Amino Acids and Peptides. XXIV.[1] Preparation and Antinociceptive Effect of [D-Ala^2,(*N*-Me)Phe^4]Enkephalin Analog-Poly(Ethylene Glycol) Hybrids

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Hybrids of amino-poly(ethylene glycol) (aPEG) and [D-Ala^2,(*N*-Me)Phe^4]enkephalin analogs, H–Tyr-D-Ala–Gly-(Me)Phe–aPEG, H–Tyr–D-Ala–Gly–(Me)Phe–Leu–aPEG and H–Tyr–D-Ala–Gly–(Me)Phe–D-Leu–aPEG, were prepared by the solution method and their antinociceptive properties were examined in comparison with those of the peptides. H–Tyr–D-Ala–Gly–(Me)Phe–OH and H–Tyr–D-Ala–Gly–(Me)Phe–Leu–OH themselves at intracerebroventricular (i.c.v.) doses of 10—30 nmol/animal produced an antinociceptive effect which was less potent than that of i.c.v. morphine, 3 μg/animal, and H–Tyr–D-Ala–Gly–(Me)Phe–D-Leu–OH did not have any marked effect. However, the antinociceptive effects of H–Tyr–D-Ala–Gly–(Me)Phe–Leu–OH and H–Tyr–D-Ala–Gly–(Me)Phe–D-Leu–OH were remarkably potentiated by hybrid formation with aPEG to levels higher than that of 3 μg/mouse of morphine, and the effect lasted at least 120 min. In contrast, the effect of H–Tyr–D-Ala–Gly–(Me)Phe–OH was rather diminished by hybrid formation. In view of the low toxicity and weak immunogenic properties of aPEG, the hybrids could be useful in therapy of patients for relieving chronic and severe pain.

Keywords enkephalin; poly(ethylene glycol) hybrid; antinociceptive effect; analgesic

The development of drug-support conjugates as improved drug delivery systems (DDS) is useful for enhancing the therapeutic effect. Poly(ethylene glycol) (PEG) is an appropriate support for some bioactive reagents because of its low toxicity and weak immunogenicity. Previously we synthesized amino-PEG(aPEG) hybrids of Leu-enkephalin (LEnk) and [D-Ala^2]LEnk and examined their biological activities; the hybrids exhibited more potent antinociceptive effects than their parent peptides.[2] In addition to the replacement of Gly^2 with D-Ala(DAla),[3] *N*-alkylation of Phe was also reported as a strategy to enhance the opioid activity of enkephalin analogs.[4]

To obtain more potent analgesics, hybrids of [DAla^2]-LEnk analogs containing *N*-methylated Phe ((Me)Phe) and D-Leu (DLeu) were prepared. Hybrids of the [DAla^2, (Me)Phe^4]LEnk analogs Tyr–DAla–Gly–(Me)Phe–aPEG, Tyr–DAla–Gly–(Me)Phe–Leu–aPEG and Tyr–DAla–Gly–(Me)Phe–DLeu–aPEG were prepared and their antinociceptive properties were examined in mice, in comparison with those of the analogs themselves. For the preparation of the hybrids, PEG #4000 (M.W. 3000—3700) was converted to aPEG according to the procedure reported by Pillai and Mutter.[5] The amino content of aPEG was determined by titration using methyl orange as an indicator, and it ranged from 0.26 to 0.56 meq/g among lots. PEG #4000 was selected because we speculated that it might not be so bulky as to hinder the binding of the peptide portion to its receptor. β-Endorphin,[6] a potent analgesic containing the Enk sequence, has a molecular weight of 3294, which does not differ much from that of the LEnk–aPEG(#4000) hybrid.

H–Tyr–DAla–Gly–(Me)Phe–OH and its aPEG hybrid were synthesized as shown in Fig. 1. The phenolic hydroxyl group of Tyr was protected with a Bzl group during the synthesis. Boc–DAla–OH and H–Gly–OBzl were coupled by the mixed anhydride method using isobutyl chloroformate,[7] followed by trifluoroacetic acid (TFA) treatment to remove the Boc group. Boc–Tyr(Bzl)–OH and H–DAla–Gly–OBzl were coupled by the mixed anhydride method, followed by hydrazine treatment to give a protected tripeptide hydrazide, Boc–Tyr(Bzl)–DAla–Gly–$NHNH_2$. The hydrazide was reacted with H–(Me)Phe–OH by the azide method[8] to form a protected tetrapeptide, Boc–Tyr(Bzl)–DAla–Gly–(Me)Phe–OH, followed by hydrogenation to remove the Bzl group. The resulting tetrapeptide was coupled with aPEG by the dicyclohexylcarbodiimide/1-hydroxybenzotriazole (DCC/HOBt) method[9] to form a hybrid. The resulting hybrid was treated with TFA to remove the Boc group and purified by LH-20 column chromatography and RP-HPLC successively. The peptide content of the hybrid was calculated from

