(2:1) was added to the incubation mixture, and the whole was extracted with $CHCl_3$. The extracts were evaporated, and the residue was chromatographed on a Sephadex LH-20 column with $CHCl_3$–hexane (3:1) as the eluent. The more polar peak eluted from the column was collected and the solvent was evaporated. The residue was purified on HPLC (Finepak-SIL, 0.46 × 25 cm) with 15% 2-propanol in hexane as the eluent. The major peak eluted between 8.5 and 12 mL was collected and purifed again on the same HPLC system. The major peak eluted between 6.7 and 7.3 mL was collected and subjected to mass spectrometry.

Incorporation of Vitamin D_3 Derivatives into HL-60 Cells. HL-60 cells (1×10^7) were incubated at 37 °C for 1 h in serum-free RPMI 1640 medium with 3.2 nM 25-(OH)[^{3}H]D_3 (**1a'**) or 25-(OH)[^{3}H]D_3 endoperoxides (**2a'** and **3a'**) in the presence or absence of a 10000-fold excess of 25-(OH)D_3 or 25-(OH)D_3 endoperoxides, respectively. After incubation, cells were washed three times with Ca^{2+}- and Mg^{2+}-free PBS containing 10% heat-inactivated calf serum (Chiba Serum Institute, Chiba, Japan) and sonicated with an ultrasonic disruptor in a solution containing 10 mM Tris/HCl, pH 7.4, 2 mM EDTA, 0.5 mM dithiothreitol, and 0.1 M KCl. The cytosol and the chromatin fractions were prepared as previously reported.[8] Radioactivity in the cytosol fraction was determined after treatment with hydroxylapatite to separate protein-bound 25-(OH)[^{3}H]D_3 and 25-(OH)[^{3}H] endoperoxides from the respective free radiosiotope.

Measurement of Phagocytic Activity. Phagocytic activity was measured according to the method of Collins et al.[10] The procedure is described in the preceding paper.[1]

Binding Assay. The procedure of the binding assay is described in the preceding paper.[1]

Measurement of Bone-Resorbing Activity. Sixteen-day pregnant mice, ddY strain (Shizuoka Laboratory Animal Center, Shizuoka, Japan), were injected subcutaneously with 50 μCi of $^{45}CaCl_2$ (New England Nuclear, Boston, MA), and 1 day later, they were sacrificed and the fetuses were isolated. The fetal calvaria were excised and divided into paired halves by dissecting along the midsagittal suture.[11] Each half calvaria was cultured for 24 h at 37 °C under 5% CO_2 in air in 0.5 mL of BGJ_b medium (GIBCO, Grand Island, NY) containing 1 mg/mL of bovine serum albumin (Fraction V, Sigma). After preculture for 24 h, each half calvaria was transferred to fresh medium with (treated) or without (control) vitamin D_3 derivatives and cultured for an additional 6 days. At the end of the culture period, the bones were digested overnight with 0.1 mL of HCl. ^{45}Ca in the medium and bone extracts was counted by a liquid scintillation counter. Results were expressed as the treated/control ratio and calculated by the following formula:

$$^{45}\text{Ca release (\%)} = \frac{^{45}\text{Ca in medium}}{^{45}\text{Ca in medium} + ^{45}\text{Ca in bone}} \times 100$$

$$\text{T/C ratio} = \frac{^{45}\text{Ca release (\%) from the treated calvaria}}{^{45}\text{Ca release (\%) from the control calvaria}}$$

Statistical difference was tested by student's t test.

Registry No. **2a**, 96999-64-3; **2a'**, 97011-22-8; **3a**, 96999-65-4; **3a'**, 96999-66-5; **4a**, 96999-67-6; **6a**, 96999-68-7; 25-(OH)[26,27-^{3}H]D_3, 71595-10-3.

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New Bidentates as Full Inhibitors of Enkephalin-Degrading Enzymes: Synthesis and Analgesic Properties

Marie-Claude Fournié-Zaluski,† Annie Coulaud,‡ Romaine Bouboutou,† Pierre Chaillet,‡ Jocelyne Devin,† Gilles Waksman,† Jean Costentin,‡ and Bernard P. Roques*†

Département de Chimie Organique, UA 496 CNRS et U 266 INSERM, U.E.R. des Sciences Pharmaceutiques et Biologiques, 75006 Paris, and Laboratoire de Pharmacodynamie, U.E.R. de Médecine et Pharmacie, Université de Rouen, 76800 Saint Etienne du Rouvray, France. *Received October 15, 1984*

New compounds were designed to fully inhibit the in vitro metabolism of enkephalins, ensured by three different metallopeptidases. For this purpose, bidentate ligands as hydroxamate and *N*-hydroxy-*N*-formylamino groups were selected as highly potent metal coordinating agents and introduced on Phe-Gly and Phe-Ala related structures. Compounds corresponding to the general formula $HC(O)N(OH)CH_2CH(CH_2Ph)CONHCH_2COOH$ (compound **7**) and $HN(OH)C(O)CH_2CH(CH_2Ph)CONHCH(R)COOH$ (compound **11**, R = H; compound **13**, R = CH_3) behave as full inhibitors of the three enzymes, with IC_{50}'s in the nanomolar range for enkephalinase, from 0.3 μM to 1 nM for dipeptidylaminopeptidase, and in the micromolar range for a biologically relevant aminopeptidase. Two diastereoisomers of the most active inhibitor **13** were separated by HPLC and their stereochemistry was assigned by ^{1}H NMR spectroscopy. Both isomers were efficient as enkephalinase blockers, but only the *RS* isomer, designated kelatorphan, was able to strongly inhibit aminopeptidase and dipeptidylaminopeptidase. Intracerebroventricular injection in mice of these mixed inhibitors, especially kelatorphan, led to naloxone reversible analgesic responses (hot-plate test) that were slightly better than those produced by a mixture of thiorphan and bestatin, two potent inhibitors of enkephalinase and aminopeptidase, respectively. Kelatorphan was also more efficient in potentiating the analgesia induced by a subanalgesic dose of Met-enkephalin. All these results support a physiological role in pain transmission for enkephalinase and a probably synaptic aminopeptidase M.

Psychic dependence, which is a specific side effect of narcotics, could be related to slow changes in normal homeostasis following an overstimulation of brain receptors involved in behavioral control. Likewise, the respiratory depression caused by high doses of morphine and surrogates is probably due to an overstimulation of opioid receptors located on bulbar respiratory neurons.[1] A large number of opioids have been synthesized with the aim of eliminating these major side effects, but no potent analgesic thus far described has proven to be completely free

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‡ Laboratoire de Pharmacodynamie.

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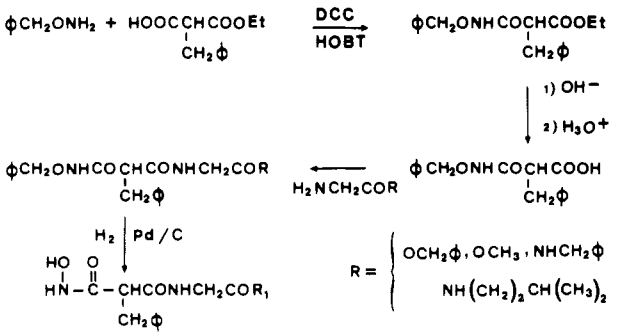

Figure 1. Scheme for the synthesis of *N*-[2(*RS*)-[(hydroxyamino)carbonyl]-1-oxo-3-phenylpropyl]glycine (1) and its analogues.

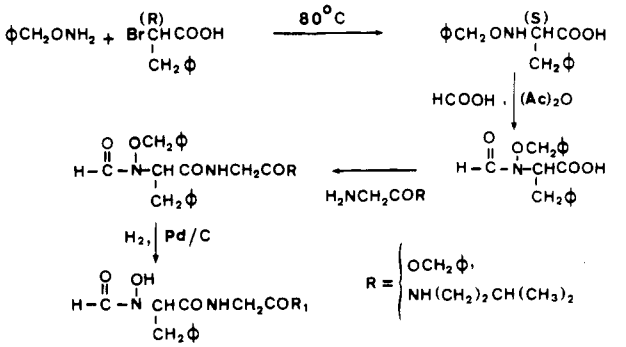

Figure 2. Scheme for the synthesis of *N*-formyl-*N*-hydroxy-L-phenylalanylglycine (5) and its analogues.

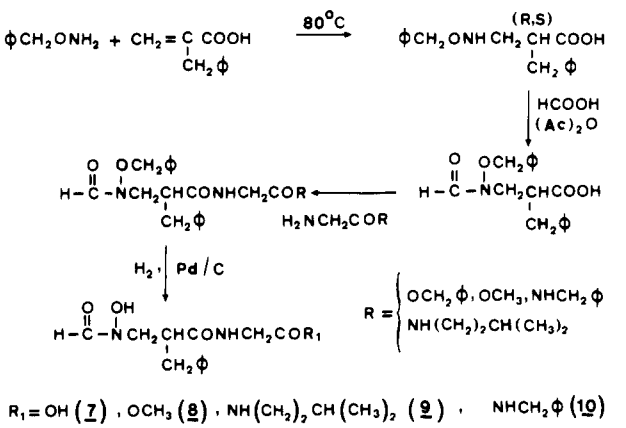

Figure 3. Scheme for the synthesis of *N*-[3(*RS*)-(*N*-formyl-*N*-hydroxyamino)-2-benzyl-1-oxopropyl]glycine (7) and its analogues.

from these serious side effects.

