

## Amino Acids and Peptides. XIX.<sup>1)</sup> Preparation of Enkephalin–Poly(ethylene glycol) Hybrid and Evaluation of Its Analgesic Activity

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The poly(ethylene glycol) hybrid of Leu-enkephalin (Tyr–Gly–Gly–Phe–Leu) was prepared and its analgesic activity was examined. Poly(ethylene glycol) #4000 was converted to amino-poly(ethylene glycol) and coupled with the *N*<sup>α</sup>-protected pentapeptide, followed by trifluoroacetic acid treatment to give the hybrid. The hybrid was soluble in water or various organic solvents. The analgesic activity of Leu-enkephalin was markedly potentiated by hybrid formation with poly(ethylene glycol).

**Keywords** poly(ethylene glycol) hybrid; enkephalin; enkephalin hybrid; opioid

Since poly(ethylene glycol) (PEG) is stable, has low toxicity, is bioinert and is only weakly immunogenic, it seems to be a promising candidate for a drug-carrier. Recently we found that the hybrid formation of some oligopeptides with PEG resulted in potentiation of the biological activity of the peptides.<sup>2)</sup> This paper describes the preparation of a PEG hybrid of Leu-enkephalin and evaluation of its analgesic activity.

For the preparation of the hybrid, PEG #4000 (MW, 3000–3700) was used. The molecular weight of enkephalin–PEG #4000 hybrid is similar to that of  $\beta$ -endorphin (MW 3294), which has potent analgesic activity. We speculate that the PEG #4000 portion of the hybrid, which has similar molecular weight to  $\beta$ -endorphin, does not hinder the binding of the enkephalin portion to its receptor. PEG #4000 was converted to amino-PEG (APEG) according to the procedure reported by Pillai and Mutter.<sup>3)</sup> The PEG was tosylated and reacted with potassium phthalimide, followed by hydrazine treatment to give APEG. The APEG was purified by carboxymethyl cellulose column chromatography using ammonium acetate buffer as an eluent and by Dowex 50 (H<sup>+</sup> form) column chromatography using 3% ammonia as an eluent. The amino content of the APEG was determined by titration.

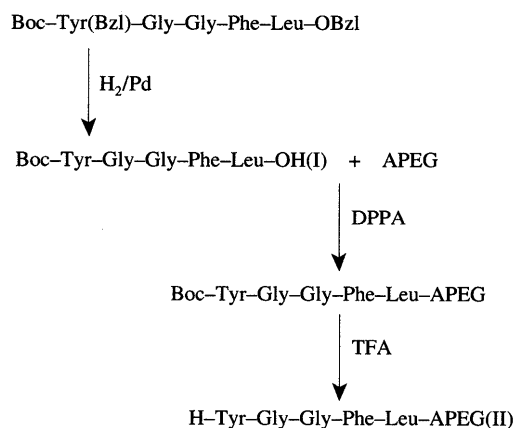


Fig. 1. Synthetic Scheme for (Leu-Enkephalin)–APEG Hybrid  
DPPA, diphenylphosphoryl azide; TFA, trifluoroacetic acid.

PEG hybrid of Leu-enkephalin was prepared as shown in Fig. 1. Boc–Tyr(Bzl)–Gly–Gly–Phe–Leu–OBzl<sup>4)</sup> was hydrogenated to give Boc–Tyr–Gly–Gly–Phe–Leu–OH(I). APEG was coupled with I by the diphenylphosphoryl azide (DPPA) method,<sup>5)</sup> followed by trifluoroacetic acid (TFA) treatment to give a PEG hybrid, H–Tyr–Gly–Gly–Phe–Leu–APEG (II) which was purified by Sephadex column chromatography and reverse-phase high-performance liquid chromatography (RP-HPLC). The Leu-enkephalin content of the hybrid was 0.35 mmol/g. The hybrid was soluble in water, dimethylformamide (DMF), methanol (MeOH), dichloromethane (DCM), and a mixture of MeOH–DCM (1/1).