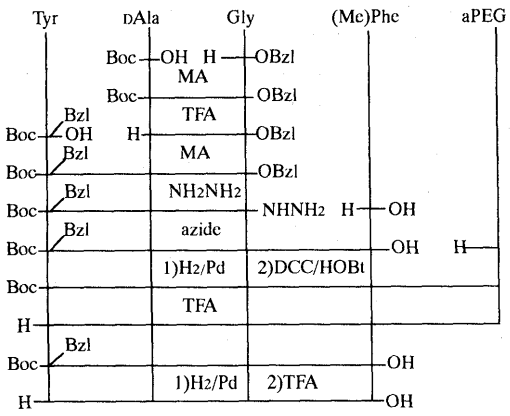

Fig. 1. Synthetic Scheme for Tyr–DAla–Gly–(Me)Phe–OH and Its aPEG Hybrid

MA: mixed anhydride method.

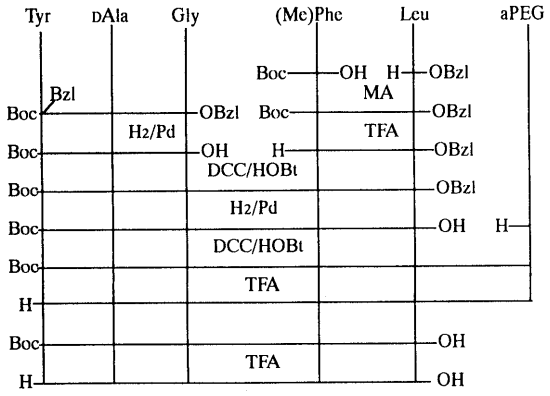

Fig. 2. Synthetic Scheme for [DAla^2, (Me)Phe^4]LEnk and Its aPEG Hybrid

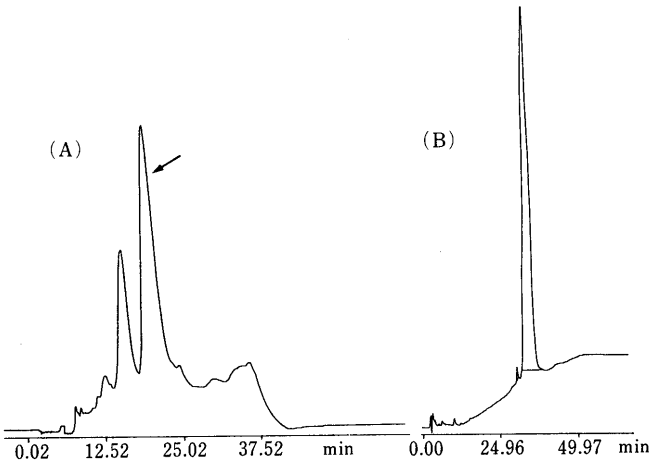

Fig. 3. HPLC Profile of Crude Tyr–DAla–Gly–(Me)Phe–Leu–aPEG (A) and Purified Tyr–DAla–Gly–(Me)Phe–Leu–aPEG (B)

(A) Semi-preparative HPLC. Column, YMC AQ-303 (4.6 × 250 mm). Flow rate, 1.0 ml/min. Solvent, A) H_2O (0.1% TFA); B) CH_3CN (0.1% TFA). Gradient, A/B: 30/70→50/50 (40 min).
(B) Column, YMC SH-343-5 (20 × 250 mm). Flow rate, 10 ml/min. Solvent, A) H_2O (0.1% TFA); B) CH_3CN (0.1% TFA). Gradient, A/B: 35/65→50/50 (30 min).

amino acid analysis of an acid hydrolysate of the hybrid and was 202 μmol/g. H–Tyr–DAla–Gly–(Me)Phe–OH was prepared from its protected tetrapeptide, Boc–Tyr(Bzl)–DAla–Gly–(Me)Phe–OH, by hydrogenation and TFA treatment. [DAla^2,(Me)Phe^4]LEnk–aPEG hybrid was prepared as shown in Fig. 2. Boc–Tyr(Bzl)–DAla–Gly–OBzl was hydrogenated to remove Bzl groups. Boc–(Me)Phe–OH and H–Leu–OBzl were coupled by the mixed anhydride method and the product was treated with TFA to give H–(Me)Phe–Leu–OBzl. Boc–Tyr–DAla–Gly–OH and H–(Me)Phe–Leu–OBzl were coupled by the DCC/HOBt method, followed by hydrogenation to remove the benzyl group. The product was coupled with aPEG by the DCC/HOBt method, followed by TFA treatment to give H–Tyr–DAla–Gly–(Me)Phe–Leu–aPEG ([DAla^2,(Me)Phe^4]LEnk). The final product was purified by Sephadex G-25 column chromatography and RP-HPLC successively. The peptide content of the hybrid was 390 μmol/g. The semi-preparative and analytical HPLC charts of the product are shown in Fig. 3. The PEG-containing material tended to give a relatively broad peak in HPLC, presumably because of the heterogeneous