The discovery of endogenous opioid peptides in the brain[2] which are associated with both a heterogeneity of binding sites[3-5] and well-defined metabolic pathways might offer new approaches to the prevention or treatment of addiction following analgesic use. In vitro studies performed with rat brain slices showed that three metalloenzymes are involved in enkephalin degradation: (1) a neutral metalloendopeptidase, EC 3.4.24.11,[6] designated enkephalinase,[7] cleaving the Gly[3]–Phe[4] bond, (2) an aminopeptidase activity[8] releasing the N-terminal tyrosine, and a dipeptidylaminopeptidase cleaving the Gly[2]–Gly[3] amide bond.[9] The importance of enkephalinase is supported by the analgesic effects induced by administration of potent inhibitors such as carboxyalkyl dipeptides,[10,11] thiorphan,[12,13] and retrothiorphan.[14] These compounds were prospectively synthesized after an investigation of the requisite recognition subsites of enkephalinase.[15,16]

The analgesic activity induced by intracerebroventricular (icv) administration of the aminopeptidase inhibitor bestatin suggested that this enzyme could also be physiologically involved in the enkephalin degradation.[17] Therefore, it was of interest to design compounds able to inhibit the three enkephalin-degrading enzymes belonging to the group of metallopeptidases. As shown by crystallographic studies on carboxypeptidase A[18] and thermolysin,[19] a mechanism of inhibition of these enzymes utilizes the binding of a coordinating agent to the Zn metal ion in the active site. Bidentate groups like hydroxamate or *N*-formyl-*N*-hydroxyamino groups are potent metal coordinating agents.[20] Bidentate ligand-containing compounds already have been reported as efficient inhibitors of enkephalinase[21] or aminopeptidase.[22] Therefore, we have assumed that the strength of the binding to the metal atom should be sufficient to counterbalance the expected loss of binding energy due to an imperfect fit of a given inhibitor within the active sites of three different enzymes. The synthesis and biochemical and pharmacological properties of such molecules, which are theoretically able

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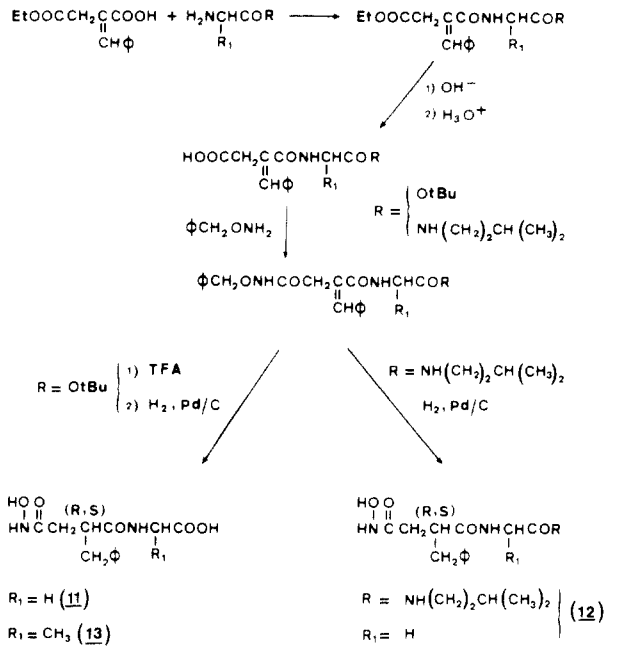

Figure 4. Scheme for the synthesis of *N*-[3(*RS*)-[(hydroxyamino)carbonyl]-2-benzyl-1-oxopropyl]glycine (11) and its derivatives.

to completely inhibit enkephalin metabolism, are reported in this paper.

Results and Discussion

Synthesis. A series of inhibitors corresponding to *N*-[2(*RS*)-[(hydroxyamino)carbonyl]-1-oxo-3-phenylpropyl]glycine (1) and its derivatives **2–4** were synthesized in four steps with use of monoethyl benzylmalonate as starting material (Figure 1). This compound, obtained by partial saponification of diethyl benzylmalonate,[23] was used as a racemic mixture. The coupling step with *O*-benzylhydroxylamine was followed by an alkaline hydrolysis. A second coupling step, by means of dicyclohexylcarbodiimide (DCC), with glycine esters or amides led to the fully protected inhibitors. Removal of the protecting benzyl groups was done by catalytic hydrogenation.

N-formyl-*N*-hydroxy-L-phenylalanylglycine (**5**) and its amide derivative **6** were also obtained in four steps from (*R*)-2-bromo-3-phenylpropanoic acid[24] (Figure 2). Thus, nucleophilic displacement of the halogen atom by *O*-benzylhydroxylamine, followed by N-formylation of the benzyloxyamino group, led to *N*-formyl-*N*-(benzyloxy)-L-phenylalanine. This compound was coupled to a suitable glycine derivative, and the synthesis was completed by deprotective catalytic hydrogenation.

The related series of inhibitors corresponding to *N*-[3-(*RS*)-(*N*-formyl-*N*-hydroxyamino)-2-benzyl-1-oxopropyl]glycine (**7**) and its analogues **8–10** were obtained by a similar method (Figure 3). Various glycine derivatives were coupled with 3-[(benzyloxy)amino]-2-benzylpropanoic acid, which corresponds to a N,N-disubstituted derivative of β-phenylalanine. This intermediate was synthesized as a racemic mixture by conjugate addition of *O*-benzylhydroxylamine to benzylacrylic acid[25] followed by N-formylation of the hydroxylamino group. The deprotection step was also performed by catalytic hydrogenation.

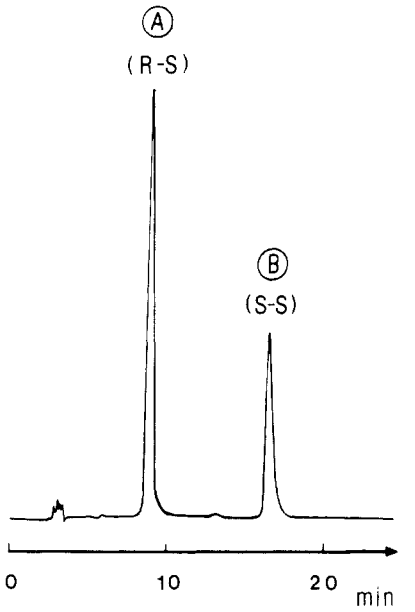

Figure 5. HPLC separation of the two stereoisomers of compound **13**. Peak A = *N*-[3(*R*)-[(hydroxyamino)carbonyl]-2-benzyl-1-oxopropyl]-L-alanine. Peak B = *N*-[3(*S*)-[(hydroxyamino)carbonyl]-2-benzyl-1-oxopropyl]-L-alanine. Solvent: CH_3CN/2% acetic acid (15/85). Detection at 260 nM.

The simplest way to prepare the last series of inhibitors corresponding to *N*-[3(*RS*)-[(hydroxyamino)carbonyl]-2-benzyl-1-oxopropyl]glycine (11) and its derivatives **12** and **13** would be to condense appropriate *tert*-butyl ester or amide derivatives of amino acids to ethyl 3-carboxy-4-phenylbutanoate, followed by introduction of the hydroxyamino group. However, this method is hampered by difficulties in the preparation of the required butanoic acid derivative and, above all, by the isomerization process encountered during the alkaline treatment of substituted "succinimides".[26] Therefore, the synthesis was begun (Figure 4) with use of ethyl 2-benzylidene-3-carboxypropanoate, obtained according to Cohen and Milovanovic.[27] This latter compound was coupled with appropriate glycine or L-alanine derivatives. The ethyl ester was hydrolyzed in alkaline medium and a second coupling step with *O*-benzylhydroxylamine in the presence of DCC led to the fully protected inhibitors. Treatment with trifluoroacetic acid removed the *tert*-butyl ester of compounds **11** and **13**, and for all derivatives of this series, catalytic hydrogenation produced both a deprotection of the hydroxylamino group and saturation of the benzylidene double bond. In this series, compounds **11** and **12** were obtained as racemic forms, but compound **13** corresponds to a diastereoisomeric mixture of *RS* and *SS* isomers. Separation of these two diastereoisomers was easily performed by HPLC, as shown by the chromatographic analysis of the crude reaction product (Figure 5). The relative proportions of the two isomers was approximately A/B = 60/40, demonstrating some asymmetric induction by the L-alanine residue during catalytic hydrogenation. After HPLC separation, the configuration of the benzylsuccinyl moiety in each isomer was tentatively established by ^1H NMR spectroscopy. Indeed, ^1H NMR spectra of a great number of small peptides containing an aromatic amino acid have shown that the chemical shifts of alkyl side chain protons were greatly dependent of the relative configuration of each asymmetric carbon. A comparison

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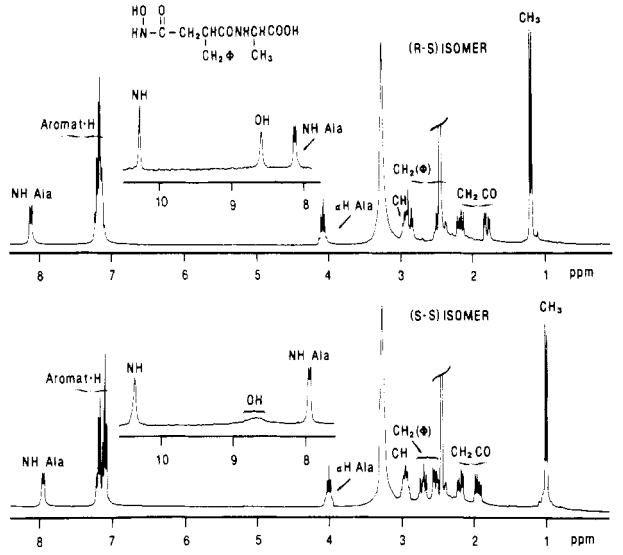

