Synthesis and Antinociceptive Activity of [D-Ala^2]Leu-Enkephalin Derivatives Conjugated with the Adamantane Moiety[1)]

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Based on the physicochemical and pharmacological properties of drugs having an adamantane skeleton, an adamantane-based moiety was evaluated as a drug carrier for poorly absorbed compounds, including peptides, active towards the central nervous system (CNS). Seven [D-Ala^2]Leu-enkephalin derivatives conjugated with an adamantane-based moiety at the C-terminus or N-terminus were prepared by the solution-phase method and their biological activities were examined. The compounds derivatized at the C-terminus through an ester or amide linkage were much more lipophilic than the parent peptide and exhibited moderate *in vitro* opioid activity (guinea-pig ileum assay). Among them, four derivatives (1, 2, 4, 5), exhibited significant antinociceptive effects in an *in vivo* assay (mouse tail-pressure test) after subcutaneous administration. This result suggests that the introduction of the lipophilic adamantane moiety into [D-Ala^2]Leu-enkephalin would improve the permeation of the poorly absorbed parent peptide through the blood-brain-barrier (BBB) without loss of antinociceptive effect.

Key words adamantane; drug carrier; brain-directed delivery; lipophilicity; chemical conjugation; enkephalin

Many potential drugs are unsuitable for therapeutic use because an optimal concentration can not be achieved at the site of action or can not be maintained for an adequate period of time. It is very important, therefore, to develop suitable delivery systems for such compounds. In the case of drugs whose site of action is in the central nervous system (CNS), the blood-brain-barrier (BBB) may restrict entry into the CNS. Therefore, brain-directed drug delivery is important,[3)] and various attempts have been made to develop delivery systems for CNS-active drugs, including polypeptides.[4)]

The pharmacological and physicochemical properties of drugs having an adamantane skeleton led us to suppose that the adamantane moiety might be useful as a brain-directed drug carrier for poorly absorbed compounds.[5)] For example, 1-aminoadamantane hydrochloride (amantadine hydrochloride) has been administered orally for the treatment of Parkinson's disease and 1-amino-3,5-dimethyl-adamantane (memantine), also an antiparkinsonism agent, has been reported to be distributed predominantly to the brain and liver after systemic or oral administration.[6)] From the physicochemical point of view, adamantane is expected to have high thermodynamic activity as well as lipophilicity due to its tricyclodecane cage structure, and some adamantane derivatives do have high solubility in both *n*-heptane and water. Attempts were made in the 1960s to use adamantane and related compounds as a lipophilicity-enhancing moiety for a hypoglycemic agent,[7a)] an anabolic steroidal agent,[7b)] and a nucleoside.[7c)]

In this article we report the synthesis of [D-Ala^2]Leu-enkephalin derivatives conjugated with adamantane-based moieties and we discuss their ability to penetrate the BBB on the basis of their biological effects.

The [D-Ala^2]Leu-enkephalin derivatives described in this report were prepared by solution-phase peptide synthesis. In order to introduce an adamantane-based moiety into the peptide, commercially available alcohols (1- and 2-adamantanol, 1-adamantanemethanol and 1-adamantaneethanol), an amine (1-adamantanamine), and two acylation reagents containing the 1-adamantane moiety were used. Since 1-adamantyl and 2-adamantyl esters are used as acid-labile carboxyl protecting groups in the field of peptide chemistry,[8)] all syntheses were carried out using protecting groups removable by catalytic hydrogenolysis; the Z group as an α-amino protecting group and the Bzl group for the side-chain hydroxyl group of Tyr.

First, four derivatives, **1**—**4**, conjugated with an adamantane-based moiety at the C-terminus through a simple ester linkage were prepared according to the scheme outlined in Chart 1. The C-terminal dipeptide unit, H–Phe–Leu–X (X: various adamantane-based moieties), was prepared by the Np active ester[9)] condensation of Z–Phe–OH and H–Leu–X followed by removal of the Z group by catalytic hydrogenolysis. The N-terminal tripeptide unit, Z–Tyr(Bzl)–D-Ala–Gly–$NHNH_2$, was prepared by the mixed anhydride (MA) method[10)] from Z–Tyr(Bzl)–OH with a TFA-treated sample of Z(OMe)–D-Ala–Gly–OMe, which was prepared by the MA method, followed by hydrazine treatment. Then [3+2] condensation by the azide method[11)] gave the protected [D-Ala^2]-Leu-enkephalin derivatives. Final removal of the Z and Bzl groups by catalytic hydrogenolysis gave the crude peptide derivatives, which were purified by silica gel column chromatography or chromatography on Diaion HP-20.

Derivative **5** conjugated with 1-adamantanamine through an amide linkage was also prepared *via* the route shown in Chart 1 and purified by silica gel column chromatography. This derivative had been prepared by Tomatis *et al.* in 1979 as a lipophilic enkephalin derivative,[12)] but its biological activities have not been fully evaluated.

