on two "Prep Pak" columns to give **22** (0.5 g) as the faster running component. This material was dissolved in EtOAc and treated with a solution of maleic acid (250 mg) in MeOH. The solution was concentrated to give a white precipitate, which was filtered off and crystallized from a mixture of MeOH and EtOAc to give **22** maleate as a white powder: yield 0.25 g (1.2%); mp 151–152 °C; ^{1}H NMR (D_2O) δ 7.40 (m, 5 H, aromatic), 6.46 (d, J = 7 Hz, 1 H, C*H*=CPh), 5.00 (d, J = 4.5 Hz, 1 H, C*H*NMe_2), 6.3 (s, 2 H, maleic acid), 2.97 (br s, 1 H, =CHC*H*<), 2.70 (s, 6 H, NMe_2), 2.68 (br s, 1 H, Me_2NCC*H*<), 2.10–1.70 (m, 6 H, remaining CH_2); UV λ_{max} (EtOH) 236 nm (ϵ 13 300). Anal. ($C_{16}H_{21}N\cdot C_4H_4O_4$) C, H; N: calcd, 4.1; found, 5.0. MS Calcd for $C_{16}H_{21}N$, 227. 1674 (M^+); found, 227.1677.

Registry No. 1·HCl, 75590-28-2; 4, 35242-17-2; 5, 16917-83-2; 6, 83435-88-5; 8, 66720-22-7; 11·HCl, 66720-27-2; 12, 83435-89-6; 13, 2394-47-0; 14, 2404-39-9; 17, 16917-84-3; 18, 83435-90-9; 19, 83435-91-0; 20·HCl, 83435-92-1; 21·HCl, 83435-93-2; 22·HCl, 83435-96-5; 22 maleate, 83435-95-4; 23·HCl, 83435-97-6; 24·HCl, 83435-98-7; 25, 83435-99-8; 26, 83436-00-4; 27·HCl, 83436-01-5; 28·HCl, 83436-02-6; 29·HCl, 83436-03-7; 30, 83436-04-8; 31, 83436-05-9; 32, 83436-06-0; 33, 83436-07-1; 34, 83436-08-2; 35, 83436-09-3; 36, 83447-47-6; 37, 83436-10-6; 38, 83436-11-7; 39, 83436-12-8; 40 maleate, 83436-14-0; 41, 83436-15-1; 42 maleate, 83436-17-3; 43, 83436-18-4; 44, 83436-19-5; 45, 83436-20-8; 46, 83436-21-9; 47, 83436-22-0; 48, 83436-23-1; 49 maleate, 83436-25-3; 50·HCl, 83436-26-4.

New Carboxyalkyl Inhibitors of Brain Enkephalinase: Synthesis, Biological Activity, and Analgesic Properties

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New carboxyalkyl compounds derived from Phe-Leu and Phe-Ala were synthesized and checked as inhibitors of "enkephalinase", a metalloendopeptidase cleaving the Gly^3–Phe^4 bond of enkephalins from mouse striatal membranes. Differential recognition of both brain enkephalinase and angiotensin-converting enzyme (ACE) catalytic sites by these carboxylalkyl compounds lead to potent ($K_1 \approx 0.5$ μM), competitive and selective inhibitors of the enkephalin-degrading enzyme. The most interesting compound, *N*-[(*RS*)-2-carboxy-3-phenylpropanoyl]-L-leucine (**3**, $K_1 = 0.34$ μM), is 10 000 times more potent on enkephalinase than on ACE activities. Intracerebroventricular (icv) injection of **3** in mice leads to a high potentiation of the analgesic effect of the exogenously administered D-Ala^2-Met-enkephalin, evidencing the in vivo inhibition of enkephalinase. Moreover, icv administration of **3** alone induces a dose-dependent analgesia in mice measured on both hot-plate and writhing tests. In the former assay, the ED_{50} was approximately 10 μg per animal, slightly higher than that of thiorphan. All the antinociceptive effects were antagonized by naloxone, demonstrating the involvement of enkephalins in analgesia and their in vivo protection from enkephalinase by **3**. The described compounds can be considered as first examples of a new series of analgesics and potentially psychoactive agents.

A large number of results,[1,2] including the recently reported release of enkephalins following tooth pulp stimulation,[3] evidenced the involvement of the endogenous pentapeptides, Tyr-Gly-Gly-Phe-Met (Met-E) and Tyr-Gly-Gly-Phe-Leu (Leu-E), in the regulation of nociceptive stimuli through a specific interaction with opiate receptors, most probably of μ subtypes.[4] According to their assumed neurotransmitter role, these endogenous peptides seem to be quickly removed from the synaptic cleft through the action of degrading enzymes. Various brain peptidases, including aminopeptidases[5-8] and the two peptidyldipeptide hydrolases, "enkephalinase"[9-13] and angiotensin-converting enzyme (ACE),[14] are able to degradate enkephalins by cleavage of the Gly^3–Phe^4 bond. However, the relevance of enkephalinase in the physiological regulation of enkephalinergic transmission is supported by (1) its regional distribution and subcellular localization, which parallel those of opiate receptors and correlate with enkephalins content;[15] (2) the changes in enzymatic activity following chronic morphine treatment;[9] and (3) the strong analgesic activity of enkephalin analogues protected from degradation by enkephalinase.[16]

Enkephalinase is a Zn metalloenzyme whose specificity is essentially ensured by specific interactions of the released C-terminal dipeptides Phe-Met or Phe-Leu.[17] So, enkephalinase contains an hydrophobic S_1' subsite, specific for aromatic residues, and a lipophilic S_2' subsite without a marked selectivity except its aversion for proline and