Figure 6. ^{1}H NMR spectra of the two stereoisomers of compound **13**. (a) Spectrum of *N*-[3(*R*)-(hydroxyamino)carbonyl-2-benzyl-1-oxopropyl]-L-alanine = kelatorphan. (b) Spectrum of *N*-[3(*S*)-[(hydroxyamino)carbonyl]-2-benzyl-1-oxopropyl]-L-alanine.

of the spectra of L-Phe-L-Ala and D-Phe-L-Ala showed that the methyl group of L-Ala was more shielded ($\delta(CH_3)$ 1.08) in the D-Phe-L-Ala isomer than in the natural dipeptide L-Phe-L-Ala ($\delta(CH_3)$ 1.18). This effect is probably due to a difference in the mean orientation of the chains in the two diastereoisomers. In D-Phe-L-Ala, the CH_3 group is shielded by the ring current of Phe, whereas in the natural dipeptide, it has the same chemical shift as in any peptide containing alanine.[28] The spectra of the two separate isomers of compound **13** (Figure 6a,b) show large chemical shift differences for hydroxyamino and amido NH groups and for the alanine side chain. In the spectrum of the most abundant isomer (HPLC, peak A), the methyl group is more deshielded ($\delta(CH_3)$ 1.18, Figure 6a) than the methyl signal of the second isomer ($\delta(CH_3)$ 0.99, Figure 6b). Consequently, the proposed structure for isomer A is *N*-[3(*R*)-[(hydroxyamino)carbonyl]-2-phenyl-1-oxopropyl]-L-alanine, which is analogous to the natural dipeptide L-Phe-L-Ala. Obviously isomer B, which corresponds to D-Phe-L-Ala is assumed to be *N*-[3(*S*)-[(hydroxyamino)carbonyl]-2-phenyl-1-oxopropyl]-L-alanine.

Inhibitory Potencies of Bidentate Inhibitors on Enkephalinase Activity. The inhibitory potency of the four series of bidentates was tested vs. enkephalinase, purified from rat kidney, with use of [^{3}H]-D-Ala^2-Leu-enkephalin as substrate (K_m = 30 ± 10 μM). As shown before,[29] this tritiated substrate, being resistant to aminopeptidase and dipeptidylaminopeptidase, was more useful for comparison of enkephalinase activity in different preparations. Thus it can be observed that similar results were obtained by use of striatal mouse brain preparation in place of rabbit kidney enzyme (not shown here).

The results presented in Table I show that among the compounds synthesized, those containing a free carboxyl group (compounds **1**, **5**, **7**, **11**, and **13**) behave as highly potent enkephalinase inhibitors. Except for compound **5**, which presents an IC_{50} value in the micromolar range, the other compounds have IC_{50} values between 1.4 and 12 nM. The inhibitory potency of the latter vs. enkephalinase is in the same range as thiorphan[12] or retrothiorphan.[14] Moreover, compounds **1**, **11**, and **13** exhibit similar inhibitory potencies despite the fact that the metal binding hydroxamate group HN(OH)CO is located in different positions relative to the S_1' subsite. (The nomenclature used for the individual amino acids residues (P_1, P_1', P_2', etc.) of an inhibitor or substrate and for the subsites (S_1, S_1', S_2', etc.) of the enzyme is that of Schechter and Berger[30].) In contrast, compound **5**, which contains the bidentate *N*-formyl-*N*-hydroxyamino group in a position similar to the hydroxamate agent in **1**, displays a much lower activity. However, the increased separation of the metal coordinating group from the aromatic side chain in **7** leads again to a highly potent inhibitor.

The affinities for enkephalinase of all these compounds were compared to those of the corresponding derivatives containing monofunctional metal binding groups. The two carboxyalkyl analogues **14** and **15**[11] have IC_{50} values in the micromolar range while the *N*-hydroxy peptide **16** is about 10 times less potent. The greatly increased activity of compounds **1**, **7**, **11**, and **13** as compared to that of the corresponding monofunctional compounds strongly suggests that the former interact as bidentate ligands with the zinc atom of the catalytic site. By contrast, compound **5** seems to act only as a monodentate ligand. In the absence of crystallographic data on enkephalinase, this result cannot be explained at the molecular level.

On the other hand, the affinity for enkephalinase is greatly dependent of the nature of the C-terminal group of the inhibitor. Although enkephalinase has been described as an endopeptidase,[6] it is noteworthy that compounds bearing a free C-terminal carboxyl group are the most potent inhibitors. Thus, esterification by a methyl group (compounds **2** and **8**) or amidation by a lipophilic chain (compounds **3**, **4**, **6**, **9**, **10**, and **12**) decreases the activity by approximately 2 orders of magnitude. These features, already observed in other series of enkephalinase substrates or inhibitors,[15,31] reinforce the assumption that enkephalinase is able to act as an exopeptidase or an endopeptidase. Finally, it is of major interest to notice that all of the present enkephalinase inhibitors showed low inhibitory potency for angiotensin converting enzyme (IC_{50} > 10 000 nM).[32]

Inhibitory Potency of Bidentate Inhibitors on Dipeptidylaminopeptidase and Aminopeptidase Activities. [^{3}H]-Leu-enkephalin was used as substrate to determine the inhibitory potency of the studied compounds on a dipeptidylaminopeptidase[9] and on a "bestatin"-sensitive aminopeptidase purified from rat brain. The enzymatic properties (K_m value, inhibition by various compounds, etc.) of the membrane-bound aminopeptidase from rat brain were found to be identical with those of aminopeptidase M associated with neutral metalloendopeptidase in rabbit kidney.[33] Therefore this latter enzyme was used routinely in the present study.

The inhibitory potencies against these two enzymatic activities are summarized in Table II. In contrast to enkephalinase, a potent dipeptidylaminopeptidase inhibition was essentially restricted to bidentates bearing an

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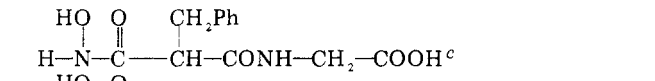
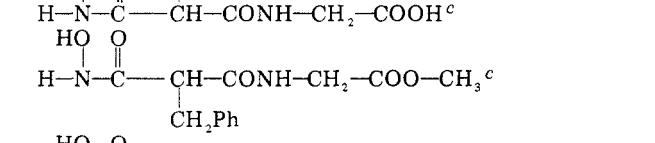
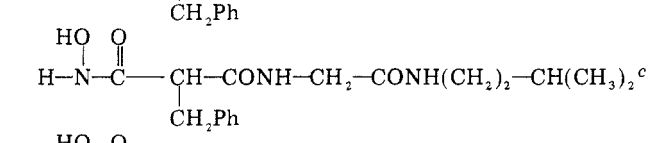
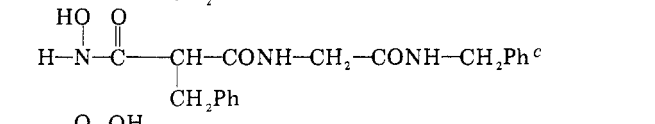
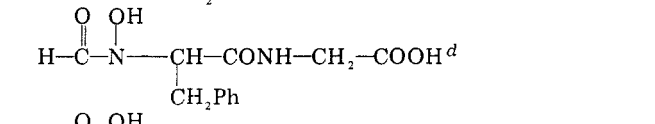
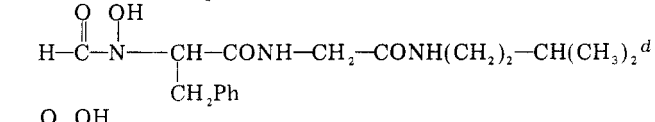
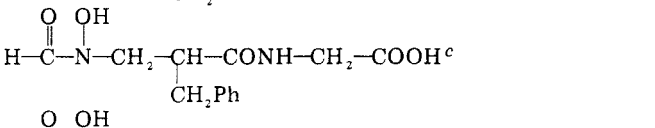
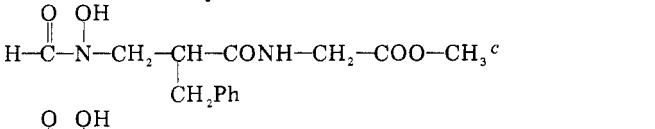
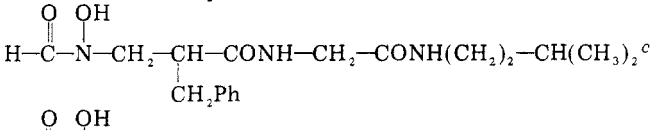
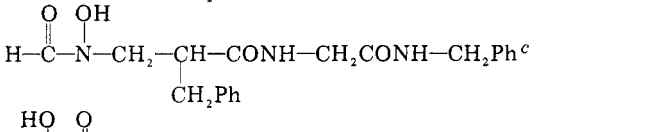
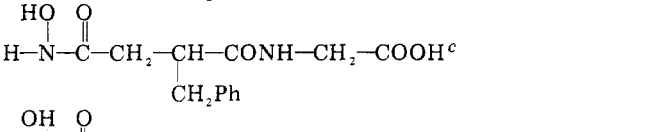
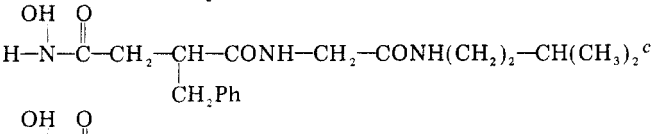
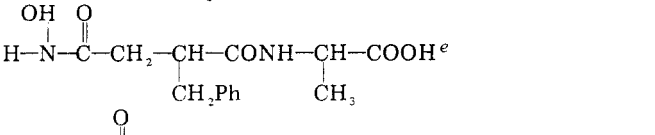
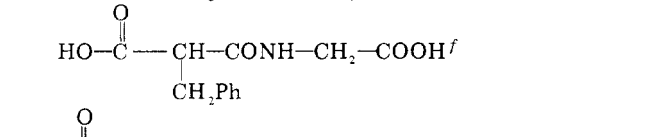
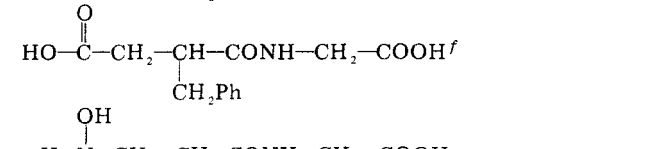
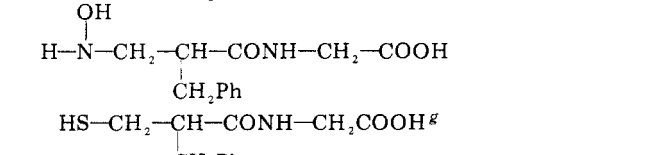
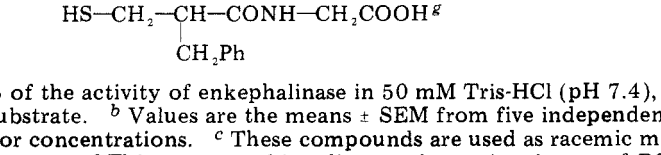
Table I. Inhibitory Potency of Hydroxamates and *N*-Formyl-*N*-hydroxy Peptides on Enkephalinase Activity[a]