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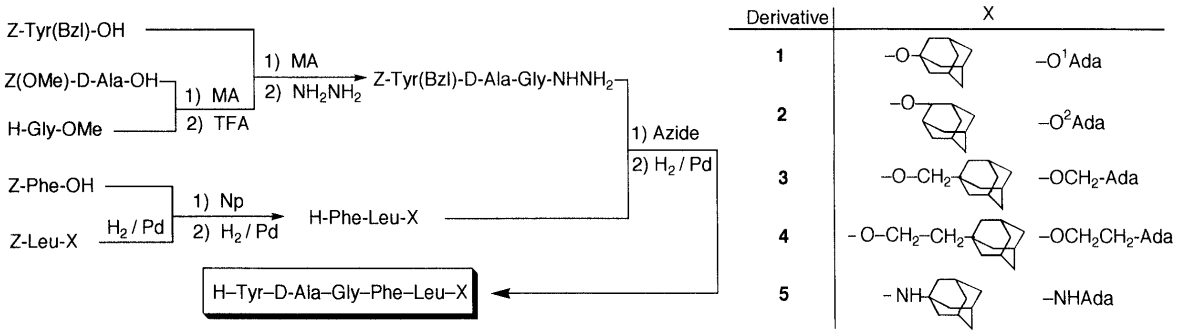

Chart 1. Synthetic Scheme for Derivatives **1**—**5**

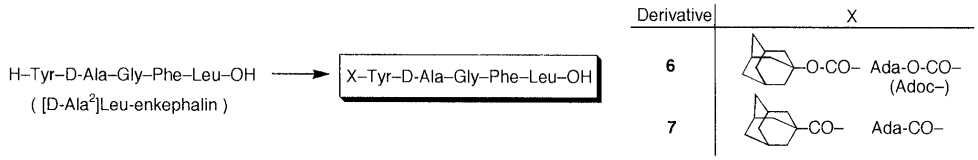

Chart 2. Synthetic Scheme for Derivatives **6** and **7**

Table 1. Physicochemical Data for [D-Ala^2]Leu-enkephalin Derivatives

Compound No.	Yield (%)	mp (°C)	$[\alpha]_D$[a] (°)	Rf[b]	t_R[c] (min)	Elemental analysis	Calcd / Found	FAB-MS $[M+H]^+$	Amino acid analysis[d]
1	85	110—115	+11.3 (c=0.2)	0.58	23.5	$C_{39}H_{53}N_5O_7 \cdot 2H_2O$	C, 63.31; H, 7.77; N, 9.47 C, 63.56; H, 7.66; N, 9.05	704.0	Tyr 0.88, Ala 1.03, Gly 1.13 Phe 1.05, Leu 1.00
2	52	115—120	+13.6 (c=0.2)	0.58	22.9	$C_{39}H_{53}N_5O_7 \cdot 3H_2O$	C, 61.80; H, 7.84; N, 9.24 C, 62.02; H, 7.54; N, 8.96	704.0	Tyr 0.92, Ala 1.00, Gly 1.06 Phe 1.05, Leu 1.00
3	56	196—201	+16.5 (c=0.2)	0.62	25.4	$C_{40}H_{55}N_5O_7 \cdot H_2O$	C, 65.28; H, 7.81; N, 9.52 C, 65.12; H, 7.61; N, 9.39	718.0	Tyr 0.94, Ala 1.05, Gly 1.00 Phe 1.05, Leu 1.00
4	50	117—122	+12.1 (c=0.3)	0.59	28.3	$C_{41}H_{57}N_5O_7 \cdot 1.5H_2O$	C, 64.88; H, 7.97; N, 9.23 C, 64.61; H, 7.72; N, 8.91	732.0	Tyr 0.98, Ala 1.03, Gly 1.00 Phe 1.07, Leu 1.00
5	41	141—145	+32.5 (c=0.2)	0.56	17.4	$C_{39}H_{54}N_6O_6 \cdot 2H_2O$	C, 63.39; H, 7.91; N, 11.37 C, 63.76; H, 7.85; N, 11.21	703.0	Tyr 1.00, Ala 1.03, Gly 1.04 Phe 1.07, Leu 1.00
6	65	205—208	+2.3 (c=0.2)	0.34	23.6	$C_{40}H_{53}N_5O_9 \cdot 4H_2O$	C, 58.59; H, 7.50; N, 8.59 C, 58.48; H, 7.51; N, 8.16	770.0[e]	Tyr 0.94, Ala 0.98, Gly 1.00 Phe 1.04, Leu 0.95
7	62	210—214	+6.5 (c=0.2)	0.38	20.3	$C_{40}H_{53}N_5O_8 \cdot 3H_2O$	C, 61.13; H, 7.57; N, 8.91 C, 60.85; H, 7.18; N, 8.75	770.0[f]	Tyr 0.98, Ala 1.00, Gly 1.00 Phe 1.01, Leu 0.97

a) Optical rotations were measured at 25 °C in 20% AcOH (**1**—**4**), in DMF (**5**), or in MeOH (**6**, **7**). *b*) $CHCl_3$–MeOH–H_2O (8 : 3 : 1) system. *c*) HPLC conditions [column, Cosmosil 5C18 (4 × 150 mm); gradient system, 0.1% TFA containing TFA/H_2O 30—70% in 40 min; flow rate, 1 ml/min; detected at 275 nm]. *d*) Ala has been D-configuration. *e*) $[M+Na]^+$. *f*) $[M+K]^+$.