TABLE I. Comparison of the Antinociceptive Effect of Authentic Peptides and Their Hybrids

Drug	Dose (/mouse)	n	Antinociceptive effect (AUC)
Morphine	3 μg	28	618±65
H–Tyr–DAla–Gly–(Me)Phe–OH	10 nmol	7	320±63
	30 nmol	7	390±94
H–Tyr–DAla–Gly–(Me)Phe–aPEG	10 nmol	6	349±78
	30 nmol	6	394±59
H–Tyr–DAla–Gly–(Me)Phe–Leu–OH	10 nmol	7	313±92
	30 nmol	7	370±88
H–Tyr–DAla–Gly–(Me)Phe–Leu–aPEG	10 nmol	7	885±76[a]
	30 nmol	7	878±81[a]
H–Tyr–DAla–Gly–(Me)Phe–DLeu–OH	3 nmol	7	83±35
	10 nmol	7	113±52
	30 nmol	7	126±95
H–Tyr–DAla–Gly–(Me)Phe–DLeu–aPEG	10 nmol	6	803±47[a]
	30 nmol	7	997±32[a]

a) $p<0.01$, compared with corresponding authentic peptide at the dosage.

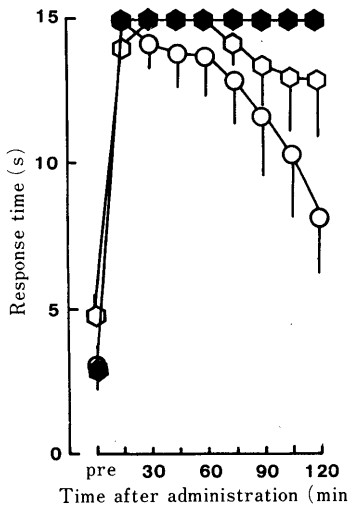

Fig. 4. Antinociceptive Effects of Hybrid and Morphine Administered i.c.v. in the Tail Pinch Method

Morphine 3 μg/mouse (○), Tyr–DAla–Gly–(Me)Phe–DLeu–aPEG 10 nmol (⬡) and 30 nmol/mouse (⬢).

molecular weight of PEG. The DLeu^5 analog, H–Tyr–DAla–Gly–(Me)Phe–DLeu–aPEG, was prepared in the same way (Fig. 2) and its peptide content was 218 μmol/g.

Antinociceptive effects of the synthetic peptides and the hybrids were examined in mice by the tail-pinch method[10] and the results are shown in Table I. H–Tyr–DAla–Gly–(Me)Phe–OH or H–Tyr–DAla–Gly–(Me)Phe–Leu–OH at doses of 10—30 nmol/animal, intracerebroventricularly (i.c.v.) administered, produced an antinociceptive effect, but less potent than that of morphine, 3 μg/animal. No appreciable antinociceptive effect of H–Tyr–DAla–Gly–(Me)Phe–DLeu–OH was found up to the dose of 30 nmol/animal. Thirty nmol/animal of H–Tyr–DAla–Gly–(Me)Phe–aPEG, like the peptide itself, produced a weak antinociceptive effect. H–Tyr–DAla–Gly–(Me)Phe–Leu–aPEG, 10 and 30 nmol/animal, and H–Tyr–DAla–Gly–(Me)Phe–DLeu–aPEG, 10 and 30 nmol/animal, however, produced remarkable antinociception; the potency of these compounds was higher than that of 3 μg/animal of morphine, and the effect lasted at

Table II. Recoveries of Tyr in Chymotryptic Digests of LEnk and LEnk–aPEG Hybrid

Enzyme (munit)	Substrate	
	LEnk (0.5 μmol) (%)	LEnk–aPEG (0.5 μmol)[a] (%)
12.5	23	0
125.0	46	28

a) LEnk content.

least 120 min (Fig. 4).