compd	Zn^{2+} S_1' S_2'	IC_{50},[b] nM
1		12 ± 1
2		≃1.000
3		850 ± 50
4		750 ± 40
5		≃1.500
6		≃30.000
7		10 ± 1
8		350 ± 50
9		800 ± 50
10		≃1.200
11		1.4 ± 0.4
12		600 ± 50
13		2.0 ± 0.5
14		700 ± 80
15		800 ± 80
16		>1.000
thiorphan		4 ± 0.5

[a] Concentration inhibiting 50% of the activity of enkephalinase in 50 mM Tris-HCl (pH 7.4), 25 °C, with 20 nM of [^{3}H]-D-Ala^2-Leu-enkaphalin as substrate. [b] Values are the means ± SEM from five independent experiments computed by log probit analysis of five inhibitor concentrations. [c] These compounds are used as racemic mixture of *R* and *S* isomers. [d] These compounds are pure *S* isomers. [e] This compound is a diastereoisomeric mixture of *RS* and *SS* isomers. [f] From ref 11. [g] From ref 12.

Table II. Inhibitory Potency of Hydroxamates and *N*-Formyl-*N*-hydroxy Peptides on Dipeptidylaminopeptidase and Aminopeptidase Activities[a]

compd	structure	IC_{50},[b] nM dipeptidylaminopeptidase	IC_{50},[b] nM aminopeptidase
1	$HN(OH)C(O)C(CH_2Ph)HCONHCH_2COOH$	$3.5 \pm 0.5 \times 10^{-5}$	$1.0 \pm 0.5 \times 10^{-4}$
5	$HC(O)N(OH)C(CH_2Ph)HCONHCH_2COOH$	$2.32 \pm 0.25 \times 10^{-4}$	$2.5 \pm 0.4 \times 10^{-5}$
7	$HC(O)N(OH)CH_2C(CH_2Ph)HCONHCH_2COOH$	$3.05 \pm 0.07 \times 10^{-7}$	$8.5 \pm 1.5 \times 10^{-7}$
11	$HN(OH)C(O)CH_2C(CH_2Ph)HCONHCH_2COOH$	$2.1 \pm 0.5 \times 10^{-8}$	$2.0 \pm 0.2 \times 10^{-6}$
13	$HN(OH)C(O)CH_2C(CH_2Ph)HCONHC(CH_3)HCOOH$	$2.10 \pm 0.08 \times 10^{-9}$	$7.1 \pm 0.2 \times 10^{-7}$
bestatin			$5 \pm 1 \times 10^{-7}$

[a] Concentration inhibiting 50% of the activity of rat brain dipeptidylaminopeptidase and rabbit kidney aminopeptidase in 50 mM Tris-HCl (pH 7.4), with [^{3}H]-Leu-enkephalin (10 nM) as substrate. [b] Values are the mean ± SEM from six independent experiments computed by log probit analysis of five inhibitor concentrations.

additional methylene group between the P_1' residue and the metal binding moiety, as shown by the weak affinity of compounds **1** and **5**. Moreover, the latter enzyme seems to be more selective than enkephalinase, since inhibitors containing the (hydroxyamino)carbonyl group, such as compounds **11** and **13**, are ca. 100-fold more potent (IC_{50} in the nanomolar range) than inhibitor **7**, which possesses a *N*-formyl-*N*-hydroxyamino group in the same position (IC_{50} = 0.3 μM). Finally, comparison of the inhibitory potency of compounds **11** and **13** indicates a significant preference for an alanine in place of a glycine residue as the putative P_2' moiety.

The inhibitory potency of the various bidentates are relatively lower on the aminopeptidase activity than on the two other studied enzymes (Table II). The most active inhibitors **7**, **11**, and **13** with IC_{50} in the micromolar range also contain the longest linking chain between the metal binding group and the P_1' residue. The replacement of the Gly residue in compound **11** by an alanine moiety in compound **13** leads also to an increased activity on aminopeptidase, but this effect is weaker than on dipeptidylaminopeptidase.

Comparison of the Inhibitory Potency of the Two Diastereoisomers of Bidentate 13 on the Three Enkephalin-Degrading Enzymes. The chromatographic separation of the two diasteroisomers of compound **13** permits an analysis of the influence of the stereochemistry of the benzylsuccinyl moiety on the three enkephalin-degrading activities. In general, enzymes interact preferentially with compounds exhibiting stereochemical orientations of side chains similar to that of the natural substrates. Thus, experiments with dipeptides and carboxyalkyl inhibitors have shown that the higher inhibitory potencies were observed for compounds having absolute configuration analogous to that of a L–L dipeptide expected to interact with S_1'–S_2' subsites. However, the study of mercaptoalkyl inhibitors of enkephalinase[34] has shown that, the higher the affinity for this enzyme, the smaller is the influence of the structural parameter. Indeed, in the thiorphan series, enkephalinase inhibitors display similar activities regardless of the configuration of the P_1' moiety.[34,35] In the bidentate series, it was important to verify the influence of this structural parameter, because it was possible that the expected increased rigidity of the inhibitor structure through bis-coordination of the metal atom might be responsible for differences in the inhibitory potency by restricting the spatial orientation of the benzyl side chain. Furthermore, although the structural characteristics of the active sites of aminopeptidase and dipeptidylamino peptidase are still unknown, the study of the inhibitory potencies of the two diastereoisomers of **13** may indicate if this compound binds to these enzymes through interactions of the benzyl and methyl side chains with S_1' and S_2' subsites, respectively, or, in a reverse manner, with the S_1 and S_2 subsites.

Table III. Inhibitory Potency of the Two Stereoisomers of Compound **13** on the Three Enkephalin-Degrading Activities

enzyme	K_i[a] "*RS*" isomer[b]	K_i[a] "*SS*" isomer
aminopeptidase[c]	$3.8 \pm 0.5 \times 10^{-7}$	$2.9 \pm 0.5 \times 10^{-5}$
dipeptidylaminopeptidase[c]	$0.9 \pm 0.1 \times 10^{-9}$	$1.0 \pm 0.5 \times 10^{-7}$
enkephalinase[d]	$1.7 \pm 0.6 \times 10^{-9}$	$1.8 \pm 0.5 \times 10^{-9}$

[a] Values are the mean ± SEM from five independent experiments computed by log probit analysis of five inhibitor concentrations. [b] *RS* isomer = kelatorphan. [c] Concentration inhibiting 50% of the enzymatic activities, with 10 nM of [^{3}H]-Leu-enkephalin as substrate. [d] Concentration inhibiting 50% of enkephalinase activity, with 20 nM of [^{3}H]-D-Ala^2-Leu-enkephalin as substrate.

As shown in Table III, enkephalinase is equally inhibited by the *RS* and *SS* forms of compound **13**. This is consistent with the existence of a very large S_1' subsite in this enzyme.[13] Moreover, the flexibility of the methylene spacer bearing the bidentate group may confer upon the inhibitor more degrees of freedom allowing the benzyl side chain to fit the S_1' subsite regardless of stereochemistry.

By contrast, the two other enzymatic activities are inhibited differently by the two stereoisomers. For dipeptidylaminopeptidase and aminopeptidase, the *RS* isomer is more active than the *SS* one. This indicates that, for these two enzymes, the benzyl moiety interacts with subsites bearing stereospecific requirements. Furthermore, this result suggests that compound **13** interacts with S_1' and S_2' subsites in the three enzymes. This assumption seems to be supported by the increased affinity for aminopeptidase of Z-D-Phe-NHOH as compared to the L-Phe isomer,[21] if the aromatic moieties of these compounds are assumed to interact with the S_1'–S_2' subsites. A more complete study of the subsite specificity will be required before a model for the interaction of this inhibitor with dipeptidylaminopeptidase or aminopeptidase active sites can be proposed.

In conclusion, the "*R*" isomer of compound **13**, designated "kelatorphan" (*N*-[3(*R*)-[(hydroxyamino)-carbonyl]-2-benzyl-1-oxopropyl]-L-alanine), can be considered as the first complete inhibitor of the three enkephalin-degrading activities.[36] This feature is illustrated in Figure 7, which shows the inhibition curves of kelatorphan on the three purified enzymes.