Derivatives **6** and **7**, conjugated with an adamantane-based moiety at the N-terminus through a carbamate or an amide linkage, were prepared by the acylation of [D-Ala^2]Leu-enkephalin as shown in Chart 2. In order to prevent undesired acylation of the free hydroxyl function of Tyr, the active esters 1-adamantyloxycarbonyl-*N*-hydroxysuccinimide (Adoc-OSu) and 1-adamantanecarbonyl-*N*-hydroxysuccinimide (Ada-OSu) were newly prepared and used as acylation reagents. Two derivatives, **6** and **7**, were obtained and purified to homogeneity by silica gel column chromatography.

The purity of each derivative was ascertained by TLC, amino acid analysis after acid hydrolysis, elemental analysis and FAB-MS. In addition, analytical HPLC confirmed the high purity (>98%) of each derivative. The physicochemical data for **1**—**7** are collected in Table 1.

The partition coefficient (log PC) of each derivative in the *n*-octanol/water system is listed in Table 2. As expected, the derivatives prepared here were much more lipophilic than [D-Ala^2]Leu-enkephalin (−0.22 in the *n*-octanol/water system). It is noteworthy that the derivatives conjugated at the C-terminus (**1**—**5**) had fairly high log PC values compared to the derivatives conjugated at the N-terminus (**6**, **7**).

Next, the inhibitory effect of the synthetic [D-Ala^2]Leu-enkephalin derivatives on electrically evoked contraction of guinea-pig ileum (GPI) was determined according to the method of Kosterlitz and Waterfield.[13] The results, including those for reference compounds (morphine hydrochloride, Met- and Leu-enkephalin, and [D-Ala^2]Leu-enkephalin), are summarized in Table 2. The derivatives (**1**—**4**) conjugated with an adamantane-based moiety at the C-terminus through an ester linkage exhibited similar inhibitory potency to [D-Ala^2]Leu-enkephalin and morphine hydrochloride. Significant differences in inhibitory potency were not observed among these derivatives in terms of the connecting position of adamantane (1-adamantane *vs.* 2-adamantane) or the connecting chain-

Table 2. Partition Coefficient (log PC) and Bio-activity of Synthetic Derivatives

Compound	log PC (octanol–water)	*In vitro* assay (GPI)			*In vivo* assay (tail pressure)
		n	ED_{50} (M)[b]	Relative potency	
Morphine·HCl		43	$2.47 \pm 0.27 \times 10^{-8}$	1	
Met-enkephalin		15	$2.85 \pm 0.44 \times 10^{-8}$	0.87	
Leu-enkephalin		12	$3.64 \pm 0.66 \times 10^{-8}$	0.68	
[D-Ala^2]Leu-enkephalin	−0.22, 0.75[a]	24	$2.03 \pm 0.40 \times 10^{-8}$	1.22	−
1	2.12	10	$4.08 \pm 1.13 \times 10^{-8}$	0.61	+
2	2.00	6	$5.43 \pm 2.01 \times 10^{-8}$	0.45	+
3	2.69	10	$1.01 \pm 0.95 \times 10^{-7}$	0.25	−
4	2.39[a]	6	$5.02 \pm 0.94 \times 10^{-8}$	0.49	+
5	2.73	16	$1.42 \pm 0.32 \times 10^{-8}$	1.74	+
6	0.96	—	$> 10^{-5}$	—	
7	0.46	—	$> 10^{-5}$	—	

a) Determined in an *n*-butanol–water system. *b*) Each ED_{50} value is shown as (mean ± S.E.M.).

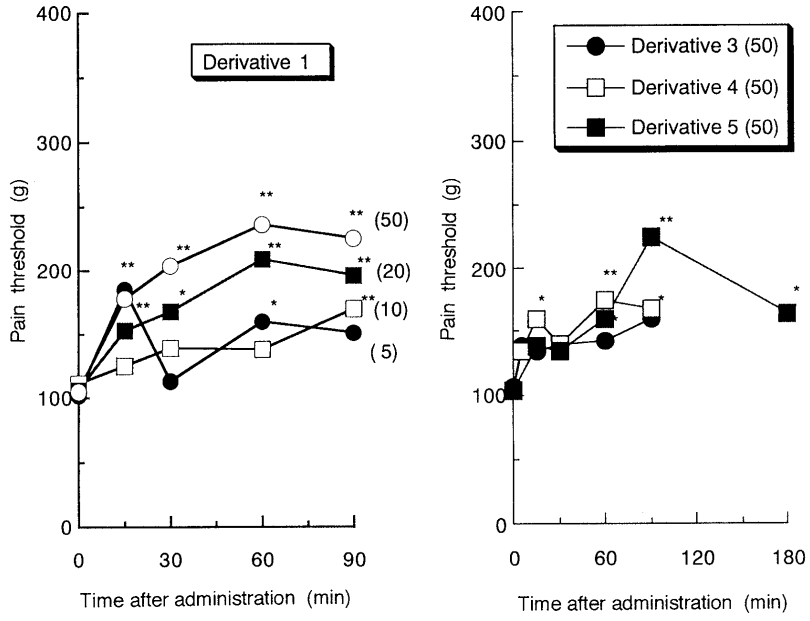

Fig. 1. Antinociceptive Activity of (A) Derivative **1** and (B) Derivatives **3**—**5**