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Table I. Inhibitory Potency of Phe-Ala, Phe-Leu, and Related Carboxyalkyl Compounds on Enkephalinase and ACE Activities from Mouse Striatum

no.	compound	IC_{50},[a] μM enkephalinase[b]	IC_{50},[a] μM ACE[c]
1	$^+H_3NCH(CH_2Ph)CONHCH(CH_3)COO^-$	1.0 ± 0.2	>1000
2	$^+H_3NCH(CH_2Ph)CONHCH[CH_2CH(CH_3)_2]COO^-$	20.0 ± 3.0	~700
3	$^-OOCCH(CH_2Ph)CONHCH[CH_2CH(CH_3)_2]COO^-$[d]	0.7 ± 0.1	>1000
4	$^-OOCCH_2CH(CH_2Ph)CONHCH[CH_2CH(CH_3)_2]COO^-$[d]	0.8 ± 0.1	20 ± 0.2
5	$^-OOCCH_2NHCH(CH_2Ph)CONHCH(CH_3)COO^-$	0.5 ± 0.2	4 ± 0.8
6	$^-OOCCH_2NHCH(CH_2Ph)CONHCH[CH_2CH(CH_3)_2]COO^-$	2.0 ± 0.6	2 ± 0.5
7	$^-OOCCH_2CH_2NHCH(CH_2Ph)CONHCH[CH_2CH(CH_3)_2]COO^-$	11.0 ± 2.0	>100
8	$^-OOCCH_2NH\overset{*}{C}H(CH_2Ph)CONHCH[CH_2CH(CH_3)_2]COO^-$[e]	18.0 ± 4.0	
9	$^-OOCCH_2NHCH(CH_2Ph)CONH\overset{*}{C}H[CH_2CH(CH_3)_2]COO^-$[e]	11.0 ± 1.0	
10	$^-OOCCH_2NH\overset{*}{C}H(CH_2Ph)CONH\overset{*}{C}H[CH_2CH(CH_3)_2]COO^-$[e]	>100	

[a] The values are the mean ± SEM from triplicate experiments computed as described under Experimental Section. [b] Concentration inhibiting 50% of the activity of mouse striatal enkephalinase (P_2 fraction) in Tris-HCl, pH 7.4, 50 mM, 25 °C, with [^{3}H]Leu-enkephalin (40 nM) as substrate. [c] Concentration inhibiting 50% of the activity of mouse striatal ACE (P_2 fraction) in Tris-HCl, pH 7.4, 50 mM, 37 °C, with Hip-His-Leu (1 mM) as substrate. [d] These compounds are studied as diastereoisomeric mixtures of *RS* and *SS* isomers. [e] The asterisk refers to a D-amino acid.

hydrophilic amino acids.[17,18] In contrast, the S_1' subsite of ACE does not exhibit a significant preference for an aromatic side chain, whereas compounds bearing a C-terminal proline are well recognized by the S_2' one.[19]

Starting from these differences between enkephalinase and ACE, we have attempted to design potent inhibitors displaying a high selectivity for the enkephalin degrading enzyme, which is an essential prerequisite for their possible use in therapeutics.

Numerous inhibitors of metalloenzymes contain a carboxy group able to coordinate the metallic cation. Thus, benzylsuccinic acid is a good inhibitor of carboxypeptidase A,[20] while L-glutaryl-L-proline[19] or *N*-(1-carboxy-3-phenylpropyl)-L-Ala-L-Pro[21] are very potent and selective inhibitors of ACE. Furthermore, in these compounds the position of the carboxy group able to interact with the Zn atom is critical.[19]

Starting from these features, a series of enkephalinase inhibitors was prepared[22] using as models the dipeptides Phe-Leu and Phe-Ala, which are, respectively, 350 and 1000 times more potent on enkephalinase than on ACE activities.[17] These new compounds are characterized by a benzyl residue as the P_1' group and a methyl or isopropyl moiety as the P_2' component and contain alkyl chains of varying length ended by a carboxy substituent able to interact with the catalytic site. The synthesis and inhibitory potency of these carboxyalkyl inhibitors both on enkephalinase and ACE from mouse striatal membranes are reported in this paper. Moreover, the analgesic properties on mice of the most potent and selective compound, *N*-[(*RS*)-2-carboxy-3-phenylpropanoyl]-L-leucine (**3**), was evaluated using different assays.

Results and Discussion

Synthesis. Two types of carboxy derivatives were prepared to explore the catalytic site of enkephalinase: *N*-carboxyalkyl dipeptides and *N*-carboxyalkanoyl amino acids. A general procedure for the synthesis of carboxymethyl amino acids was proposed by Korman and Clarke,[23] but extension of this method to the preparation and purification of *N*-carboxyalkyl dipeptides appeared difficult to perform. For this reason, we preferred transposing the method of synthesis of α,α′-aminodicarboxylic acids described by Karrer et al.[24] to the preparation of *N*-carboxyalkyl dipeptides. A similar technique was recently used by Miyazawa in the synthesis of *N*-carboxymethyl amino acids.[25] The carboxymethyl dipeptides **5**, **6**, and **8–10** (Table I) were obtained by reaction between the trifluoroacetate salt of a dipeptide methyl ester and ethyl bromoacetate in the presence of triethylamine, followed by alkaline hydrolysis of the intermediate diester. The carboxyethyl dipeptide **7** was obtained by a similar procedure, using ethyl bromopropionate in place of ethyl bromoacetate. All the compounds of this series were obtained with a full optical purity, since it is well known that the introduction of a carboxyalkyl chain on the amino terminal group of dipeptides does not modify the stereochemistry of the two asymmetric α carbons. The lack of racemization in the different steps of synthesis was easily evidenced by ^{1}H NMR spectroscopy (270 MHz).

The carboxyalkanoyl derivatives **3** and **4** were synthesized by coupling L-leucine methyl ester with ethyl hydrogen benzylmalonate and β-ethyl hydrogen benzylsuccinate, respectively.