The antinociceptive effect of these two hybrids seems to be attributable to the peptide portion in the hybrids since aPEG itself did not possess any antinociceptive effect. As demonstrated in our previous report,[2a] i.c.v. LEnk–aPEG hybrid was capable of producing antinociception but LEnk was not, presumably reflecting the greater stability of LEnk–aPEG hybrid to enzymatic degradation. Unexpectedly, the LLeu epimer, H–Tyr–DAla–Gly–(Me)Phe–Leu–OH produced antinociception, whereas no appreciable antinociception by the DLeu epimer, H–Tyr–DAla–Gly–(Me)Phe–DLeu–OH was observed. However, when these peptides formed hybrids with aPEG, the hybrid with DLeu epimer showed a marked antinociceptive effect, and the effect of LLeu epimer was also intensified by the hybrid formation.

To examine the stability of a PEG hybrid to enzymatic degradation, LEnk–aPEG hybrid (H–Tyr–Gly–Gly–Phe–Leu–aPEG),[2] was treated with chymotrypsin and the recovery of Tyr in a digest was compared with that of LEnk. As shown in Table II, hydrolysis at the Tyr bond in the hybrid was slower than that in LEnk.

The low toxicity and low immunogenicity of aPEG, and the potentiation and prolongation of the antinociceptive effect of opioid peptides by hybrid formation with aPEG imply that the hybrids may be useful in the therapy of patients for relieving chronic and severe pain.

Experimental

Melting points are uncorrected. Solvent systems for ascending thin-layer chromatography (TLC) on Silica gel G (type 60, Merck) are indicated as follows: Rf^1 = BuOH–AcOH–H_2O (4:1:5, upper phase), Rf^2 = BuOH–pyridine–AcOH–H_2O (4:1:1:2), Rf^3 = $CHCl_3$–MeOH–H_2O (8:3:1, lower phase), Rf^5 = $CHCl_3$–AcOH–MeOH (90:2:8). Synthetic peptides were hydrolyzed in 6 N HCl at 110 °C for 24 h and PEG-peptide hybrids were hydrolyzed for 48 h. Amino acid compositions of acid hydrolysates were determined with a Hitachi 835 amino acid analyzer. Rotations were measured with a JASCO DIP-360 polarimeter. RP-HPLC was conducted with a Waters 600 on YMC Pack AQ-ODS-5 column using a mixture of 0.1% TFA-containing CH_3CN/H_2O as an eluent. Positive ion mass spectra were measured with a Hitachi Model M-1000 quadrupole mass spectrometer.

aPEG PEG #4000 (M.W. 3000—3700, Nacalai Tesque Co. Ltd.) was converted to aPEG according to the procedure reported by Pillai and Mutter.[5] aPEG was purified as reported in the previous paper.[2a]

Boc-DAla–Gly–OBzl Boc-DAla–OH (1.9 g, 10 mmol) and H–Gly–OBzl tosylate (3.4 g, 10 mmol) were reacted in DMF (20 ml) by the mixed anhydride method in the usual manner.[7] The product was precipitated from AcOEt/petroleum ether. Yield 2.8 g (83%), mp 93—97 °C, Rf^3 0.83, $[\alpha]_D^{22}$ +28.7° (c=1.0, MeOH). *Anal.* Calcd for $C_{17}H_{24}N_2O_5$: C, 60.69; H, 7.21; N, 8.33. Found: C, 60.97; H, 7.39; N, 8.24. Amino acid ratios in an acid hydrolysate: Ala 1.00, Gly 1.02 (average recovery 97%).

Boc–Tyr(Bzl)–DAla–Gly–OBzl Boc–DAla–Gly–OBzl (1 g, 3 mmol) and anisole (0.1 ml) was dissolved in TFA (1 ml) and the solution was stirred for 1 h at 0 °C. Chilled ether/petroleum ether (1/1) was added and the precipitate was washed with the same solvent. The precipitate was coupled with Boc–Tyr(Bzl)–OH (1.5 g, 4 mmol) in DMF by the mixed anhydride method in the usual manner.[7] The product was purified by precipitation from MeOH/petroleum ether. Yield 2.8 g (78%), mp 140—145 °C, Rf^3 0.93, $[\alpha]_D^{24}$ +24.0° (c=1.0, MeOH). *Anal.* Calcd for $C_{33}H_{39}N_3O_7$: C, 67.20; H, 6.68; N, 7.13. Found: C, 67.34; H, 6.69; N, 7.14. Amino acid ratios in an acid hydrolysate: Tyr 0.87, Ala 1.00, Gly 1.02 (average recovery 87%).