The values in parentheses are the doses in mg/kg s.c. Each point is the mean value of the pain threshold from 10 mice. (*) and (**) show $p < 0.05$ and $p < 0.01$ against the pre-drug value, respectively.

length between the peptide part and the adamantane moiety (none *vs.* methylene *vs.* ethylene). The derivative **5**, conjugated at the C-terminus through an amide linkage, was the most potent of the tested compounds and its relative potency with respect to [D-Ala^2]Leu-enkephalin was comparable to that reported by Tomatis *et al.*[12] In contrast, the derivatives (**6**, **7**) conjugated at the N-terminus were not active at the dose of 10^{-5} M. This is consistent with the fact that the free amino group of the N-terminal Tyr is involved in the interaction with opiate receptors.[14]

Antinociceptive activity was examined for the derivatives (**1**—**5**) which were active in the *in vitro* assay, using the tail-pressure method[15] in mice. The tested peptide derivatives were administered by subcutaneous injection. As shown in Fig. 1, **1** exhibited an antinociceptive effect in a dose-dependent manner in the range of 5—50 mg/kg and the effect reached a maximum level at 60—90 min after administration. Compounds **2**, **4** and **5** exhibited significant antinociceptive effects, but were less potent than **1**. In contrast, **3** and the parent compound [D-Ala^2]Leu-enkephalin were ineffective (maximum doses; 50 mg/kg). As controls, 1-adamantanol, saline, and saline plus *N*,*N*-dimethylacetamide (DMA), showed no effects. The responses of **1** and **5** in both *in vitro* and *in vivo* assays were naloxone-reversible.

As to the *in vivo* stability, the degradation rates of **1** and **5** were examined *in vitro* using mouse brain homogenates. The degradation rate of **1** was faster than that of **5**, as shown in Fig. 2.

Leu-enkephalin (Tyr–Gly–Gly–Phe–Leu) shows an antinociceptive effect after direct intracisternal injection, but is inactive after peripheral administration.[16] This is mainly due to its instability to proteolytic enzymes and also its poor ability to permeate through the BBB. Similarly, the *in vivo* results of the tested derivatives were not correlated with the *in vitro* results, presumably for similar reasons. However, the *in vivo* results (Fig. 1) strongly suggest that the antinociceptive effects of [D-Ala^2]Leu-enkephalin derivatives conjugated with an

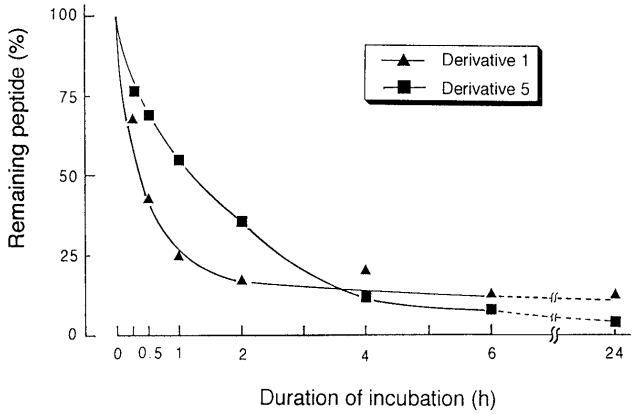

Fig. 2. Rate of Degradation of Derivatives **1** and **5** by Mouse Brain Homogenates

adamantane moiety at the C-terminus reflect the enhanced lipophilicity of these derivatives. The log PC values (2.0—2.7) in **1**—**5** were sufficient to allow penetration through the BBB, *i.e.*, in the range of 1.5 to 2.5.[17] The duration of the antinociceptive effect of derivative **5** may reflect the greater elimination half-life of this derivative in mouse brain homogenates (Fig. 2). In another paper,[18] our group has shown that the introduction of an adamantane moiety at the C-terminus did not significantly improve the *in vivo* stability of **1**, but the distribution of **1** in the brain was increased during intravenous infusion in comparison with that of [D-Ala^2]Leu-enkephalin. The parent peptide, [D-Ala^2]Leu-enkephalin, was formed more rapidly and to a greater extent from **1** in rat brain homogenates than in rat plasma.[18] It is conceivable, therefore, that a part of **1** penetrates into the brain through the BBB in an intact form, then both **1** and [D-Ala^2]Leu-enkephalin formed by the action of enzyme(s) in the brain act on the opioid receptor(s).

In order to enhance the lipophilicity sufficiently to allow passage across the BBB and also to improve the *in vivo* stability, the incorporation of sterically hindered amino acids with long alkyl chains or expanded cyclic systems (a "lipidic" amino acid or a "fatty" amino acid) into the peptide chain was reported.[19] Introduction of such an amino acid into the peptide chain has led to highly potent analogues of many bioactive peptides. Attempts to tether lipidic amino acids or oligomers as a drug carrier with hydrophilic compounds have also been reported.[20] Our approach may be effective in designing a brain-directed drug, as the derivatives described here were covalently conjugated with a highly lipophilic moiety through an enzymatically labile ester or amide linkage. In conclusion, the high lipophilicity and thermodynamic activity and the low toxicity of the adamantane moiety favor its application as a drug carrier for CNS drug delivery.