Ethyl hydrogen benzylmalonate, prepared by partial saponification of diethyl benzylmalonate, and β-ethyl hydrogen benzylsuccinate, obtained following the strategy of Cohen et al.,[26] were used as their racemic forms. Consequently, as opposed to the carboxyalkyl dipeptides, the

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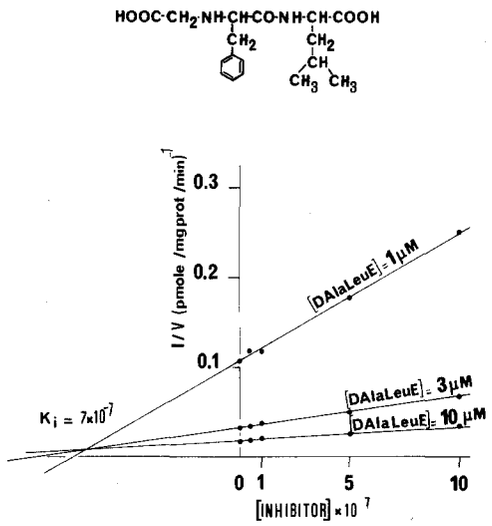

Figure 1. Dixon plots of the inhibition of enkephalinase from mouse striata by compound **6**, using mixtures of [^{3}H]-D-Ala^2-Leu-enkephalin and cold D-Ala^2-Leu-enkephalin at the indicated concentrations. Each point represents the mean of triplicate experiments. K_M = 16 μM for D-Ala^2-Leu-enkaphalin.

two carboxyalkanoyl amino acids **3** and **4** were studied as diastereoisomeric mixtures.

Inhibitory Potencies of Carboxyalkyl Derivatives on Enkephalinase Activity from Mouse Striatum. As shown in Table I, the introduction on Phe-Leu, Phe-Ala, or related structures of a carboxy group increases the inhibitory potency toward enkephalinase and ACE (compounds **3**–**6**). According to a full competitive inhibition mode against both peptidases exhibited by compound **6** (Figure 1), this feature can be related to the coordination of the COOH with the Zn atom located in the catalytic site of both brain peptidases. However, this effect being stronger in the case of ACE, the enkephalinase specificity of compounds **4**–**6** is tightly decreased as compared to their dipeptide precursors. This could be related to the binding strength of the coordinating group, which minimizes the preferential interaction of the side chains with the specific active subsites of enkephalinase or ACE.

Likewise, at the dipeptide level, replacement of L-Ala in **1** by L-Leu in **2** leads to a 20-fold decrease in potency, whereas the same change only gives a 3 times lower activity from **5** to **6**.

The compared activities of the four *N*-carboxymethyl dipeptides **6** and **8**-**10**, differing only by the stereochemistry of the amino acids Phe and Leu, show that the absolute configuration of the two residues is less critical in these compounds than it is in dipeptides. Indeed, the configurational change of a single residue in dipeptides leads to a 100-fold decrease in activity,[17] while the same change in the inhibitors **8** or **9** causes only a 10-fold one. These differences between inhibitors and dipeptides can be easily explained by taking into account that for dipeptides, only two important stabilizing interactions, involving the side chains of Phe and Leu, occur within the enzyme active site. Therefore, each change of absolute configuration at the P_1' or P_2' level leads to the disappearance of half of these stabilizing factors. In the inhibitors, there is an additional binding to the enzyme arising from the carboxy group, and, consequently, a single stereochemical change leads only to the loss of about a third of the three interactions. Obviously, in **10**, the loss of two assumed binding components produces once more, at least, a 100 times decreased affinity for enkephalinase.

The relatively good inhibitory potency exhibited by dipeptides like Phe-Ala is mainly due to hydrophobic interactions but could be reinforced by additional binding of the amino group under its neutral or protonated form with either a glutamic residue or the Zn atom located in the catalytic site.

Relationships between Enkephalinase Inhibition and Position of the Zn Chelating Carboxy Group in Inhibitors. The results of Table I show that whatever the position of the carboxy group in the chain, the inhibitory potencies on enkephalinase activity remain in the same range, whereas ACE recognition is strongly modulated by the length of the chain bearing the COOH group. Therefore, as far as ACE is concerned, the variation of IC_{50} values reported in Table I for compounds **3**–**7** are in fair agreement with that occuring in the carboxyalkanoyl-L-proline series.[19] Thus, compounds **5** and **6**, characterized by a chain length corresponding to that of glutarylproline, display better IC_{50} values than **4** or **7**. The very large changes in inhibitory potency between enkephalinase and ACE regarding the position of the carboxylate group in the chain could be explained by the binding strength involving the terminal COOH groups.

In the case of ACE, which behaves as a true carboxydipeptidase, ionic interactions of the P_2' carboxylate with a guanidinium group of the enzyme may be as strong as the Zn chelation by the COOH group belonging to the chain. Therefore, strong ACE inhibition could require dual interaction of the two COOH groups, which obviously depends of the distance between them.

In contrast enkephalinase exhibits exodipeptidase and endopeptidase activities.[27] So, in the enzyme, the metal binding strength of inhibitors could be predominant, leading to a relatively weak distance dependency in inhibitory potency provided that the coordination step can occur without drastic hindrance in enkephalinase recognition by the neighboring hydrophobic P_1' residue.

Furthermore, it can be noticed that in the potent carboxyalkyl dipeptides **5** and **6**, the NH group of Phe could interact by hydrogen bonding to the donor group of the enzyme involved in the catalytic process. This putative interaction has been proposed to play a major stabilizing role in the recently developed ACE carboxy inhibitors derived from L-Ala-L-Pro.[21] Such a hydrogen bond seems to be more important for the binding of inhibitors to ACE than to enkephalinase. Indeed, although Phe-Ala and Phe-Leu are badly recognized by ACE, the derived compounds **5** and **6** exhibit inhibitory potencies similar to that of the selective ACE inhibitor *N*-(carboxymethyl)-Ala-Pro (IC_{50} = 2.4 μM),[21] structurally related to the active dipeptide Ala-Pro (IC_{50} = 230 μM).[28] Such very large increases in activity toward ACE could support a hydrogen-bond formation at the level of the secondary amine.