Boc–Tyr(Bzl)–DAla–Gly–N_2H_3 $NH_2NH_2 \cdot H_2O$ (0.9 ml, 19 mmol) was added to a solution of Boc–Tyr(Bzl)–DAla–Gly–OBzl (3.7 g, 6.37 mmol) in DMF/MeOH (1/1, 40 ml) and the mixture was stirred for 16 h. The solvent was removed *in vacuo* and the residue was washed repeatedly with MeOH and H_2O in a mortar. The material was precipitated from DMF/ether. Yield 2.1 g (65%), mp 168 °C, Rf^3 0.67, $[\alpha]_D^{22}$ +7.4° (c=1.0, DMF). *Anal.* Calcd for $C_{26}H_{35}N_6O_6$: C, 60.79; H, 6.88; N, 13.64. Found: C, 60.47; H, 6.98; N, 13.89.

Boc–Tyr(Bzl)–DAla–Gly–(Me)Phe–OH A 5.7 N HCl/dioxane solution (2.1 ml, 12 mmol) and isoamyl nitrite (2.1 ml, 12 mmol) were added to a solution of Boc–Tyr(Bzl)–DAla–Gly–N_2H_3 (2 g, 4 mmol) in DMF (30 ml) at −30 °C and the mixture was stirred for 15 min, followed by neutralization with Et_3N (1.7 ml, 12 mmol). The mixture was added to a solution of H–(Me)Phe–OH (0.41 g, 2.3 mmol) and Et_3N (0.33 ml, 2.3 mmol) in DMF (10 ml) and the whole was stirred for 2 d in a cold room. The solvent was removed *in vacuo* and the residue was extracted with 5% Na_2CO_3 and AcOEt. The aqueous layer was washed with AcOEt and acidified with citric acid. The resulting precipitate was washed with H_2O in a mortar and dried. The material was precipitated from DMF/ether. Yield 0.64 g (42%), mp 161—167 °C, Rf^3 0.68, $[\alpha]_D^{24}$ +23.4° (c=1.0, MeOH). *Anal.* Calcd for $C_{36}H_{44}O_6N_4 \cdot 4H_2O$: C, 58.99; H, 7.44; N, 7.65. Found: C, 59.33; H, 7.20; N, 7.65. Amino acid ratios in an acid hydrolysate: Tyr 0.89, Ala 0.96, Gly 1.00, (Me)Phe 0.86 (average recovery 81%).

H–Tyr–DAla–Gly–(Me)Phe–OH Boc–Tyr(Bzl)–DAla–Gly–(Me)Phe–OH (120 mg, 0.18 mmol) was hydrogenated over Pd catalyst in MeOH (10 ml) for 6 h. The catalyst was filtered off and the solvent was removed *in vacuo*. The residue was treated with TFA (1 ml) containing 10% anisole. A chilled mixture of ether and petroleum ether was added and the precipitate was collected by centrifugation, followed by purification by RP-HPLC. The product was lyophilized from HCl-containing H_2O to afford the hydrochloride. Yield 58 mg (66%), Rf^3 0.32, $[\alpha]_D^{20}$ +22.0° (c=1.0, H_2O). MS m/z: 471 $(M+H)^+$. Amino acid ratios in an acid hydrolysate: Tyr 0.86, Ala 1.00, Gly 1.07, (Me)Phe 0.86 (average recovery 80%).

H–Tyr–DAla–Gly–(Me)Phe–aPEG Boc–Tyr(Bzl)–DAla–Gly–(Me)Phe–OH (600 mg, 0.91 mmol) was hydrogenated over Pd catalyst in MeOH (25 ml) for 6 h. The catalyst was removed by filtration and the solvent was removed *in vacuo*. Yield 520 mg, Rf^3 0.33. The material was dissolved in a mixture of DMF and DCM (1/1, 5 ml) and DCC (206 mg, 1 mmol) and HOBt (149 mg, 1.1 mmol) were added to the solution at −10 °C. The mixture was stirred for 10 min, then combined with aPEG (amino content: 0.28 meq/g, 110 mg, 0.03 meq.) solution (DMF/DCM: 1/1, 1 ml) and the whole was stirred for 2 d in a cold room. The solvent was removed *in vacuo* and the residue was purified by Sephadex LH-20 column (3.2 × 140 cm) chromatography using MeOH as an eluent. Yield 137 mg, Rf^3 0.54. Amino acid ratios in an acid hydrolysate: Tyr 0.99, Ala 1.04, Gly 1.00, (Me)Phe 0.73. The product (100 mg) was treated with TFA (1 ml) containing m-cresol (0.05 ml) and anisole (0.05 ml) at 0 °C for 1 h. Petroleum ether was added and the resulting precipitate was washed with petroleum ether and dried. The material was purified by Sephadex G-25 column (3.2 × 140 cm) chromatography using 1% acetic acid as an eluent, followed by purification by RP-HPLC. Yield 37 mg, fluffy powder, Rf^3 0.36. Amino acid ratios in an acid hydrolysate: Tyr 0.87, Ala 1.00, Gly 1.03, (Me)Phe 0.80. Peptide content: 202 μmol/g.