Experimental

Melting points were determined with a Yanagimoto microapparatus and are uncorrected. Optical rotations were determined with a Union PM-201 polarimeter. HPLC was conducted with a Hitachi L-6200 apparatus. Amino acid ratios in acid hydrolysates were determined with a Hitachi 8500 model amino acid analyzer. FAB-MS were recorded on a JEOL JMS-D 300 spectrometer.

TLC was performed on precoated Silica gel 60 F_{254} plate (1 × 8 cm, 0.25 mm thickness, Merck) and bands were visualized by the following methods: UV light, I_2 vapor, and ninhydrin. *Rf* values on TLC refer to the following solvent systems (v/v): Rf_1 AcOEt–*n*-hexane (2 : 8), Rf_2 $CHCl_3$–MeOH–H_2O (8 : 3 : 1, upper phase), Rf_3 $CHCl_3$–MeOH (20 : 1).

Adoc-F[21] was purchased from Kokusan Chemical Works, Ltd. (Tokyo, Japan). Authentic [D-Ala^2]Leu-enkephalin was purchased from Peptide Institute Inc. (Osaka, Japan).

Synthesis of H–Tyr–D-Ala–Gly–Phe–Leu–OX (1—4). Z–Leu–O^{1}Ada (Typical Procedure) 1-Adamantanol (1.90 g, 12.4 mmol), DCC (2.60 g, 12.4 mmol) and DMAP (0.15 g, 1.24 mmol) were added to an ice-chilled solution of Z–Leu–OH (3.00 g, 11.3 mmol) in distilled CH_2Cl_2 (30 ml). The reaction mixture was stirred for 24 h at 4 °C, then the formed urea-derivative was removed by filtration, and the filtrate was concentrated *in vacuo*. The residue was dissolved in AcOEt (50 ml) and the solution was washed with 5% Na_2CO_3, 10% citric acid, and H_2O successively. It was dried over Na_2SO_4, and the AcOEt was removed by evaporation. The residue was purified by silica gel column chromatography with $CHCl_3$. The fractions containing the desired material were combined and concentrated to dryness to give Z–Leu–O^{1}Ada as an oil; 3.10 g (69%), Rf_1 0.56, FAB-MS *m/z* 400.0 $[M+H]^+$ (Calcd for $C_{24}H_{34}NO_4$, 400).

The following compounds were prepared similarly:

Z–Leu–O^{2}Ada: Yield 81% (oil), Rf_1 0.56, FAB-MS *m/z* 400.0 $[M+H]^+$ (Calcd for $C_{24}H_{34}NO_4$, 400).

Z–Leu–OCH_2Ada: Yield 74% (oil), Rf_1 0.54, FAB-MS *m/z* 414.2 $[M+H]^+$ (Calcd for $C_{25}H_{36}NO_4$, 414).

Z–Leu–OCH_2CH_2Ada: Yield 46% (oil), Rf_1 0.58, FAB-MS *m/z* 428.0 $[M+H]^+$ (Calcd for $C_{26}H_{38}NO_4$, 428).

Z–Phe–Leu–O^{1}Ada (Typical Procedure) Z–Phe–O^{1}Ada (6.20 g, 15.5 mmol) was dissolved in THF (30 ml) containing AcOH (1 ml) and hydrogenated over 5% Pd–C (2.0 g) for 5 h. The catalyst was removed by filtration, and the filtrate was evaporated *in vacuo*. The residue was dissolved in DMF (30 ml) containing Et_3N (2.14 ml, 15.5 mmol) and Z–Phe–ONp (4.64 g, 15.5 mmol) was added. The reaction mixture was stirred for 48 h at 15 °C, and the DMF was removed by evaporation *in vacuo*. The residue was dissolved in AcOEt (50 ml) and this solution was washed with 5% Na_2CO_3, 10% citric acid, and H_2O successively. It was dried over Na_2SO_4 and concentrated, and the residue was further purified by silica gel column chromatography with $CHCl_3$. The solvent of the desired fractions was removed by evaporation and the residue was triturated with *n*-hexane to give a powder; yield 4.25 g (51%), mp 105—106 °C, Rf_1 0.26, $[\alpha]_D^{25}$ −28.7° (*c* = 1, MeOH). *Anal.* Calcd for $C_{33}H_{42}N_2O_5$: C, 72.50; H, 7.74; N, 5.12. Found: C, 72.40; H, 8.00; N, 5.13.

Z–Phe–Leu–O^{2}Ada: Yield 72%, mp 75—78 °C, Rf_1 0.25, $[\alpha]_D^{25}$ −22.9° (*c* = 0.8, MeOH). *Anal.* Calcd for $C_{33}H_{42}N_2O_5$: C, 72.50; H, 7.74; N, 5.12. Found: C, 72.28; H, 7.95; N, 4.90.

Z–Phe–Leu–OCH_2Ada: Yield 99% (oil), Rf_1 0.30, FAB-MS *m/z* 561.3 $[M+H]^+$ (Calcd for $C_{34}H_{45}N_2O_5$, 561).

Z–Phe–Leu–OCH_2CH_2Ada: Yield 100% (oil), Rf_1 0.30, FAB-MS *m/z* 575.0 $[M+H]^+$ (Calcd for $C_{35}H_{47}N_2O_5$, 575).