Pharmacological Activity of the Selective Inhibitor *N*-[(*RS*)-2-Carboxy-3-phenylpropanoyl]-L-leucine (3). As expected, the large differential interaction of carboxyalkyl inhibitors with enkephalinase and ACE leads to specific compounds, as *N*-[(*RS*)-2-carboxy-3-phenylpropanoyl]-L-leucine (**3**), which acts as a full competitive inhibitor of enkephalinase with a K_I = 0.34 μM.[29]

This compound is at least 10 000-fold more potent against enkephalinase than against ACE and, thus, represents the first example of a new series of highly selective enkephalinase inhibitors different from (1) thiorphan and

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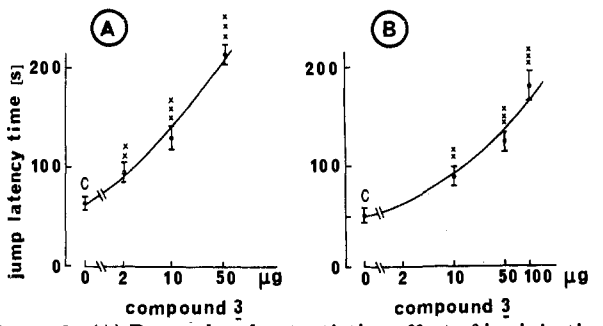

Figure 2. (A) Dose-related potentiating effect of icv injections of the enkephalinase inhibitor **3** on the analgesic response (hot-plate test, temperature = 55 ± 0.5 °C) induced by D-Ala²-Met-enkephalin (50 µg/10 µL per mouse, icv) in mice (n = 16): Cut-off time, 240 s; C = controls; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. (B) Dose-related analgesic responses (hot-plate test, temperature = 55 ± 0.5 °C) induced by icv injections of the enkephalinase inhibitor **3** in mice (n = 10): Cut-off time, 240 s; C = controls; ** = $p < 0.01$; *** = $p < 0.001$.

related compounds,[22,30] (2) phosphoryl-Leu-Phe,[31] and (3) hydroxamic acids[32] and barbiturates.[33] Therefore, it was of great interest to check the pharmacological activities of **3**.

In Vivo Inhibition of Brain Enkephalinase. According to their rapid degradation by brain peptidases, intracerebroventricular administration of Met-enkephalin or Leu-enkephalin leads to weak and transient analgesia.[34] Therefore, numerous enkephalin derivatives protected from these brain degrading enzymes were prepared, and their analgesic potency was shown to be roughly correlated with their metabolic stability.[16] According to its enkephalinase susceptibility, the morphine-like peptide D-Ala²-Met-enkephalin (DAME) is unable to produce an analgesic effect at the low dose (50 ng per mouse) used in this experiment. By contrast, icv injection of the same dose of DAME with increasing concentrations of **3** leads to a strong and dose-related analgesia in mice (Figure 2A). This potentiating effect, already found using thiorphan,[30] clearly demonstrates the protection of DAME from enkephalinase degradation by **3**.

Analgesic Effects of 3 on Mice. In order to definitely prove the action of **3** at the level of enkephalinergic synapses, it was necessary to check the analgesic responses exhibited by icv injection of this compound alone.

In order to compare this new inhibitor with thiorphan, we chose the icv route to circumvent possible differences in pharmacokinetic properties of both inhibitors. Figure 2B shows the dose-dependent increase of the jump latency time following icv administration of **3**.

The involvement of opiate receptors in this analgesic response is evidenced by the blocked effect of an intraperitoneal injection of naloxone (1 mg/kg) 10 min before administration of **3**. As already observed, naloxone alone behaves in the hot-plate test as an hyperalgesic drug.[29,35]

Mouse Writhing Test. Inhibition of the writhings produced in mice by the intraperitoneal injection of phenylbenzoquinone is a classical method to check analgesic substances.[36] As shown in Table II, icv administration of 10 µg of **3** significantly reduced the numbers of writhings. As in the hot plate test, this antinociceptive effect is antagonized by naloxone.

Table II. Analgesic Activity of the Enkephalinase Inhibitor 3 on the Mouse Writhing Test

inhibitor	writhing numbers
controls (n = 12)	23.5 ± 3.6
compound 3 (n = 12)	11.3 ± 2.3**
controls + naloxone (1 mg/kg, ip)[b]	31.2 ± 2.9
3 + naloxone (1 mg/kg, ip)[b]	29.7 ± 3.0

[a] Compound 3 was intracerebroventricularly injected at a single concentration (10 µg per mouse in 10 µL of saline) 10 min after phenylbenzoquinone was ip administered, and 10 min later, the number of writhings was counted during 10 min. [b] Naloxone was ip injected 10 min before icv administration of 3 or saline. ** = $p <$ 0.01.

Conclusion

As a result of the presence of a Zn atom in its catalytic site, enkephalinase can be inhibited by carboxyalkyl compounds bearing suitable P_1' components such as a benzyl group. Moreover, the large differences in the active sites of ACE and enkephalinase allowed us to design a potent and highly specific inhibitor, *N*-[(*RS*)-2-carboxy-3-phenylpropanoyl]-L-leucine (**3**). This compound acts in vitro and in vivo as a full competitive inhibitor of the enkephalin peptidyl dipeptide hydrolase and displays analgesic properties in mice after icv administration. As compared to thiorphan, **3** exhibits a slightly lower activity on the hot-plate test, since at the same icv administered dose (30 µg per mouse, n = 12) the increase in the jump latency time (in seconds) was 135 ± 6 for thiorphan and 112 ± 4 for **3**, respectively (controls = 54 ± 6). The strong analgesic activity of compound **3** as regard to its relatively low inhibitory potency against enkephalinase could be related to inhibition of other peptidases involved in antinociceptive transmission. It is well known that several aminopeptidases are able to degradate enkephalins. Although preliminary results indicate a low IC_{50} value of compound **3** for a set of membrane-bound aminopeptidases, one cannot exclude that **3** displays a high inhibitory potency on the specific aminopeptidase implicated in enkephalin metabolism.