Moreover, it is interesting to observe that the potency of bestatin for inhibition of the release of tyrosine from

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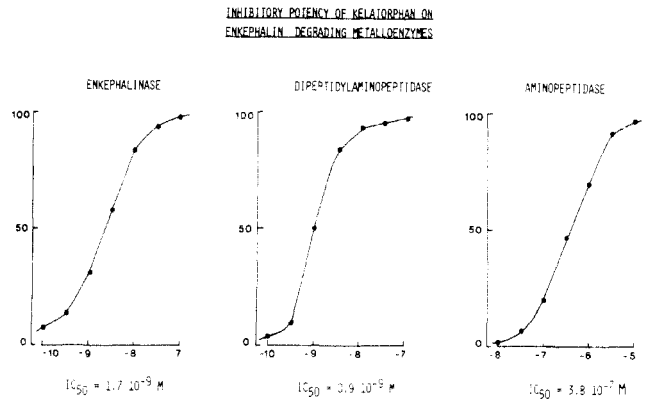

Figure 7. Inhibitory potency of kelatorphan on the three enkephalin-degrading metallopeptidases. IC_{50} values represent the concentration of kelatorphan inhibiting 50% of the degradation of 20 nM of [^{3}H]-D-Ala^2-Leu^5-enkephalin for enkephalinase and 10 nM of [^{3}H]-Leu^5-enkephalin for dipeptidylaminopeptidase and aminopeptidase.

enkephalins is almost identical ($IC_{50} \sim 0.9$ μM) with that for inhibition of purified aminopeptidase or on various other aminopeptidases from mouse brain membrane preparations. In contrast, kelatorphan is about 200-fold less potent ($IC_{50} \sim 90$ μM) vs. aminopeptidases from brain homogenate than vs. purified enzyme ($IC_{50} \sim 0.4$ μM), suggesting that this new inhibitor is more selective than bestatin toward the biologically relevant enkephalin-degrading aminopeptidase. As shown in the next section, this observation is supported by the similar analgesic effects produced by kelatorphan or by a combination of bestatin and thiorphan since the potencies of kelatorphan and thiorphan vs. enkephalinase are identical.

Antinociceptive Activities of Bidentate Inhibitors. The occurrence of an antinociceptive potency of the newly synthesized bidentates can be tested in vivo either by evaluation of their individual analgesic effects or by potentiation of the analgesia induced by peptidase-sensitive enkephalins.

As already reported,[11,12,14,36-42] the own analgesic effects of enkephalin-degrading enzyme inhibitors can be easily demonstrated by use of various nociceptive stimuli such as mouse hot plate at 55 °C, mouse writhing induced by ip administration of phenylbenzoquinone, electrical stimulation of the mouse tail, or foot pressure in rats. In these tests, enkephalinase inhibitors, as well as the aminopeptidase inhibitor bestatin,[37,38] raise the nociceptive threshold and naloxone lowers it.[43,12] Moreover, all these inhibitor-induced antinociceptive effects are reversed by prior administration of naloxone. These features clearly indicate that an enkephalin release is induced by the preceding nociceptive stimuli and that the analgesic effects produced by the inhibitors are related to the interaction

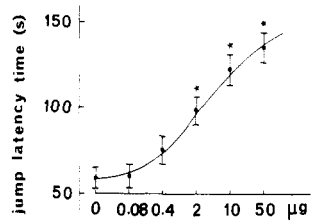

Figure 8. Analgesic effect of kelatorphan in the hot-plate test: dose–response curve. Kelatorphan was injected icv at the indicated doses in a constant volume (10 μL) of saline (n = 10). Temperature = 55 ± 0.5 °C. (*) Statistical comparison with control; $p < 0.001$.

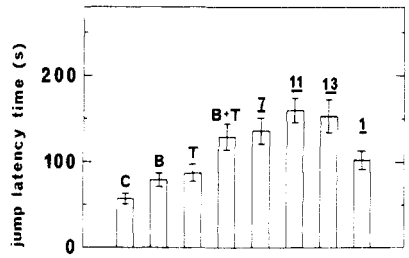

Figure 9. Comparative analgesic effects of various inhibitors in the hot-plate test. All the compounds were injected icv (10 μg) with 10 μL of saline; n = 12. C, control; B, bestatin; T, thiorphan. The numbers 1, 7, 11, and 13 correspond to inhibitor numbering in the text.

of the released endogenous enkephalins protected from degrading enzymes with opioid receptors.

As often underlined, peptidase inhibitors are inactive on mouse tail-flick and on mouse tail-withdrawal tests.[12,41,44] These features are likely due to a lack or an insufficient release of enkephalins induced by these intense but short nociceptive stimuli. According to this assumption, thiorphan was found active in the tail-flick test in rats exposed to inescapable stress,[45] a situation well-known to cause a release of endogenous enkephalins.[46] Another explanation may be that, in tail-flick and tail-withdrawal tests, opioid peptides less sensitive to enkephalinase such as β-endorphin or dynorphin should be preferentially involved in pain regulation. On the other hand, the protecting efficiency of the various inhibitors can be measured by coadministration of these compounds with subanalgesic doses of peptidase-sensitive enkephalins. Obviously in this case, peptidase inhibitors are able to potentiate the antinociceptive action of exogenously administered enkephalins on tail-flick and tail-withdrawal tests.

Hot-Plate Test. This simple assay permits the measurement of both the individual antinociceptive effect of a peptidase inhibitor as well as the time course of the induced analgesia. Intracerebroventricular injection (in constant 10-μL volume) was chosen as the route of administration in order to minimize pharmacokinetic differences in the ability of the tested inhibitors to cross the blood–brain barrier.

As shown in Figure 8, a dose-dependent effect, significant for doses up to 2 μg per mouse, was observed with kelatorphan. The maximum effect was obtained with 50 μg per animal followed by a plateau at higher concentration.

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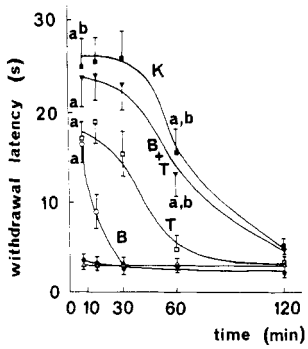

Figure 10. Comparative analgesic effects of kelatorphan, bestatin, and thiorphan, in the tail-withdrawal test. The inhibitors (10 μg) were administered icv with a subanalgesic dose of Met[5]-enkephalin (100 μg), 7 mn 30 before the first measurement. Statistical comparison: a = with saline, b = with bestatin or thiorphan; $p < 0.001$. B, bestatin; T, thiorphan; K, kelatorphan.

In addition, the analgesic properties of compound **13** and of the most active bidentates **1**, **7**, and **11** were compared at similar dosages (10 μg per mouse) with that of bestatin and thiorphan individually and in combination. Compound **13**, corresponding to a diastereoisomeric mixture was used, instead of kelatorphan, to ensure homogeneous results since compounds **1**, **7**, and **11** were studied under their racemic forms. Under these conditions, all of the inhibitors tested exhibited analgesic effects (Figure 9). In comparison to thiorphan or bestatin alone, compound **1** is as effective and compounds **7**, **11**, and **13** are significantly more potent than the two former inhibitors. Furthermore, compounds **7**, **11**, and **13** are statistically as effective as the combination of bestatin and thiorphan. All inhibitor-induced analgesic responses were prevented by prior administration of naloxone (1 mg/kg ip).

Tail-Withdrawal Test. On this second test, two series of experiments were performed. As in the tail-flick test, no analgesic effect was observed after icv administration of enkephalin degrading enzyme inhibitors, including bidentates (not shown here).

Analgesic responses were obtained only when the inhibitors were coadministered with a subthreshold dose of Met-enkephalin (100 μg icv). Figure 10 shows the time course of the analgesia induced by kelatorphan, bestatin, thiorphan, and a combination of bestatin and thiorphan in the presence of Met-enkephalin. Under these conditions (10 μg of each inhibitor), the coadministration of Met-enkephalin and bestatin led to a significant but transient, e.g., less than 30 min, duration of analgesia. The coadministration of Met-enkephalin and thiorphan led to a lengthening of the withdrawal latency, which was still significant after 1 h. As shown in Figure 10, the combination of bestatin and thiorphan induced a durable and effective analgesia even at a time (>60 min) where bestatin or thiorphan alone were inactive. This potentiating effect, which has been reported previously,[40,47] could be attributed to the simultaneous inhibition of both enzymes, assuming that the enkephalin-degrading efficiency of each peptidase is enhanced by inhibition of the other. Recent biochemical experiments from our own laboratories have confirmed that the levels of Tyr or Tyr-Gly-Gly in rat striatal slices are enhanced by selective inhibition of enkephalinase or aminopeptidase, respectively.[48] Finally, as expected, kelatorphan was significantly more active than the mixture of bestatin and thiorphan during the first hour after administration.

Two conclusions can be derived from these experiments.

(i) On the hot plate test three bidentate inhibitors were as effective as the combination of bestatin and thiorphan. These three compounds **7**, **11**, and **13** are highly potent inhibitors of enkephalinase and dipeptidylaminopeptidase and are also able to inhibit aminopeptidase with potencies similar to that of bestatin.

(ii) The analgesic effect induced by coadministration of Met-enkephalin and kelatorphan on the tail-withdrawal test is of particular interest. Indeed, as shown in Figure 10, kelatorphan may be able to induce a slightly more effective analgesia than the mixture of bestatin and thiorphan during a 1-h period. Owing to its high inhibitory potency against the three peptidases, this result suggests that the three enzymatic activities may be implicated in the metabolism of enkephalins and that the greater efficacy of kelatorphan as compared to bestatin + thiorphan could be due to inhibition of the dipeptidylaminopeptidase. However, both in vivo and in vitro experiments designed to show protection of exogenous or endogenous Met-enkephalin[48] seem to suggest only a minor role for the dipeptidylaminopeptidase in the metabolism of enkephalins. The synthesis of a highly potent and specific inhibitor of this latter enzymatic activity will be necessary in order to clarify this issue.