Z(OMe)–D-Ala–Gly–OMe This compound was prepared by the mixed anhydride method in a usual manner. The product was extracted with AcOEt and the organic layer was washed with 5% Na_2CO_3, 10% citric acid, and H_2O, then dried over Na_2SO_4 and evaporated *in vacuo*. The residue was triturated with ether to give a powder, which was recrystallized from AcOEt with ether; yield 58%, mp 99—101 °C, Rf_2 0.75, $[\alpha]_D^{25}$ +17.3° (*c* = 1, MeOH). *Anal.* Calcd for $C_{15}H_{20}N_2O_6$: C, 55.55; H, 6.22; N, 8.64. Found: C, 55.58; H, 6.22; N, 8.72.

Z–Tyr(Bzl)–D-Ala–Gly–OMe A mixed anhydride [prepared from Z–Tyr(Bzl)–OH (3.81 g, 11.1 mmol) in THF (25 ml)] was combined with a solution of a TFA-treated sample of Z(OMe)–D-Ala–Gly–OMe (3.00 g, 9.25 mmol) in DMF (40 ml) containing Et_3N (2.56 ml, 18.5 mmol). The reaction mixture was stirred for 2 h at 4 °C, and the solvent was removed by evaporation *in vacuo*. The residue was dissolved in AcOEt (45 ml) and the solution obtained was washed with 5% Na_2CO_3, 10% citric acid, and H_2O. It was dried over Na_2SO_4 and concentrated, and the residue was triturated with isopropyl ether to give a solid, which was recrystallized from MeOH with ether; yield 3.31 g (65%), mp 157—161 °C, Rf_2 0.75, $[\alpha]_D^{25}$ +24.9° (*c* = 1, MeOH). *Anal.* Calcd for $C_{30}H_{33}N_3O_7$: C, 65.80; H, 6.07; N, 7.67. Found: C, 65.33; H, 6.06; N, 7.60.

Z–Tyr(Bzl)–D-Ala–Gly–NHNH$_2$ A solution of Z–Tyr(Bzl)–D-Ala–Gly–OMe (3.20 g, 5.80 mmol) in DMF (40 ml) was treated with 80% hydrazine hydrate (2.32 ml, 58 mmol) for 24 h. The DMF was removed

by evaporation *in vacuo*, and the residue was triturated with EtOH to give a precipitate, which was recrystallized from DMF with EtOH; yield 3.15 g (98%), mp 171—174 °C, Rf_2 0.61, $[\alpha]_D^{25}$ −21.2° ($c=1$, DMF). *Anal.* Calcd for $C_{29}H_{33}N_5O_6 \cdot H_2O$: C, 61.58; H, 6.24; N, 12.38. Found: C, 61.73; H, 6.09; N, 12.86.

Z–Tyr(Bzl)–D-Ala–Gly–Phe–Leu–O^{1}Ada (Typical Procedure) Z–Phe–Leu–O^{1}Ada (1.00 g, 1.83 mmol) was dissolved in THF (20 ml) containing AcOH (1 ml) and hydrogenated over 5% Pd–C (1 g) for 5 h. The catalyst was removed by filtration, the filtrate was concentrated *in vacuo* and the residue was dissolved in DMF (30 ml). The azide [prepared from Z–Tyr(Bzl)–D-Ala–Gly–$NHNH_2$ (1.30 g, 2.38 mmol)] in DMF (15 ml) and Et_3N (0.70 ml, 5.00 mmol) were added to the above DMF solution of the amino component. The reaction mixture was stirred for 24 h at 4 °C, then the DMF was removed by evaporation *in vacuo*. The residue was triturated with H_2O to give a solid, which was washed with 5% $NaHCO_3$, 10% citric acid and H_2O in a batchwise manner. Recrystallization from MeOH with ether gave an analytically pure product; yield 1.67 g (85%), mp 136—139 °C, Rf_2 0.78, $[\alpha]_D^{25}$ −26.8° ($c=1$, DMF). *Anal.* Calcd for $C_{54}H_{65}N_5O_9 \cdot 0.5H_2O$: C, 69.21; H, 7.17; N, 7.47. Found: C, 69.29; H, 7.23; N, 7.33.

Z–Tyr(Bzl)–D-Ala–Gly–Phe–Leu–O^{2}Ada: Yield 68%, mp 150—153 °C, Rf_2 0.78, $[\alpha]_D^{25}$ −24.8° ($c=1$, DMF). *Anal.* Calcd for $C_{54}H_{65}N_5O_9$: C, 69.88; H, 7.06; N, 7.55. Found: C, 69.84; H, 7.13; N, 7.58.

Z–Tyr(Bzl)–D-Ala–Gly–Phe–Leu–OCH_2Ada: Yield 38% (after silica gel column chromatography), mp 158—162 °C, Rf_3 0.34, $[\alpha]_D^{25}$ −17.2° ($c=0.5$, DMF), *Anal.* Calcd for $C_{55}H_{67}N_5O_9 \cdot 0.5H_2O$: C, 69.45; H, 7.21; N, 7.36. Found: C, 69.61; H, 7.29; N, 7.79.