Nevertheless, as already observed for other enkephalinase inhibitors,[30] **3** was found inactive on the rat tail-flick test where naloxone does not produce hyperalgesia. This feature could indicate that the efficiency of enkephalinase inhibitors requires long-time nociceptive stimuli able to induce a large release of endogenous enkephalins at the synaptic cleft level. Taking into account the large distribution of opiate receptors in the brain that could be associated with the wide range of pharmacological effects elicited by opioid substances, one might consider enkephalinase inhibitors as potential new analgesics and psychoactive agents.

Experimental Section

Biological Tests. Assay for Enkephalinase Activity. The enkephalinase activity was checked following the procedure previously described.[16,30] The particulate P_2 fractions of mouse striata, after being preincubated for 15 min at 25 °C, were incubated in a total volume of 0.1 mL of 0.1 M Tris buffer (pH 7.4)

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at the same temperature during 20 min in the presence of 10 nM of [^{3}H]Leu-enkephalin, 0.1 mM puromycin, and with or without various concentrations of inhibitors. The incubations were stopped by adding 25 μL of 0.2 N HCl, and ^{3}H-labeled metabolites were separated from intact [^{3}H]Leu-enkephalin by Porapak column chromatography (Waters).[13] Enkephalinase activity was evaluated as the difference between [^{3}H]Tyr-Gly-Gly formed in incubations performed in the absence and in the presence of inhibitors. Kinetic measurements were performed with the new substrate [^{3}H]D-Ala^2-Leu-enkephalin[37] as previously described.[29] IC_{50} values were computed from log probit plots with four to five concentrations of inhibitors.

Assay for Angiotensin-Converting Enzyme Activity. Hydrolysis of the substrate Hip-His-Leu (from Bachem, Switzerland) was studied following the procedure of Yang and Neff.[38] The particulate P_2 fractions of mouse striata were incubated in a total volume of 0.1 mL of 0.1 M Tris buffer (pH 7.4) at 37 °C for 30 min with 1 mM substrate and various concentrations of inhibitors. The reaction was terminated by adding 0.4 mL of 2 N NaOH, followed by the addition of 3 mL of water. The liberated His-Leu was detected by adding 0.1 mL of (1%) phthalaldehyde in methanol, followed 4 min later by 0.2 mL of 6 N HCl. The samples were centrifuged at 12000*g* for 10 min, and the fluorescence of the supernatant was measured with a MPF 44 A Perkin-Elmer fluorimeter (excitation 365 nm, emission 495 nm). A calibration curve for His-Leu was prepared by adding increasing concentrations of His-Leu to 0.1 mL of 0.1 M Tris buffer (pH 7.4) containing the denatured enzyme source.

Pharmacological Assays. Male Swiss albino mice (CD1, Charles River, 24–26 g) were used. All drugs were injected intracerebroventricularly, except naloxone, which was administered intraperitoneally. The icv injections were performed according to the method of Haley and McCormick[39] under a 10-μL volume. Drugs were dissolved in saline, and when NaOH was necessary for dissolving, the solution pH was thereafter adjusted as close as possible to 7 with HCl. Control animals were injected with saline.

Hot Plate Test. This test was derived from that of Eddy et al.[40] Ten minutes after the icv injection of the agent to be tested, the animals were set on a plate heated to 55 ± 0 °5, and the jump latency was measured, up to 240 s (cutoff time).

Phenylbenzoquinone Test. This test was derived from that of Siegmund et al.[41] Ten minutes after the icv injection of the agent to be tested, the animals were injected ip with a phenylbenzoquinone solution, and 10 min later abdominal writhings were counted during 10 min. For that purpose, phenylbenzoquinone (Sigma Chemical Co.) was dissolved in absolute alcohol (2 mg in 0.5 mL); then this solution was adjusted to 10 mL with distilled water, and the extemporaneously prepared resulting solution was kept at 37 °C during the experiments and injected at this temperature.

Statistical Comparisons. Means were compared to respective controls by Student's *t* test.

Chemistry. The protected amino acids, dipeptides Phe-Leu and Phe-Ala, are from Bachem (Switzerland). The purity of all the synthesized compounds was checked (1) by thin-layer chromatography on silica gel plates (Merck) [solvent systems (v/v): A, $CHCl_3$/MeOH/H_2O (9:1:saturated); B, BuOH/AcOH/H_2O (4:1:1); C, $CHCl_3$/MeOH/AcOH (9:1:0.5); D, $CHCl_3$/Et_2O (5:5)] and (2) by HPLC on a reverse-phase μ-Bondapak C_{18} column (Waters) with CH_3CN/NH_4AcO buffer (pH 4.2) as solvent. The eluted peaks were monitored at 220 nm.

The structure of all the synthesized compounds was confirmed by ^{1}H NMR spectroscopy (Bruker WH 270 MHz) in Me_2SO-d_6 solution (5×10^{-3} M). Complete assignment of ^{1}H NMR signals was performed by classical double resonance experiments. Chemical shifts (in parts per million ± 0.02) relative to HMDS as internal reference were reported only for the final compounds **5–10**. Melting points of the crystallized products are reported uncorrected. Analyses were given for the most relevant compounds, except for the trifluoroacetate salts, which are too highly hygroscopic.