H–(Me)Phe–Leu–OBzl Boc–(Me)Phe–OH (3.9 g, 14 mmol) and H–Leu–OBzl tosylate (5.5 g, 14 mmol) was coupled in DMF by the mixed anhydride method in the usual manner.[7] Yield 6.6 g (97%), oily material, Rf^5 0.78. The material (6 g) was treated with TFA (5 ml) containing anisole (0.5 ml) at 0 °C for 1 h and the product was precipitated by addition of a chilled mixture of ether/petroleum ether (1/1). Yield 4.4 g (95%), mp 98—106 °C, Rf^5 0.41, $[\alpha]_D^{24}$ +46.5° (c=1.0, MeOH). Amino acid ratios in an acid hydrolysate: (Me)Phe 1.00, Leu 1.28 (average recovery 85%). For elemental analysis, the TFA salt was converted to free dipeptide. The TFA salt (100 mg) was suspended in 3% Na_2CO_3

and extracted with AcOEt. The AcOEt was removed *in vacuo*. Yield 69 mg (90%). *Anal.* Calcd for $C_{23}H_{30}N_2O_3$: C, 72.21; H, 7.92; N, 7.32. Found: C, 71.97; H, 7.69; N, 7.24.

Boc-Tyr-DAla-Gly-(Me)Phe-Leu-OBzl Boc-Tyr(Bzl)-DAla-Gly-OBzl (1 g, 1.7 mmol) was hydrogenated over Pd catalyst in MeOH in the usual manner to give Boc-Tyr-DAla-Gly-OH. Yield 0.67 g (95%), Rf^5 0.17. The product (600 mg, 1.5 mmol) was coupled with H-(Me)Phe-Leu-OBzl (1.1 g, 2.9 mmol) in DMF by the DCC/HOBt method in the usual manner.[9] The product was purified by silica gel column (1.9×12 cm) chromatography using MeOH/$CHCl_3$ as an eluent. Fractions of 2% MeOH/$CHCl_3$ eluate were collected and the solvent was removed. The residue was precipitated from MeOH/petroleum ether. Yield 480 mg (41%), mp 156—159 °C, Rf^3 0.74, $[\alpha]_D^{24}$ +34.7° (c=1.0, MeOH). *Anal.* Calcd for $C_{42}H_{55}N_5O_9$: C, 65.17; H, 7.18; N, 9.05. Found: C, 65.16; H, 7.36; N, 9.32. Amino acid ratios in an acid hydrolysate: Tyr 0.92, Ala 1.00, Gly 1.00, (Me)Phe 0.90, Leu 1.14 (average recovery 90%).

Boc-Tyr-DAla-Gly-(Me)Phe-Leu-OH Boc-Tyr-DAla-Gly-(Me)Phe-Leu-OBzl (300 mg, 0.44 mmol) was hydrogenated over Pd catalyst in MeOH in the usual manner. The product was lyophilized from dioxane to give a powder. Yield 238 mg (92%), mp 173—175 °C, Rf^5 0.40, $[\alpha]_D^{24}$ +46.2° (c=1.0, MeOH). *Anal.* Calcd for $C_{35}H_{49}N_5O_7 \cdot 2H_2O$: C, 58.39; H, 7.43; N, 9.73. Found: C, 58.48; H, 7.10; N, 9.63. Amino acid ratios in an acid hydrolysate: Tyr 0.89, Ala 1.00, Gly 1.14, (Me)Phe 0.90, Leu 1.09 (average recovery 94%).

H-Tyr-DAla-Gly-(Me)Phe-Leu-OH Boc-Tyr-DAla-Gly-(Me)Phe-Leu-OH (63 mg, 0.1 mmol) was treated with TFA (1 ml) containing 10% anisole for 1 h in an ice-bath. A chilled mixture of ether and petroleum ether (1/1) was added and the precipitate was collected by centrifugation, followed by purification by RP-HPLC. The product was lyophilized from HCl-containing H_2O to afford the hydrochloride. Yield 42 mg (74%), Rf^3 0.32, $[\alpha]_D^{24}$ +10.4° (c=1.0, H_2O). MS m/z: 584 $(M+H)^+$. Amino acid ratios in an acid hydrolysate: Tyr 0.89, Ala 1.00, Gly 1.11, (Me)Phe 0.90, Leu 1.02 (average recovery 80%).