Z–Tyr(Bzl)–D-Ala–Gly–Phe–Leu–OCH_2CH_2Ada: Yield 51% (after silica gel column chromatography), mp 148—151 °C, Rf_3 0.31, $[\alpha]_D^{25}$ −13.3° ($c=0.6$, DMF). *Anal.* Calcd for $C_{56}H_{69}N_5O_9 \cdot 1.5H_2O$: C, 68.41; H, 7.38; N, 7.12. Found: C, 68.44; H, 7.09; N, 7.46.

H–Tyr–D-Ala–Gly–Phe–Leu–O^{1}Ada (1) (Typical Procedure) Z–Tyr(Bzl)–D-Ala–Gly–Phe–Leu–O^{1}Ada (250 mg, 0.27 mmol) dissolved in THF (30 ml) containing AcOH (1 ml) was hydrogenated over 5% Pd–C (500 mg) for 5 h. The catalyst was removed by filtration, and the filtrate was concentrated *in vacuo*. The residue was dissolved in $CHCl_3$–MeOH (10 : 1, 2 ml) and subjected to silica gel column chromatography (1.5 × 10 cm) with the same solvent system. The fractions containing the desired product were pooled and the solvent was removed by evaporation *in vacuo*. The residue was lyophilized from 5% AcOH to give a fluffy powder; yield 85 mg (85%).

Derivative **2** was similarly purified by silica gel column chromatography after hydrogenolysis. Derivatives **3** and **4** were purified on a column of Diaion HP-20 after hydrogenolysis; namely, the crude product after hydrogenolysis was dissolved in 5% AcOH (25 ml) and applied to a column of HP-20 (2.5 × 15 cm), which was eluted with 5% AcOH (200 ml), followed by a gradient system formed from 5% AcOH (250 ml) and CH_3CN (300 ml). The main peak fraction was pooled and the solvent was removed by lyophilization to give a fluffy powder.

Physicochemical data for derivative **1** together with derivatives **2**—**7** are collected in Table 1.

Synthesis of H–Tyr–D-Ala–Gly–Phe–Leu–NHAda (5) This compound has been prepared by Tomatis *et al.*[12] as mentioned in the text, though the formula given by them is not correct. Data for the protected peptides used in the preparation of this derivative are collected below.

Z–Leu–NHAda: Prepared by the MA method and recrystallized from AcOEt with petroleum ether; yield 84%, mp 103—105 °C, Rf 0.44, $[\alpha]_D^{25}$ −23.3° ($c=1$, MeOH). *Anal.* Calcd for $C_{24}H_{34}N_2O_3$: C, 72.33; H, 8.60; N, 7.03. Found: C, 72.02; H, 8.78; N, 6.82.

Z–Phe–Leu–NHAda: Prepared by the Np method and recrystallized from DMF with AcOEt; mp 125—127 °C, Rf_1 0.15, $[\alpha]_D^{25}$ −32.0° ($c=1$, MeOH). *Anal.* Calcd for $C_{33}H_{43}N_3O_4 \cdot 0.5H_2O$: C, 71.45; H, 8.00; N, 7.58. Found: C, 71.57; H, 8.08; N, 7.61.

Z–Tyr(Bzl)–D-Ala–Gly–Phe–Leu–NHAda: Prepared by the azide method and recrystallized from DMF with MeOH; mp 145—149 °C, Rf_2 0.69, $[\alpha]_D^{25}$ −26.5° ($c=1$, DMF). *Anal.* Calcd for $C_{54}H_{66}N_6O_8 \cdot 0.5H_2O$: C, 69.28; H, 7.28; N, 8.98. Found: C, 69.25; H, 7.14; N, 8.85.

H–Tyr–D-Ala–Gly–Phe–Leu–NHAda: This derivative was prepared from the protected peptide by hydrogenolysis in the same manner as described for the preparation of derivatives **1**—**4**. Purification was done by silica gel column chromatography with $CHCl_3$–MeOH (10 : 1); yield 41%.

Syntheses of Adoc–Tyr–D-Ala–Gly–Phe–Leu–OH (6) and Ada–Tyr–D-Ala–Gly–Phe–Leu–OH (7) [D-Ala^2]Leu-enkephalin was prepared by solution-phase peptide synthesis using [3+2] condensation of the N-terminal tripeptide unit Z–Tyr(Bzl)–D-Ala–Gly–$NHNH_2$ and the C-terminal dipeptide unit H–Phe–Leu–OBzl, which was derived from Z(OMe)–Phe–Leu–OBzl.[22] After removal of the protecting groups (Z, Bzl) by catalytic hydrogenolysis, the partially purified pentapeptide, H–Tyr–D-Ala–Gly–Phe–Leu–OH, was confirmed to coincide with an authentic sample on HPLC and was used for the acylation without further purification.

Adoc–OSu: Adoc–F[21] (1.0 g, 5.05 mmol) was dissolved in AcOEt (20 ml) and to this solution, HOSu (0.58 g 5.05 mmol) and Et_3N (1.4 ml, 10.1 mmol) were added. The reaction mixture was stirred for 1 h at room temperature, then washed with H_2O (×5) and dried over Na_2SO_4. The AcOEt was removed by evaporation *in vacuo* and the residue was triturated with petroleum ether to give crystals; yield 1.08 g (74%), mp 143—144 °C. *Anal.* Calcd for $C_{15}H_{19}NO_5$: C, 61.42; H, 6.53; N, 4.78. Found: C, 61.29; H, 6.70; N, 4.77.