The following abbreviations are used: THF, tetrahydrofuran; MeOH, methanol; EtOAc, ethyl acetate; DCC, *N,N*-dicyclohexylcarbodiimide; HOBT, 1-hydroxybenzotriazole; TFA, trifluoroacetic acid; Et_2O, diethyl ether; BuOH, 1-butanol; AcOH, acetic acid. The trifluoroacetate salts of L-Phe-L-Ala-OCH_3, L-Phe-L-Leu-OCH_3, D-Phe-L-Leu-OCH_3, and L-Phe-D-Leu-OCH_3 were synthesized following the described procedures.[42,43]

***N*-[(*RS*)-2-Carbethoxy-3-phenylpropanoyl]-L-leucine Methyl Ester.** To a solution of 1 g (15 mmol) of ethyl hydrogen benzylmalonate in THF (15 mL), cooled at 0 °C, were added successively 0.82 g (4.5 mmol) of L-leucine methyl ester hydrochloride and 0.65 mL of triethylamine in $CHCl_3$ (15 mL), 0.79 g (4.5 mmol) of 1-hydroxybenzotriazole in THF (10 mL), and 1.02 g (4.95 mmol) of *N,N*-dicyclohexylcarbodiimide in $CHCl_3$ (10 mL). After 1 h at 0 °C, the mixture was stirred at room temperature overnight. After filtration of dicyclohexylurea (DCU) and evaporation of the solvents, the residue was dissolved in EtOAc and washed successively with 10% citric acid (3 × 20 mL), water (20 mL), 10% $NaHCO_3$ (3 × 20 mL), water (20 mL), and, finally, with saturated NaCl (20 mL). The organic layer was dried over Na_2SO_4 and evaporated in vacuo. This procedure is designated as "standard treatment". An oily product was obtained (0.9 g, 85%): R_f (A) 0.70. Anal. ($C_{19}H_{27}NO_5$), C, H, N.

***N*-[(*RS*)-2-Carboxy-3-phenylpropanoyl]-L-leucine (3).** To a solution of 0.70 g (2 mmol) of *N*-[2-carbethoxy-3-phenylpropanoyl]-L-leucine methyl ester in acetone (5 mL) was added, at 0 °C, 4.3 mL of 1 N NaOH. The mixture was stirred at 0 °C for 1 h and at room temperature for 3 h. The solution was concentrated in vacuo, diluted with 10 mL of water, washed with Et_2O (10 mL), acidified to pH 2 with 2 N HCl, and extracted with Et_2O (3 × 10 mL). The etheral layer was dried over Na_2SO_4 and evaporated in vacuo. This treatment is designated as "standard procedure for alkaline hydrolysis". A white solid, recrystallized from Et_2O, was obtained (0.60 g, 95%): mp 84 °C; R_f (B) 0.60; ^{1}H NMR δ 0.62 and 0.79 (Leu CH_3), 1.27 and 1.43 (Leu β-CH_2), 1.04 and 1.56 (Leu γ-CH), 2.91 (CH_2-Ph), 3.54 ppm (C*H*-CH_2Ph), 4.10 (Leu α-CH), 7.14 (Ph), 8.19 and 8.29 (NH). Anal. ($C_{16}H_{21}NO_5$), C, H, N.

***N*-[(*RS*)-3-Carbethoxy-2-benzylpropanoyl]-L-leucine Methyl Ester.** To a solution of β-ethyl benzylsuccinate[26] (0.43 g, 1.82 mmol) in anhydrous THF (10 mL), cooled at 0 °C, were added successively a mixture of L-leucine methyl ester hydrochloride (0.33 g, 1.82 mmol) and triethylamine (0.255 mL) in $CHCl_3$ (15 mL), a solution of HOBT (0.28 g, 1.82 mmol) in anhydrous THF (10 mL), and a solution of DCC (0.375 g, 1.82 mmol) in $CHCl_3$ (10 mL). After 1 h at 0 °C, the mixture was allowed to come to room temperature and stirred overnight. The reaction was worked up following the "standard treatment" and yielded 0.57 g (86%) of an oily product: R_f (C) 0.92. Anal. ($C_{20}H_{29}NO_5$) C, H, N.

***N*-[(*RS*)-3-Carboxy-2-benzylpropanoyl]-L-leucine (4).** To a solution of the preceding compound (0.266 g, 0.77 mmol) in MeOH (5 mL), cooled to 0 °C, was added 1.54 mL of 1 N NaOH. The mixture was stirred at 0 °C for 1 h and at room temperature for 2 h. The reaction was treated following the "standard procedure for alkaline hydrolysis". A white solid, recrystallized from Et_2O, was obtained (0.205 g, 84%): mp 164 °C; R_f (C) 0.62; ^{1}H NMR δ 0.68 and 0.79 (Leu CH_3), 1.21 and 1.60 (Leu γ-CH), 1.30 and 1.43 (Leu β-CH_2) 2.44 and 2.97 (CH_2-Ph), 2.72 (C*H*-CH_2Ph), 2.03 and 2.44 (CH_2COOH), 4.06 and 4.14 (Leu α-CH), 7.16 (Ph), 8.07 and 8.15 (NH). Anal. ($C_{17}H_{23}NO_5$) C, H, N.

***N*-(Carbethoxymethyl)-L-phenylalanyl-L-alanine Methyl Ester.** To a suspension of L-Phe-L-Ala-OCH_3 trifluoroacetate (0.50 g, 1.37 mmol) in anhydrous benzene (15 mL) were added, at 0

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°C, 0.20 mL (1.37 mmol) of triethylamine and a solution of ethyl bromoacetate (0.25 g, 1.37 mmol) in 5 mL of benzene. The mixture was refluxed for 12 h. The organic layer was washed with water (2 × 5 mL) and neutralized with 0.15 N NaOH. After decantation, the benzene layer was washed with water and dried over Na_2SO_4. An oily product was obtained (0.28 g, 61%): R_f (A) 0.71. Anal. ($C_{17}H_{24}N_2O_5$) C, H, N.