The antinociceptive effect of the hybrid of Leu-enkephalin with APEG, administered intracerebroventricularly (i.c.v.), was examined by the tail-pinch method. The antinociceptive effect of the hybrid was compared with that of Leu-enkephalin on a molar basis. Prior to the assay, the analgesic effect of PEG and APEG was examined and no effect was observed. Leu-enkephalin did not produce any

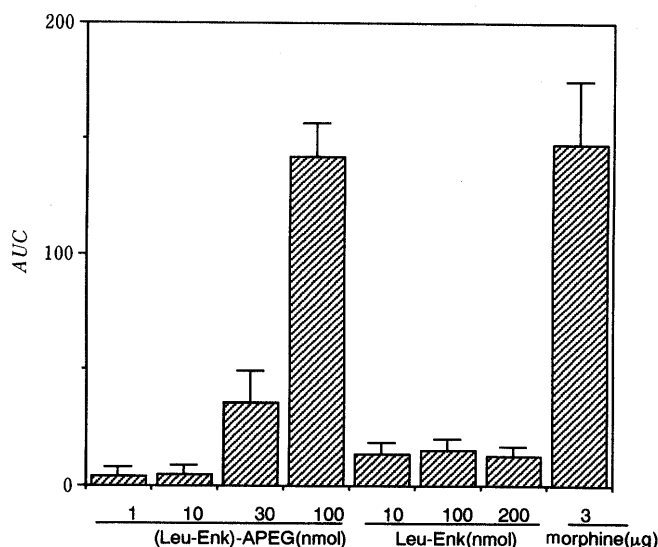


Fig. 2. Analgesic Effect of i.c.v. Administered (Leu-Enk)–APEG Hybrid Examined by the Tail Pinch Method  
AUC: area under the curve.

appreciable antinociception at doses up to 200 nmol/animal; however, the hybrid at doses of 30 and 100 nmol/animal exhibited a remarkable antinociceptive effect, and the effect of 100 nmol/animal of the hybrid is equipotent to that of 3  $\mu$ g (about 10 nmol)/animal i.c.v. morphine (Fig. 2). Thus, the antinociceptive effect of the hybrid is more potent than that of Leu-enkephalin. Although the mechanism underlying the production of potent antinociception by the hybrid remains to be established, it is possible that the enzymatic digestion of the hybrid is slower than that of Leu-enkephalin and the binding of the hybrid to receptors is more stable than that of Leu-enkephalin. In fact, it is reported that Leu-enkephalin is very rapidly degraded enzymatically to an inactive metabolite.<sup>6)</sup>

The PEG-(Leu-enkephalin) hybrid should be a promising lead compound for developing effective analgesics.

#### Experimental

Melting points are uncorrected. The solvent system for ascending thin-layer chromatography on Silica gel G (type 60, E. Merck) was as follows:  $R_f^3 = \text{CHCl}_3\text{-MeOH-H}_2\text{O}$  (8:3:1, lower phase). Synthetic peptides were hydrolyzed in 6N HCl at 110°C for 20 h and PEG-peptide hybrid was hydrolyzed for 48 h. Amino acid compositions of acid hydrolysates were determined with a Hitachi 835 amino acid analyzer. RP-HPLC was conducted with Waters 600 on YMC Pack AQ-ODS-5 using a mixture of 0.1% TFA-containing  $\text{CH}_3\text{CN-H}_2\text{O}$  as an eluent.

**APEG** APEG was prepared from PEG#4000 according to the procedure reported by Pillai and Mutter.<sup>3)</sup> The product was purified by CM-cellulose column chromatography using 0.001–0.05M  $\text{NH}_4\text{OAc}$  or Dowex 50 column chromatography using 3% AcOH as an eluent. Amino content was determined by titration using methyl orange as an indicator. Amino contents of various lots of hybrids were in the range of 0.26–0.56 meq/g.