Conclusion

This paper shows that the incorporation of strong metal binding components into Phe-Gly and Phe-Ala related structures allowed us to obtain potent inhibitors of three different metallopeptidases. Among these enzymes, enkephalinase and a membrane-bound brain aminopeptidase similar to kidney aminopeptidase M are involved in enkephalin catabolism as shown by the naloxone reversible analgesic effects induced by kelatorphan.

Nevertheless, the possible clinical use of these inhibitors will depend upon several factors, among them being the strength of the induced analgesia, the development of pharmacokinetically acceptable forms, and a clear demonstration of a lack of adverse side effects. In any case, inhibitors of enkephalin metabolism are useful tools in the investigation of the physiological role of these neuromodulators in different brain areas. These studies have proved difficult due to the low tonic activity of the enkephalinergic system. According to its high inhibitory potency against all enkephalin-degrading enzymes, kelatorphan could be more effective than previously synthesized inhibitors for such investigations.

Experimental Section

Biological Tests. Materials. [^{3}H]-Leu-enkephalin (35 Ci/mmol) was obtained from New England Nuclear and [^{3}H]-Tyr[1]-D-Ala-Leu-enkephalin (32 Ci/mmol) was from the Centre d'Etude Atomique (CEA, France). DEAE-Sepharose CL6B, AH Sepharose 4B, and Sephadex G200 were purchased from Pharmacia. Bestatin was a generous gift of Roger Bellon Laboratories (France).

Purification of Enkephalin-Degrading Enzymes. Enkephalinase (Neutral Endopeptidase, EC 3.4.24.11) and Aminopeptidase M from Rabbit Kidney. Enkephalinase from rat brain and rabbit kidney were identical,[6] and this enzyme was purified to homogeneity from the latter organ either by the method of Almenoff and Orlowski[49] or by the technique of Kerr and Kenny.[33] A single species was observed by polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$. Both methods led to identical results regarding purity, specific activity, and effects

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of inhibitors. Moreover, the membrane-bound aminopeptidase M associated with the endopeptidase EC 3.4.24.11 was also purified by both methods as already described by Kerr and Kenny[33] but not reported by Almenoff and Orlowski.[49] The purified aminopeptidase M from rabbit kidney was inhibited by bestatin ($IC_{50} = 5 \pm 1 \times 10^{-7}$ M) and kelatorphan ($IC_{50} = 4 \pm 1 \times 10^{-7}$ M). The K_m value for Leu-enkephalin was 50 ± 5 μM.

Dipeptidylaminopeptidase and Aminopeptidase from Rat Brain. As reported recently in greater detail,[32] the membrane-bound dipeptidylaminopeptidase cleaving the Gly^2–Gly^3 bond of enkephalins[9] was purified from rat brain by slight modification of the reported methods.[9,50] The following additional steps in the purification procedure allowed us to separate the dipeptidylaminopeptidase from a broad peak of exoaminopeptidase activity releasing tyrosine from enkephalins. The fractions containing this latter activity were pooled and concentrated in an ultrafiltration cell (Amicon) and applied to an AH Sepharose 4B column (0.9 × 7 cm) equilibrated with 25 mM Tris-HCl buffer, pH 7. After washing, two exoaminopeptidases A and B were separated by using a linear gradient from 0.15 to 0.45 M NaCl. Both aminopeptidases (A and B) exhibited similar sensitivity to bestatin ($IC_{50} = 0.5 \pm 0.1$ μM) and were distinguished by the inhibitory potency of kelatorphan: IC_{50} value was 50 μM on A and 0.4 μM on B. This latter enzyme is closely related to aminopeptidase M from rabbit kidney as shown by its similar K_m value (45 ± 5 μM) for Leu-enkephalin and its similar sensitivity to various inhibitors (bestatin, kelatorphan).

Assay of Enkephalinase, Dipeptidylaminopeptidase, and Aminopeptidase Activity. The enkephalinase activity was checked following a procedure previously described.[29] The enzyme (at a final concentration of 0.9 ± 0.2 pmol/100 μL) was preincubated for 15 min at 25 °C with or without increasing concentrations of inhibitor in 100-μL total volume of 50 mM Tris-HCl buffer. [^{3}H]-D-Ala^2-Leu-enkephalin (K_m = 30 μM) was added to 20 mM final concentration and the reaction was stopped after 30 min by adding 25 μL of 0.2 N HCl. The dipeptidylaminopeptidase and the aminopeptidase activity was measured by the same procedure with use of [^{3}H]-Leu-enkephalin, at 10 nM final concentration, as substrate (K_m = 25 μM for dipeptidylaminopeptidase and K_m = 50 μM for aminopeptidase). The tritiated metabolites formed were separated on polystyrene beads.[51] Determinations of IC_{50} values and kinetic experiments were performed as already described in detail.[21]

Analgesic Tests. Male Swiss albino mice (CD_1, Charles River, 24–26 g) were used. Drugs were injected intracerebroventricularly under a 10-μL volume according to the method of Haley and McCormick.[52] Drugs were dissoved in saline or in a minimal volume of 0.1 NaOH and then neutralized with 0.1 N HCl. The hot-plate (55 ± 0.5 °C) test[53] was performed 10 min after drug administration in mice by measurements of the jump latency time.

The tail-withdrawal test[54] was performed by immersion of mouse tail in hot water (48 ± 0.5 °C). The mouse was placed horizontally in a Plexiglas box with its tail dropping vertically and the tail-flick time after immersion in hot water was recorded.

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

Chemistry. The protected amino acids were from Bachem (Switzerland). The purity of all the synthesized compounds was checked by thin-layer chromatography on silica gel plates (Merck). The following solvent systems (v/v) were used: A, $CHCl_3$/ MeOH (9:1); B, $CHCl_3$/MeOH/AcOH (9:1:0.5); C, BuOH/AcOH/H_2O (4:1:1); D, $CHCl_3$/MeOH (5:1); E, BuOH/AcOH/pyridine/H_2O (4:1:1:2); F, $CHCl_3$/MeOH (7:3); G, $CHCl_3$/MeOH (8:2). The purity of the final compounds was checked also by HPLC on a reversed-phase μ-Bondapak C_{18} column (Waters) with CH_3CN/NH_4AcO buffer (PH 4.2) as solvent. The eluted peaks were monitored at 260 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 1–13. 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; HOBT, 1-hydroxybenzotriazole; TFA, trifluoroacetic acid; Et_2O, diethyl ether; BuOH, 1-butanol; AcOH, acetic acid.

2(*RS*)-[[(Benzyloxy)amino]carbonyl]-3-phenylpropanoic Acid Ethyl Ester. Procedure A. To a solution of 3 g (13.5 mmol) of 2-(ethoxycarbonyl)-3-phenylpropanoic acid in THF (30 mL), cooled to 0 °C, were added successively 2.15 g (13.5 mmol) of *O*-benzylhydroxylamine hydrochloride and 1.9 mL of triethylamine in $CHCl_3$ (20 mL), 2.07 g (13.5 mmol) of 1-hydroxybenzotriazole in THF (20 mL), and 3.06 g (14.85 mmol) of dicyclohexylcarbodiimide in $CHCl_3$ (20 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 water (2 × 50 mL), 10% citric acid (3 × 50 mL), water (50 mL), 10% $NaHCO_3$ (3 × 50 mL), water (50 mL), and, finally, with saturated NaCl (50 mL). The organic layer was dried over Na_2SO_4 and evaporated in vacuo. An oily product was obtained (4.24 g, 98%): R_f (D) 0.75.

2(*RS*)-[[(Benzyloxy)amino]carbonyl]-3-phenylpropanoic Acid. Procedure B. To a solution of 4.2 g (12.8 mmol) of the preceding compound in ethanol (20 mL) and water (10 mL) was added at 0 °C 25 mL of 2.5 N NaOH. The mixture was stirred at 0 °C for 1 h and at room temperature for 4 h. The solution was concentrated in vacuo, diluted with 30 mL of water, washed with Et_2O (4 × 10 mL), acidified to pH 2 with 2 N HCl, and extracted with Et_2O (4 × 20 mL). The ethereal layer was washed with H_2O (20 mL), dried over Na_2SO_4, and evaporated in vacuo. The white solid obtained (2.46 g, 64%) was filtered, washed with water, and dried: mp 180 °C; R_f (E) 0.72.

***N*-[2(*RS*)-[[(Benzyloxy)amino]carbonyl]-1-oxo-3-phenylpropyl]glycine Benzyl Ester. Procedure C.** To a solution of 0.5 g (1.67 mmol) of 2(*RS*)-[[(benzyloxy)amino]-carbonyl]-3-phenylpropanoic acid in THF (10 mL) cooled to 0 °C were added successively 0.56 g (1.67 mmol) of glycine *tert*-butyl ester *p*-tosylate and 0.23 mL of triethylamine in THF (10 mL), 0.256 g (1.67 mmol) of HOBT in THF (5 mL), and 0.71 g (1.67 mmol) of *N*-cyclohexyl-*N'*-[2-(4-morpholinyl)ethyl]carbodiimide methol *p*-toluenesulfonate. After 1 h at 0 °C, the mixture was stirred at room temperature overnight. After filtration and evaporation of the solvent, the residue was taken up with 2% HCl (10 mL). The solid obtained was filtered, washed several times with water and 10% $NaHCO_3$, and dried. A white solid was obtained (0.56 g, 75%): mp 70 °C; R_f (B) 0.72. Anal. ($C_{26}H_{26}N_2O_5$) C, H, N.