***N*-(Carboxymethyl)-L-phenylalanyl-L-alanine (5).** To a cold solution of the preceding compound (0.7 g, 0.60 mmol) in MeOH (10 mL) was added 1 mL of 1 N NaOH. After 1 h at 0 °C, the mixture was stirred for 2 h at room temperature. After evaporation of MeOH in vacuo, the residue was dissolved in water (10 mL), washed with EtOAc (5 mL), and acidified to pH 2 with 1 N HCl. The obtained white solid (0.10 g, 72%) was filtered, washed with water, and dried: mp 138 °C; R_f (B) 0.37. ^{1}H NMR δ 1.21 (Ala CH_3), 3.02 (Phe β-CH_2), 3.44 (CH_2COOH), 3.90 (Phe α-CH), 4.14 (Ala α-CH), 7.23 ppm (Ph), 8.74 (NH). Anal. ($C_{14}H_{18}N_2O_5$) C, H, N.

***N*-(Carbethoxymethyl)-L-phenylalanyl-L-leucine Methyl Ester.** To a suspension of L-Phe-L-Leu-OCH_3 trifluoroacetate (0.53 g, 1.3 mmol) in anhydrous benzene (10 mL) was added, at 0 °C, 0.19 mL of triethylamine and 0.217 g (1.3 mmol) of ethyl bromoacetate in anhydrous benzene (5 mL). The mixture was refluxed for 12 h. The benzene layer was washed with water (2 × 5 mL), neutralized with 0.15 N NaOH, washed again with water (2 × 5 mL) and saturated NaCl, and finally dried over Na_2SO_4. The solvent was evaporated in vacuo, and an oily product was obtained (0.26 g, 53%): R_f (A) 0.77. Anal. ($C_{20}H_{30}N_2O_5$) C, H, N.

***N*-(Carboxymethyl)-L-phenylalanyl-L-leucine (6).** To a cold solution of the preceding compound (0.25 g, 0.66 mmol) in MeOH (5 mL) was added 1.3 mL of 1 NaOH. After 1 h at 0 °C, the mixture was stirred for 2 h at room temperature. After the solvent was evaporated in vacuo, the residue was dissolved in H_2O (10 mL), washed with EtOAc (2 × 5 mL), and acidified to pH 2 with 1 N HCl. The obtained white precipitate (0.135 g, 61%) was filtered, washed with water, and dried. Recrystallization from EtOH/H_2O (50:50): mp 208 °C; R_f (B) 0.62; ^{1}H NMR δ 0.78 (Leu CH_3), 1.44 (Leu β-CH_2 and γ-CH), 2.70 and 2.85 (Phe α-CH_2), 3.07 (CH_2COOH), 3.33 (Phe α-CH), 4.14 (Leu α-CH), 7.17 (Ph), 8.07 (NH). Anal. ($C_{17}H_{24}N_2O_5$) C, H, N.

***N*-(Carbethoxyethyl)-L-phenylalanyl-L-leucine Methyl Ester.** To a suspension of L-Phe-L-Leu-OCH_3 trifluoroacetate (0.82 g, 2 mmol) in anhydrous benzene (20 mL) were added successively triethylamine (0.44 mL) and ethyl bromopropionate (0.37 mL, 2.9 mmol). The mixture was refluxed for 12 h. The benzene layer was washed with water (2 × 5 mL), neutralized with 0.15 N NaOH, washed again with water (2 × 5 mL) and with saturated NaCl, and finally dried over Na_2SO_4. After evaporation of the solvent, an oily product was obtained (0.24 g, 32%): R_f (A) 0.75. Anal. ($C_{21}H_{32}N_2O_5$) C, H, N.

***N*-(Carboxyethyl)-L-phenylalanyl-L-leucine (7).** To a cold solution of the preceding compound (0.07 g, 0.18 mmol) dissolved in MeOH (5 mL) was added 0.36 mL of 1 N NaOH. After 1 h at 0 °C, the mixture was stirred for 2 h at room temperature. The reaction was worked up following the procedure described for compound 8. A white solid (0.03 g, 47%) was obtained, which was recrystallized from EtOH/H_2O (50:50): mp 218 °C; R_f (B) 0.46; ^{1}H NMR δ 0.70 (Leu CH_3), 1.37 (Leu β-CH_2 and γ-CH), 2.75 and 2.90 (Phe β-CH_2), 2.70 and 3.00 (CH_2CH_2COOH), 3.67 (Phe α-CH), 4.17 (Leu α-CH), 7.18 (Ph), 8.47 (NH). Anal. ($C_{18}H_{26}N_2O_5$) C, H, N.

***N*-(Carbethoxymethyl)-D-phenylalanyl-L-leucine Methyl Ester.** This compound was obtained following the procedure described for *N*-(carbethoxymethyl)-L-phenylalanyl-L-leucine methyl ester. From 0.5 g (1.23 mmol) of D-Phe-L-Leu-OCH_3 trifluoroacetate was obtained 0.27 g (51%): R_f (A) 0.72. Anal. ($C_{20}H_{30}N_2O_5$) C, H, N.

***N*-(Carboxymethyl)-D-phenylalanyl-L-leucine (8).** This compound was obtained following the procedure described for **6**. From 0.2 g (0.52 mmol) of the preceding compound was obtained 0.11 g (61%) of compound **8**, which was recrystallized from EtOH/H_2O (50:50): mp 238 °C; R_f (B) 0.32; ^{1}H NMR 0.67 (Leu CH_3), 1.17 (Leu γ-CH), 1.32 (Leu β-CH_2), 2.75 (D-Phe β-CH_2), 3.08 (CH_2COOH), 3.40 (D-Phe α-CH), 4.04 (Leu α-CH), 7.16 (Ph), 8.15 (NH). Anal. ($C_{17}H_{24}N_2O_5$) C, H, N.