H-Tyr-DAla-Gly-(Me)Phe-Leu-aPEG DCC (41 mg, 0.2 mmol) was added to a solution of Boc-Tyr-DAla-Gly-(Me)Phe-Leu-OH (110 mg, 0.16 mmol), HOBt (27 mg, 0.2 mmol) and aPEG (amino content: 0.28 meq/g) (143 mg, 40 meq) in DMF/DCM (1/1, 10 ml) at −10 °C and the mixture was stirred for 20 h in a cold room. The solvent was removed *in vacuo* and the residue was extracted with DCM/DMF (1/1). Insoluble material was removed by filtration and the filtrate was purified by Sephadex LH-20 column (3.2×140 cm) chromatography using DMF/DCM (1/1) as an eluent. Yield 176 mg, Rf^3 0.55. The material was treated with TFA (1 ml) containing anisole (0.1 ml) at 0 °C for 1 h and the TFA was removed *in vacuo*. The residue was purified by Sephadex G-50 column (3.2×140 cm) chromatography using 1% AcOH as an eluent. Yield 156 mg. The material was further purified by RP-HPLC. Yield 66 mg, fluffy powder, Rf^1 0.02, Rf^3 0.38. Amino acid ratios in an acid hydrolysate: Tyr 0.93, Ala 0.96, Gly 1.14, Phe 1.00, Leu 1.07. Peptide content: 218 μmol/g.

H-(Me)Phe-DLeu-OBzl Boc-(Me)Phe-DLeu-OBzl was prepared from Boc-(Me)Phe-OH (3.90 g, 14 mmol) and H-DLeu-OBzl tosylate (5.5 g, 14 mmol) by the mixed anhydride method in the usual manner. Yield 6.0 g (88%), oily material, Rf^3 0.76. The product (5 g, 10 mmol) was treated with TFA (5 ml) containing anisole (0.5 ml) at 0 °C for 1 h and chilled ether/petroleum ether (1/1) was added. The resulting precipitate was collected by filtration and dried. Yield 3.7 g (96%), mp 155—157 °C, Rf^5 0.37, $[\alpha]_D^{24}$ +23.4° (c=1.0, MeOH). Amino acid ratios in an acid hydrolysate: (Me)Phe 1.00, Leu 1.22 (average recovery 83%).

For elemental analysis, the TFA salt was converted to the free amine in the same way as described for preparation of H-(Me)Phe-Leu-OBzl. *Anal.* Calcd for $C_{23}H_{30}N_2O_3$: C,72.21; H, 7.92; N, 7.32. Found: C, 72.00; H,7.73; N, 7.25.

Boc-Tyr-DAla-Gly-(Me)Phe-DLeu-OBzl Boc-Tyr(Bzl)-DAla-Gly-OBzl (1 g, 1.7 mmol) was hydrogenated over Pd in MeOH in the usual manner. Yield 670 mg (95%), Rf^5 0.17. The hydrogenated material (600 mg) and H-(Me)Phe-DLeu-OBzl (1.1 g, 2.9 mmol) were coupled in DMF by the DCC/HOBt method[9] in the usual manner. The product was dissolved in $CHCl_3$ and purified by silica gel column (1.9×12 cm) chromatography. The desired material was in the 2% MeOH/$CHCl_3$ eluate. The material was precipitated from AcOEt/ether. Yield 650 mg (56%), mp 149—154 °C, Rf^3 0.84, $[\alpha]_D^{24}$ +19.2° (c=1.0, MeOH). *Anal.* Calcd for $C_{42}H_{55}O_9N_5$: C, 65.17; H, 7.18; N, 9.32. Found: C, 65.01; H, 7.18; N, 9.28. Amino acid ratios in an acid hydrolysate: Tyr 0.78, Ala 1.00, Gly 1.18, (Me)Phe 0.93, Leu 1.18 (average recovery 89%).

Boc-Tyr-DAla-Gly-(Me)Phe-DLeu-OH Boc-Tyr-DAla-Gly-(Me)Phe-DLeu-OBzl (300 mg, 0.44 mmol) was hydrogenated over Pd catalyst in MeOH in the usual manner. Yield 213 mg (83%), mp 153—155 °C, Rf^5 0.40, $[\alpha]_D^{24}$ +23.8° (c=1.0, MeOH). *Anal.* Calcd for $C_{35}H_{49}N_5O_9 \cdot 2H_2O$: C, 58.39; H, 7.43; N, 9.73. Found: C, 58.52; H, 7.30; N, 9.63. Amino acid ratios in an acid hydrolysate: Tyr 0.89, Ala 1.00, Gly 1.14, (Me)Phe 0.90, Leu 1.09 (average recovery 94%).