***N*-[2(*RS*)-[(Hydroxyamino)carbonyl]-1-oxo-3-phenylpropyl]glycine (1).** To a 10% Pd on charcoal (0.13 g) suspension in MeOH (5 mL), saturated by hydrogen, was added 0.5 g (1.12 mmol) of the preceding compound in MeOH (5 mL). The mixture was stirred for 3 h at room temperature. After filtration, the solution was evaporated in vacuo. A white solid was obtained (0.274 g, 92%): mp = 183 °C; R_f (C) 0.47; ^{1}H NMR δ 2.90 and 3.01 (CH_2Ph), 3.21 (CH), 3.62 (Gly CH_2), 7.15 (Ph), 7.92 (Gly NH), 9.00 (OH), 10.65 (NH). Anal. ($C_{12}H_{14}N_2O_5$) C, H, N.

***N*-[2(*RS*)-[[(Benzyloxy)amino]carbonyl]-1-oxo-3-phenylpropyl]glycine Methyl Ester.** This compound was obtained following procedure C. From 0.6 g (2 mmol) of 2(*R*,*S*)-[[(benzyloxy)amino]carbonyl]-3-phenylpropanoic acid and 0.25 g (2 mmol) of glycine methyl ester hydrochloride, a white solid was obtained (0.47 g, 64%): mp 107 °C; R_f (B) 0.70. Anal. ($C_{20}H_{22}N_2O_5$) C, H, N.

***N*-[2(*RS*)-[(Hydroxyamino)carbonyl]-1-oxo-3-phenylpropyl]glycine Methyl Ester (2).** A 0.4-g (1.08 mmol) sample of the preceding compound was hydrogenated following the procedure described for compound 1. A white solid was obtained (0.24 g, 80%): mp 174 °C; R_f (B) 0.44; ^{1}H NMR δ 2.92 and 2.97

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(CH_2Ph), 3.21 (CH), 3.56 (OCH_3), 3.70 (Gly CH_2), 7.14 (Ph), 8.10 (Gly NH), 8.90 (OH), 10.40 (NH). Anal. ($C_{13}H_{16}N_2O_5$) C, H, N.

Glycine Isopentylamide Trifluoroacetate. From 4 g (22.84 mmol) of *N*-(*tert*-butyloxycarbonyl)glycine and 1.99 g (22.84 mmol) of isopentylamine, the title compound was obtained, following the procedure described for 2(*R,S*)-[[(benzyloxy)-amino]carbonyl]-3-phenylpropanoic acid ethyl ester: oily product (5.3 g, 95%); R_f (C) 0.81.

A 4.84-g (19.8 mmol) sample of the preceding compound was dissolved at 0 °C in trifluoroacetic acid (30 mL). After 30 min at 0 °C, the solution was stirred for 1 h at room temperature. After evaporation in vacuo of the excess TFA, the residue was washed with Et_2O and dried. A white powder was obtained (4.38 g, 86%): R_f (C) 0.47.

***N*-[2(*RS*)-[[(Benzyloxy)amino]carbonyl]-1-oxo-3-phenylpropyl]glycine Isopentylamide.** This compound was obtained following procedure C. From 0.37 g (1.25 mmol) of 2(*R,S*)-[[(benzyloxy)amino]carbonyl]-3-phenylpropanoic acid and 0.32 g (1.25 mmol) of glycine isopentylamide trifluoroacetate, a white solid was obtained (0.47 g, 88%): mp 140 °C; R_f (B) 0.63. Anal. ($C_{24}H_{31}N_3O_4$) C, H, N.

***N*-[2(*RS*)-[(Hydroxyamino)carbonyl]-1-oxo-3-phenylpropyl]glycine Isopentylamide (3).** A 0.25-g (0.6 mmol) sample of the preceding compound was hydrogenated following the procedure described for compound 1. A white solid was obtained (0.17 g, 87%): mp 186 °C; R_f (C) 0.84; ^{1}H NMR δ 0.73 (CH_3), 1.18 (CH_2-*i*-Pr), 1.47 (CH-*i*-Pr), 2.92 and 2.97 (CH_2Ph), 2.95 (NCH_2), 3.19 (CH), 3.53 (Gly CH_2), 7.12 (Ph), 7.66 (NH), 7.93 (Gly NH), 8.83 (OH), 10.43 (HNO). Anal. ($C_{17}H_{25}N_3O_5$) C, H, N.

Glycine Benzylamide Trifluoroacetate. This compound was obtained from 4 g (22.84 mmol) of *N*-(*tert*-butyloxycarbonyl)glycine and 2.44 g (22.84 mmol) of benzylamine following procedure C. After recrystallization from EtOAC, the title compound was obtained (3.76 g, 66%): mp 77–78 °C; R_f (C) 0.84.

A 3.1-g (11.72 mmol) sample of the preceding compound was treated following the procedure described for glycine isopentylamide trifluoroacetate to give a white solid (3.12 g, 95%): R_f (C) 0.33.

***N*-[2(*RS*)-[[(Benzyloxy)amino]carbonyl]-1-oxo-3-phenylpropyl]glycine Benzylamide.** This compound was obtained following procedure C. From 0.7 g (2.34 mmol) of 2(*RS*)-[[(benzyloxy)amino]carbonyl]-3-phenylpropanoic acid and 0.65 g (2.34 mmol) of glycine benzylamide trifluoroacetate. A white solid was obtained (0.91 g, 88%): mp 143 °C; R_f (B) 0.65. Anal. ($C_{26}H_{27}N_3O_4$) C, H, N.

***N*-[2(*RS*)-[(Hydroxyamino)carbonyl]-1-oxo-3-phenylpropyl]glycine Benzylamide (4).** A 0.4-g (0.9 mmol) sample of the preceding compound was hydrogenated following the procedure described for compound 1. A white solid was obtained (0.3 g, 93%): mp 166 °C; R_f (B) 0.31; ^{1}H NMR δ 2.93 and 2.97 (CH_2Ph), 3.25 (CH), 3.63 (Gly CH_2), 4.21 (CH_2), 7.17 (Ph), 8.02 (Gly NH), 8.28 (NH), 8.84 (OH), 10.44 (HNO). Anal. ($C_{19}H_{21}N_3O_4$) C, H, N.

***N*-(Benzyloxy)-L-phenylalanine.** To a solution of 12 g (52.4 mmol) of (*R*)-2-bromo-3-phenylpropanoic acid in MeOH was added 25.8 g (210 mmol) of *O*-benzylhydroxylamine. The mixture was refluxed overnight. After evaporation, the residue was taken up in 1 N NaOH (53 mL). The remaining *O*-benzylhydroxylamine was extracted into EtOAc (3 × 20 mL). The aqueous layer was acidified to pH 2 with 1 N HCl. The white precipitate obtained (3.28 g, 23%) was filtered, washed with water, dried, and recrystallized from ether: mp 168 °C; R_f (F) 0.56. Anal. ($C_{16}H_{17}NO_3$) C, H, N.

***N*-(Benzyloxy)-*N*-formyl-L-phenylalanine.** To a cold solution of (*S*)-*N*-(benzyloxy)phenylalanine (1.5 g, 5.52 mmol) in formic acid (27.6 mL) was added acetic anhydride (2.76 mL) at 0 °C. The mixture was stirred for 3 h at 0 °C and concentrated to dryness. The residue was taken up with Et_2O and evaporated in vacuo. An oily product was obtained (1.5 g, 91%): *Rf* (D) 0.55. Anal. ($C_{17}H_{17}NO_4$) C, H, N.

***N*-(Benzyloxy)-*N*-formyl-L-phenylalanylglycine Benzyl Ester.** A 0.49-g (1.64 mmol) sample of *N*-(benzyloxy)-*N*-formyl-L-phenylalanine and 0.52 g (1.64 mmol) of glycine benzyl ester *p*-tosylate were treated following procedure A to give an oily product (0.5 g, 68%): *Rf* (B) 0.74. Anal. ($C_{26}H_{26}N_2O_5$) C, H, N.

***N*-Formyl-*N*-hydroxy-L-phenylalanylglycine (5).** A 0.48-g (1.0 m mmol) sample of the preceding compound was treated following the procedure described for 1. An oily product was obtained (0.26 g, 92%): R_f (C) 0.49; ^{1}H NMR δ 2.98 and 3.08 (Phe β-CH_2), 3.73 (Gly CH_2), 4.48 and 4.95 (Phe α-CH), 7.22 (Ph), 8.20 (Gly NH), 7.70 and 8.13 (formyl), 9.62 and 9.92 (OH). Anal. ($C_{12}H_{14}N_2O_5$) C, H, N.

***N*-(Benzyloxy)-*N*-formyl-L-phenylalanylglycine Isopentylamide.** This compound was obtained following procedure A. From 0.21 g (0.53 mmol) of *N*-formyl-*N*-(benzyloxy)-L-phenylalanine and 0.14 g (0.53 mmol) of glycine isopentylamide trifluoroacetate was obtained 0.18 g (85%) of the title compound as an oily product: R_f ($CHCl_3/CH_3OH$, 95:5) 0.32. Anal. ($C_{24}H_{31}N_3O_4$) C, H, N.

***N*-Formyl-*N*-hydroxy-L-phenylalanylglycine Isopentylamide (6).** A 0.13-g (0.3 mmol) sample of the preceding compound was hydrogenated following the procedure described for compound 1 to give an oily product (0.09 g, 88%): R_f (C) 0.85; ^{1}H NMR δ 0.83 (CH_3), 1.23 (CH_2-*i*-Pr), 1.51 (CH-*i*-Pr), 2.98 and 3.03 (Phe β-CH_2), 3.28 (CH_2), 3.63 (Gly CH_2), 4.47 and 4.95 (Phe α-CH), 7.18 (Ph), 7.68 (NH), 7.55 and 8.15 (formyl), 8.15 (Gly NH), 9.60 and 9.89 (OH). Anal. ($C_{17}H_{25}N_3O_4$) C, H, N.