***N*-(Carbethoxymethyl)-L-phenylalanyl-D-leucine Methyl Ester.** This compound was obtained following the procedure described for *N*-(carbethoxymethyl)-L-Phe-L-Leu-OCH_3. From 0.81 g (2 mmol) of L-Phe-D-Leu-OCH_3 trifluoroacetate was obtained 0.5 g (50%) of the title compound: R_f (D) 0.45. Anal. ($C_{20}H_{30}N_2O_5$), C, H, N.

***N*-(Carboxymethyl)-L-phenylalanyl-D-leucine (9).** This compound was obtained following the procedure described for **6**. From 0.156 g (0.41 mmol) of the preceding compound was obtained 0.12 g (87%) of **9**, which was recrystallized from EtOH/H_2O (50:50): mp 238 °C; R_f (B) 0.32; ^{1}H NMR δ 0.67 (Leu CH_3), 1.16 (Leu γ-CH), 1.33 (Leu γ-CH), 2.75 (Phe β-CH_2), 3.08 (CH_2COOH), 3.41 (Phe α-CH), 4.04 (Leu α-CH), 7.16 (Ph), 8.15 (NH). Anal. ($C_{17}H_{24}N_2O_5$), C, H, N.

***N*-(*tert*-Butyloxycarbonyl)-D-phenylalanyl-D-leucine Methyl Ester.** This compound was obtained following the procedure described for Boc-L-Phe-L-Leu-OCH_3. From 2 g (7.7 mmol) of Boc-D-Phe and 1.4 g (7.7 mmol) of D-Leu-OCH_3·HCl was obtained 2.61 g (82%) of the protected dipeptide, which was recrystallized from EtOAc: mp 105 °C; R_f (A) 0.76. Anal. ($C_{21}H_{32}N_2O_5$) C, H, N.

D-Phenylalanyl-D-leucine Methyl Ester Trifluoroacetate. This compound was obtained following the procedure described for L-Phe-L-Leu-OCH_3 trifluoroacetate. From 2 g (5.1 mmol) of Boc-D-Phe-D-Leu-OCH_3 was obtained 1.46 g (70%) of the title compound: mp 170 °C; R_f (B) 0.72.

***N*-(Carbethoxymethyl)-D-phenylalanyl-D-leucine Methyl Ester.** This compound was obtained following the procedure described for *N*-(carbethoxymethyl)-L-Phe-L-Leu-OCH_3. From 1.31 g (3.2 mmol) of D-Ala-D-Leu-OCH_3 trifluoroacetate was obtained 0.59 g (48%) of the title compound: R_f (A) 0.77. Anal. ($C_{20}H_{30}N_2O_5$) C, H, N.

***N*-(Carboxymethyl)-D-phenylalanyl-D-leucine (10).** This compound was obtained following the procedure described for **6**. From 0.22 g (0.58 mmol) of the preceding compound was obtained 0.17 g (87%) of compound **10**, which was recrystallized from EtOH/H_2O (50:50): mp 209 °C; R_f (B) 0.62; ^{1}H NMR δ 0.78 (Leu CH_3), 1.44 (Leu β-CH_2 and γ-CH), 2.70 and 2.85 (Phe β-CH_2), 3.07 (CH_2COOH), 3.33 (Phe α-CH), 4.14 (Leu α-CH), 7.17 (Ph), 8.07 (NH). Anal. ($C_{17}H_{24}N_2O_5$), C, H, N.

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Registry No. 1, 3918-87-4; 2, 3303-55-7; (*R*)-3, 83588-04-9; (*S*)-3, 83588-14-1; (*R*)-4, 81024-86-4; (*S*)-4, 81024-87-5; 5, 81109-96-8; 6, 81109-85-5; 7, 81109-93-5; 8, 81109-89-9; 9, 81109-87-7; 10, 81109-91-3; L-Phe-L-Ala-OCH_3 trifluoroacetate, 81109-94-6; L-Phe-L-Leu-OCH_3 trifluoroacetate, 68835-84-7; *N*-(carbethoxymethyl)-L-Phe-L-Leu-OCH_3, 81109-92-4; D-Phe-L-Leu-OCH_3 trifluoroacetate, 83588-09-4; L-Phe-D-Leu-OCH_3 trifluoroacetate, 83588-10-7; Boc-D-Phe, 18942-49-9; D-Leu-OCH_3, 23032-21-5; Boc-D-Phe-D-Leu-OCH_3, 83588-11-8; ACE, 9015-82-1; *N*-[(*R*)-2-carbethoxy-3-phenylpropanoyl]-L-leucine methyl ester, 83588-05-0; *N*-[(*S*)-2-carbethoxy-3-phenylpropanoyl]-L-leucine methyl ester, 83588-06-1; ethyl hydrogen (±)-benzylmalonate, 67682-05-7; L-leucine methyl ester hydrochloride, 7517-19-3; *N*-[(*R*)-3-carbethoxy-2-benzylpropanoyl]-L-leucine methyl ester, 83588-07-2; *N*-[(*S*)-3-carbethoxy-2-benzylpropanoyl]-L-leucine methyl ester, 83588-08-3; β-ethyl (±)-benzylsuccinate, 21307-98-2; *N*-(carbethoxymethyl)-L-phenylalanyl-L-alanine methyl ester, 81109-95-7; ethyl bromoacetate, 105-36-2; *N*-(carbethoxymethyl)-L-phenylalanyl-L-leucine methyl ester, 81109-84-4; ethyl bromopropionate, 539-74-2; *N*-(carbethoxymethyl)-D-phenylalanyl-L-leucine methyl ester, 81109-88-8; *N*-(carbethoxymethyl)-L-phenylalanyl-D-leucine methyl ester, 81109-86-6; D-phenylalanyl-D-leucine methyl ester trifluoroacetate, 83588-13-0; *N*-(carbethoxymethyl)-D-phenylalanyl-D-leucine methyl ester, 81109-90-2; enkephalinase, 70025-49-9.