H-Tyr-DAla-Gly-(Me)Phe-DLeu-OH Prepared from Boc-Tyr-DAla-Gly-(Me)Phe-DLeu-OH (63 mg, 0.1 mmol) by TFA treatment in the same way as described for the preparation of H-Tyr-DAla-Gly-(Me)Phe-Leu-OH. Yield 34 mg (61%), Rf^3 0.32, $[\alpha]_D^{24}$ +22.0° (c=1.0, H_2O), MS m/z: 584 $(M+H)^+$. Amino acid ratios in an acid hydrolysate: Tyr 0.89, Ala 1.00, Gly 1.09, (Me)Phe 0.91, Leu 1.09 (average recovery 87%).

H-Tyr-DAla-Gly-(Me)Phe-DLeu-aPEG DCC (41 mg, 0.2 mmol) was added to a solution of Boc-Tyr-DAla-Gly-(Me)Phe-DLeu-OH (110 mg, 0.16 mmol), HOBt (27 mg, 0.2 mmol) and aPEG (amino content 0.28 meq/g) (143 mg, 0.04 meq) in DMF/DCM (1/1, 5 ml) at −10 °C and the mixture was stirred for 20 h in a cold room. The solvent was evaporated *in vacuo* and the residue was purified by Sephadex LH-20 column (3.2×140 cm) chromatography using MeOH/DCM (1/1) as an eluent, followed by RP-HPLC. Yield 158 mg, Rf^3 0.42. The product (133 mg) was treated with TFA (1 ml) containing anisole (0.1 ml) at 0 °C for 1 h and the TFA was evaporated *in vacuo*. The residue was purified by Sephadex G-25 column (3.2×140 cm) chromatography using 1% AcOH as an eluent, followed by RP-HPLC on YMC Pack AQ-5 using H_2O/CH_3CN as an eluent. Yield 57 mg, Rf^3 0.38. Amino acid ratios in an acid hydrolysate: Tyr 0.93, Ala 0.96, Gly 1.14, (Me)Phe 0.88, Leu 1.03. Peptide content: 218 μmol/g.

Animals Male mice of the ddY strain weighing 18—20 g (Otsubo Exp. Animals, Nagasaki) were purchased and housed in groups of 20 animals. They were maintained in a room held at ambient temperature (22±1 °C) and relative humidity (55±5%) with free access to laboratory diet (MF, Oriental Yeast, Tokyo) and tap water. After reaching a body weight of 23 to 28 g, they were used for the experiments.

Drug Administration Morphine·HCl (Takeda, Osaka), naloxone·HCl (Sigma, St. Louis, U.S.A.), the synthetic peptides and their hybrids were dissolved in saline. Intracerebroventricular (i.c.v.) injection was done according to the method of Haley and McCormick,[11] and the dose was contained in a volume of 10 μl/mouse.

Assessment of Antinociceptive Effect The antinociceptive effect was measured by the tail pinch test (TP)[10] using an artery clip. The intensity of the pressure was set to provide a predrug latency time of 2—4 s (a cutoff time of 15 s) in the test. Measurements were made every 15 min after administration for a period of 90 min for authentic peptides or 120 min for the hybrids, and the effect was expressed as the area under the time-response curve (*AUC*), by plotting the increase in response time (s) on the ordinate and the time interval (min) on the abscissa (a cutoff time of 90 min).

Data Analyses The results were expressed as the mean±S.E. Following analysis of variance for repeated measures of the overall data to assess statistical significance, differences between the individual mean values in different groups were analyzed by the use of Dunnett's test.

Chymotrypsin Digestion of H-Tyr-Gly-Gly-Phe-Leu-aPEG LEnk-aPEG (0.6 mg, peptide content: 0.5 μmol) was dissolved in a mixture of phosphate buffer (pH 8.0, 1 ml) and chymotrypsin solution(25 m unit/ml, 1 ml) and the whole was shaken for 24 h at 37 °C. After dilution with citrate buffer (pH 2.0), the mixture was analyzed by an amino acid analyzer. LEnk (0.5 μmol) was also treated with chymotrypsin in the same way. Recovery of Tyr in each experiment is shown in Table II.

References and Notes

1) Standard abbreviations for amino acids, peptides and protecting groups are used [*Eur. J. Biochem.*, **138**, 9 (1984)]; other abbreviations include: DMF=dimethylformamide, Et_3N=triethylamine, DCM=dichloromethane.
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