Adoc–Tyr–D-Ala–Gly–Phe–Leu–OH (**6**): [D-Ala^2]Leu-enkephalin (230 mg, 0.4 mmol) in DMF (8 ml) was acylated with Adoc–OSu (120 mg, 0.41 mmol) in the presence of Et_3N (0.11 ml, 0.8 mmol). The reaction mixture was stirred for 2 h at 4 °C, then diluted with AcOEt (25 ml). The organic layer was washed with H_2O and dried over Na_2SO_4. The AcOEt was removed by evaporation *in vacuo* and the residue was purified on a silica gel column with $CHCl_3$–MeOH–H_2O (8 : 3 : 1, upper phase). The fractions containing the desired product were pooled and the solvent was removed by evaporation *in vacuo*. The residue was triturated with ether to give the desired compound; yield 135 mg (45%).

Ada–Tyr–D-Ala–Gly–Phe–Leu–OH (**7**): 1-Adamantane carboxylic acid (180 mg, 1 mmol) was converted to the *N*-hydroxysuccinimide ester with DCC (206 mg, 1 mmol) and HOSu (153 mg, 1 mmol) in DMF (5 ml), then the formed urea-derivative was removed by filtration. The filtrate was added to a solution of [D-Ala^2]Leu-enkephalin (340 mg, 0.6 mmol) in DMF (5 ml) containing Et_3N (0.17 ml, 1.2 mmol). The reaction mixture was stirred for 2 h at 4 °C, and the desired compound was isolated in the same manner as described for the preparation of **6**; yield 252 mg (58%).

Determination of Partition Coefficients *n*-Octanol and 0.01 M phosphate buffer (pH 7.0) were saturated with each other prior to use. [D-Ala^2]Leu-enkephalin and its derivatives were each dissolved in 10 ml of presaturated *n*-octanol at a concentration of *ca.* 500 μg/ml and mixed with 5 ml of phosphate buffer (pH 7.0). The mixture was vigorously shaken for 60 min with a mechanical shaker and then centrifuged at 3000 rpm. The concentration of each compound in each phase was determined by HPLC. The log PC value of derivative **4** could not be determined in the *n*-octanol/buffer system, probably due to the occurrence of ester-exchange reaction, so an *n*-butanol/buffer system was employed for this derivative and the log PC in the *n*-butanol/buffer system was determined using the same method as described above. The log PC value was calculated as the ratio of the drug concentration in the octanol phase to that in the buffer phase.

***In Vitro* Bioassay** The inhibitory effect on the electrically evoked contraction of isolated GPI was measured according to the method of Kosterlitz and Waterfield.[13]

***In Vivo* Bioassay** The antinociceptive activity of each compound was determined by the tail-pressure method in dd-Y male mice (*ca.* 20 g) after subcutaneous administration according to Hata *et al.*[15] All tested compounds were dissolved in DMA and saline (4 : 6, v/v) and injected in a volume of 100 μl of solution (1—50 mg/kg body weight).

Degradation of 1 and 5 The degradation study was performed as described by Marks *et al.*[23] Brains from dd-Y male mice (15 g) were homogenized in 160 ml of ice-cold saline and the homogenate was centrifuged at 18000 rpm for 20 min at 4 °C. The clear supernatant was ultrafiltered through a YM10 membrane (Amicon Corp.) and concentrated to approximately 25 ml. During the ultracentrigation, the temperature was maintained at 4 °C. The protein concentration in the filtrate determined according to Lowry *et al.*[24] was 14.8 mg/ml. An aliquot of 300 μl of the mouse brain filtrate was combined with the same volume of stock solution of **1** or **5** (*ca.* 40 μg/ml in phosphate buffer), and the mixture was incubated at 37 °C. At appropriate times, aliquots were withdrawn from the mixture and the amounts of the derivative were determined by HPLC. The HPLC apparatus was a Hitachi HPLC Model 655 consisting of a UV detector (655A-21), a pump control unit (655A-11), and an integrator (655-61). The chromatographic conditions were as follows: column, YMC A-312 (6 × 150 mm), column temperature,

30 °C; flow rate, 1 ml/min; UV detection, 280 nm. The mobile phase consisted of CH_3CN and 0.1% TFA. The ratios of CH_3CN and 0.1% TFA were 40 : 60 for derivative **1** and 60 : 40 for derivative **5**.

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References and Notes

1) Abbreviations used: Z, benzyloxycarbonyl; Z(OMe), *p*-methoxybenzyloxycarbonyl; Bzl, benzyl; ^{1}Ada, 1-adamantyl; ^{2}Ada, 2-adamantyl; Adoc, adamantaneoxycarbonyl; Np; *p*-nitrophenyl; OSu, *N*-hydroxysuccinimidyl; OMe, methyl ester; TFA, trifluoroacetic acid; AcOH, acetic acid; DCC, *N*,*N*-dicyclohexylcarbodiimide; DMAP, *N*,*N*-dimethylaminopyridine; AcOEt, ethyl acetate; THF, tetrahydrofuran; DMF, *N*,*N*-dimethylformamide; Et_3N, triethylamine.
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