**Boc-Tyr-Gly-Gly-Phe-Leu-OH(I)** Boc-Tyr(Bzl)-Gly-Gly-Phe-Leu-OBzl<sup>8)</sup> (500 mg, 0.63 mmol) was hydrogenated over Pd catalyst for 3 h in MeOH (10 ml). The catalyst was removed by filtration and the solvent was evaporated off. The residue was precipitated from MeOH–11.6° ( $c=1.0$ , MeOH). *Anal.* Calcd for  $\text{C}_{33}\text{H}_{45}\text{N}_5\text{O}_9 \cdot 2\text{H}_2\text{O}$ : C, 57.29; H, 7.13; N, 10.12. Found: C, 57.10; H, 6.93; N, 9.97. Amino acid ratios in an acid hydrolysate: Tyr 0.84, Gly 2.27; Phe 1.00, Leu 1.06 (average recovery 87%).

**(Tyr-Gly-Gly-Phe-Leu)-APEG(II)** DPPA (0.13 ml, 0.61 mmol) dissolved in DMF (4 ml) was added to a solution of Boc-Tyr-Gly-Gly-Phe-Leu-OH (360 mg, 0.55 mmol) and  $\text{Et}_3\text{N}$  (0.08 ml, 0.55 mmol) in DMF (5 ml) at 0°C and the mixture was stirred for 10 min. APEG (589 mg,  $\text{NH}_2$  content; 0.30 meq) dissolved in DMF (5 ml) was added to the mixture and the whole was stirred for 2 d in a cold room. The solvent was evaporated off and the residue was purified by LH 20 column ( $3.2 \times 140$  cm) chromatography using MeOH as an eluent. Fractions of 5 g were collected and the absorbance at 265 nm of each fraction was measured. Fractions 60–78 were collected and the solvent was evaporated off. Yield 747 mg.  $R_f^3$  0.66. The material (300 mg) was dissolved in a mixture of TFA (3 ml), *m*-cresol (0.15 ml) and anisole (0.15 ml) and the mixture was stirred for 1 h at 0°C. Petroleum ether was added and the precipitate was washed repeatedly with petroleum ether. The precipitate was purified by Sephadex G-25 column ( $3.2 \times 140$  cm) chromatography using 1% AcOH as an eluent. Fractions of 5 g were collected and the absorbance at 265 nm of each

fraction was measured. Two absorbance peaks were observed. Fractions of the second absorbance peak (frs. 311–355) did not contain any peptidic material. The first absorbance peak (frs. 91–125) was divided into 3 fractions [fraction I (frs. 90–100), fraction II (frs. 101–112), and fraction III (frs. 113–124)]. Fractions I, II and III gave 83 mg, 146 mg and 44 mg of peptidic materials and all gave  $R_f$  0.65 on TLC. These materials were converted to their hydrochloride by lyophilization from HCl-containing water. Amino acid ratios in acid hydrolysates of these materials gave similar results as shown below.

	Tyr	Gly	Phe	Leu
Fr. I	1.04	2.02	1.00	1.18
Fr. II	1.02	2.03	1.00	1.22
Fr. III	0.99	1.93	1.00	1.21

Thus fractions I–III were combined and Leu-enkephalin content was calculated from the average recovery of amino acids in an acid hydrolysate. Leu-enkephalin content of the hybrid was 0.35 mmol/g.

**Bioassay** Leu-enkephalin, Leu-enkephalin-APEG hybrid and morphine hydrochloride (Takeda) were dissolved in saline so that the dose was contained in a volume of 0.1 ml/10 g body weight. Leu-enkephalin, the hybrid and morphine were administered i.c.v., according to the method of Haley and McCormick.<sup>7)</sup>

Male mice of ddY strain weighing 20 to 23 g (Ohtsubo Experimental Animals) were purchased and housed in a temperature-controlled room with free access to food and water. After reaching to 25 to 30 g, they were used for experiments.

The antinociceptive effect was measured by the modified Haffner's method,<sup>8)</sup> using a cut-off time of 6 s to avoid tissue damage. The measurement was made at intervals of 15 min for 60 min after administration of test compounds. The effect was calculated as the area under the curve (AUC) by plotting the response (S) on the ordinate and elapsed time (min) on the abscissa.

Statistical significance of the differences was evaluated by the analysis of variance followed by Dunnett's analysis for individual comparisons.

#### References and Notes

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