the chain elongation progressed) in DMF and Et_3N (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)–NH$_2$, column chromatography on silica gel was employed using $CHCl_3$–MeOH–H_2O (45 : 10 : 1) as an eluant (procedure C). For the purification of Z(OMe)–(4—36)–NH$_2$ and Z(OMe)–(1—36)–NH$_2$, 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–NH$_2$, 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%), Rf_1 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 H_2O (20 ml). The pH of the solution was adjusted to 8.0 with 5% NH_4OH and 5 M NH_4F (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)	Rf_1	Formula	Analysis (%) Calcd (Found) C	H	N
Z(OMe)–(23—36)–NH$_2$	B	80	218—221	+4.3	0.30	$C_{125}H_{176}Cl_2N_{28}O_{28}S_3 \cdot 5H_2O$	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	$C_{161}H_{221}Cl_2N_{35}O_{37}S_4 \cdot 2H_2O$	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	$C_{192}H_{265}Cl_2N_{39}O_{46}S_5 \cdot 3H_2O$	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	$C_{226}H_{312}Cl_2N_{44}O_{55}S_5 \cdot 6H_2O$	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	$C_{267}H_{370}Cl_2N_{52}O_{66}S_5 \cdot 6H_2O$	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	$C_{284}H_{391}Cl_2N_{55}O_{71}S_5 \cdot 8H_2O$	56.03 (55.79)	6.74 (6.65)	12.65 (12.44)

solution was adjusted to 5.5 with 1 N 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 $7C_{18}$, 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° (*c* = 0.1, 1 M AcOH), Rf_2 0.28, Rf_3 0.33; retention time, 13.6 min in HPLC on an analytical Nucleosil $7C_{18}$ column (4.6 × 250 mm) (Fig. 3-C). Amino acid ratios in a 6 N 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 H_2O (10 ml). The pH of the solution was adjusted to 8.5 with 5% NH_4OH containing 5 M NH_4F, 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 NH_4F in H_2O (pH 8.0) as described above, then lyophilized. This sample was dissolved in H_2O (5 ml) and treated with 3% H_2O_2 (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. Rf_2 0.05, Rf_3 0.10; retention time, 12.8 min in HPLC on a Nucleosil $7C_{18}$ column (10 × 250 mm) (Fig. 3-D). Amino acid ratios in a 6 N 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%.

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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, Cl_2Bzl = 2,6-dichlorobenzyl, Np = *p*-nitrophenyl, Su = *N*-hydroxysuccinimidyl, TFA = trifluoroacetic acid, TMSOTf = trimethylsilyl trifluoromethanesulfonate, TMSBr = trimethylsilyl bromide, Et_3N = 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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