3(*RS*)-[(Benzyloxy)amino]-2-benzylpropanoic Acid. To a solution of 10 g (61.6 mmol) of benzylacrylic acid in EtOH (10 mL) was added 30 g (247 mmol) of *O*-benzylhydroxylamine. The mixture was refluxed for a week. After evaporation, the residue was taken up with 1 N NaOH (62 mL). The remaining *O*-benzylhydroxylamine was extracted into EtOAc (4 × 20 mL). The aqueous layer was acidified to pH 2. The product was extracted into EtOAc, washed with water, and dried over Na_2SO_4. After evaporation, an oil, which crystallized from Et_2O, was obtained (10 g, 57%): mp 75 °C; R_f (A) 0.47.

3(*RS*)-[*N*-(Benzyloxy)-*N*-formylamino]-2-benzylpropanoic Acid. This compound was obtained following the procedure described for *N*-(benzyloxy)-*N*-formyl-L-phenylalanine. From 5 g (17.52 mmol) of the preceding compound an oily product was obtained (5.4 g, 98%): R_f (E) 0.80.

***N*-[3(*RS*)-[*N*-(Benzyloxy)-*N*-formylamino]-1-oxo-2-benzylpropyl]glycine Benzyl Ester.** This compound was synthesized following procedure A. From 0.6 g (1.92 mmol) of (*RS*)-3-[*N*-(benzyloxy)-*N*-formylamino]-2-benzylpropanoic acid and 0.64 g (1.92 mmol) of glycine benzyl ester *p*-tosylate an oily product was obtained (0.86 g, 97%): R_f (B) 0.70. Anal. ($C_{27}H_{28}N_2O_5$) C, H, N.

***N*-[3(*RS*)-(*N*-Formyl-*N*-hydroxyamino)-1-oxo-2-benzylpropyl]glycine (7).** This inhibitor was prepared following the procedure described for compound 1. From 0.8 g (1.73 mmol) of the preceding compound, 0.45 g (92%) of the title compound was obtained: R_f (C) 0.50; ^{1}H NMR δ 2.55 and 2.77 (CH_2Ph), 2.96 (CH), ≃3.32 (CH_2), 3.60 (Gly CH_2), 7.14 (Ph), 7.73 and 8.17 (formyl), 8.23 (Gly NH). Anal. ($C_{13}H_{16}N_2O_5$) C, H, N.

***N*-[3(*RS*)-[*N*-(Benzyloxy)-*N*-formylamino]-1-oxo-2-benzylpropyl]glycine Methyl Ester.** This compound was prepared following procedure A. From 0.6 g (1.92 mmol) of 3(*R,S*)-[*N*-(benzyloxy)-*N*-formylamino]-2-benzylpropanoic acid and 0.24 g (1.92 mmol) of glycine methyl ester hydrochloride, 0.63 g (87%) of the title compound was obtained: R_f (D) 0.77. Anal. ($C_{21}H_{24}N_2O_5$) C, H, N.

***N*-[3(*RS*)-(*N*-Formyl-*N*-hydroxyamino)-1-oxo-2-benzylpropyl]glycine Methyl Ester (8).** This compound was obtained following the procedure described for compound 1. From 0.30 g (0.78 mmol) of the preceding compound, 0.23 g (98%) of the title compound was obtained as an oily product: R_f (D) 0.50; ^{1}H NMR δ 2.58 and 2.77 (CH_2Ph), 2.96 (CH), ≃3.38 (CH_2), 3.56 (OCH_3), 3.70 (Gly CH_2), 7.21 (Ph), 7.78 and 8.25 (formyl), 8.37 and 8.41 (Gly NH), 9.57 and 10.30 (OH). Anal. ($C_{14}H_{18}N_2O_5$) C, H, N.

***N*-[3(*RS*)-[*N*-(Benzyloxy)-*N*-formylamino]-1-oxo-2-benzylpropyl]glycine Isopentylamide.** This compound was obtained following procedure B. From 0.6 g (1.92 mmol) of 3(*RS*)-[*N*-(benzyloxy)-*N*-formylamino]-2-benzylpropanoic acid and 0.496 g (1.92 mmol) of glycine isopentylamide, an oily product was obtained (0.67 g, 80%): R_f (B) 0.72. Anal. ($C_{25}H_{33}N_3O_4$) C, H, N.

***N*-[3(*RS*)-(*N*-Formyl-*N*-hydroxyamino)-1-oxo-2-benzylpropyl]glycine Isopentylamide (9).** A 0.2-g (0.46 mmol) sample

of the preceding compound was hydrogenated following the procedure described for compound 1. An oily product was obtained (0.11 g, 70%): R_f (C) 0.76; ^{1}H NMR δ 0.82 (CH_3), 1.21 (CH_2-*i*-Pr), 1.49 (CH-*i*-Pr), 2.57 and 2.73 (CH_2Ph), 2.97 (CH), 2.98 (NCH_2), ≃3.30 (CH_2), 3.50 (Gly CH_2), 7.15 (Ph), 7.36 and 7.47 (NH), 7.73 and 8.18 (formyl), 10.30 (NOH). Anal. ($C_{18}H_{27}N_3O_4$) C, H, N.

***N*-[3(*RS*)-[*N*-(Benzyloxy)-*N*-formylamino]-1-oxo-2-benzylpropyl]glycine Benzylamide.** This compound was obtained following procedure A. From 0.72 g (2.3 mmol) of 3-(*RS*)-[*N*-(benzyloxy)-*N*-formylamino]-2-benzylpropanoic acid and 0.64 g (2.3 mmol of glycine benzylamide trifluoroacetate, 0.87 g (82%) of the title compound was obtained: R_f (A) 0.50. Anal. ($C_{27}H_{29}N_3O_4$) C, H, N.

***N*-[3(*RS*)-(*N*-Formyl-*N*-hydroxyamino)-1-oxo-2-benzylpropyl]glycine Benzylamide (10).** A 0.46-g (1 mmol) sample of the preceding compound was hydrogenated following the procedure described for compound 1: 0.34 g (92%) of compound 10 was obtained: R_f (A) 0.37; ^{1}H NMR δ 2.60 and 2.72 (CH_2Ph), 2.97 (CH), ≃3.33 (CH_2), 3.58 (Gly CH_2), 4.20 (NCH_2), 7.15 (Ph), 7.75 and 8.18 (formyl), 7.97 and 8.07 (NH), 8.22 (Gly NH), 9.55 and 10.05 (NOH). Anal. ($C_{20}H_{23}N_3O_4$) C, H, N.

***N*-[3-(Ethoxycarbonyl)-2-benzylidene-1-oxopropyl]glycine *tert*-Butyl Ester.** This compound was obtained following procedure A. From 0.5 g (2.14 mmol) of 3-(ethoxycarbonyl)-2-benzylidenepropanoic acid and 0.36 g (2.14 mmol) of glycine *tert*-butyl ester, an oily product was obtained (0.74 g, 96%): R_f (B) 0.83. Anal. ($C_{19}H_{25}NO_5$) C, H, N.

***N*-(3-Carboxy-2-benzylidene-1-oxopropyl)glycine *tert*-Butyl Ester.** To a solution of 2 g (5.76 mmol) of the preceding compound in EtOH (20 mL) was added at 0 °C 6.3 mL of 1 N NaOH. The mixture was stirred at 0 °C for 1 h and at room temperature for 2 h. The reaction mixture was treated following procedure B. An oily product was obtained (1.53 g, 83%): R_f (C) 0.77.

***N*-[3-[[(Benzyloxy)amino]carbonyl]-2-benzylidene-1-oxopropyl]glycine *tert*-Butyl Ester.** This compound was obtained following procedure A. From 1.24 g (3.9 mmol) of the preceding compound and 0.26 g (3.9 mmol) of *O*-benzylhydroxylamine hydrochloride, an oily compound, which crystallized slowly, was obtained: 1.1 g (67%); mp 125 °C; R_f (A) 0.69. Anal. ($C_{24}H_{28}N_2O_5$) C, H, N.

***N*-[3-[[(Benzyloxy)amino]carbonyl]-2-benzylidene-1-oxopropyl]glycine.** The preceding compound (0.5 g, 1.18 mmol) was dissolved at 0 °C in trifluoroacetic acid (1.5 mL) and stirred for 1 h at room temperature. After evaporation of the excess TFA, the residue was washed with Et_2O and dried to give 0.3 g (69%) of the title compound: mp 171 °C; R_f (C) 0.83. Anal. ($C_{20}H_{20}N_2O_5$) C, H, N.

***N*-[3(*RS*)-[(Hydroxyamino)carbonyl]-2-benzyl-1-oxopropyl]glycine (11).** This compound was obtained following the procedure described for compound 1. From 0.25 g (2.71 mmol) of the preceding compound, 0.07 g (99%) of compound 11 was obtained: R_f (C) 0.54; ^{1}H NMR δ 1.83 and 2.15 (CH_2), 2.47 and 2.78 (CH_2Ph), 3.60 (CH and Gly CH_2), 7.15 (Ph), 8.17 (Gly NH), 8.60 (NOH), 10.28 (HNO). Anal. ($C_{13}H_{16}N_2O_5$) C, H, N.

***N*-[3-(Ethoxycarbonyl)-2-benzylidene-1-oxopropyl]glycine Isopentylamide.** This compound was obtained following procedure A. From 0.35 g (1.48 mmol) of 3-(ethoxycarbonyl)-2-benzylidenepropanoic acid and 0.26 g (1.48 mmol) of glycine isopentylamide trifluoroacetate, an oily compound was obtained (0.52 g, 96%): R_f (A) 0.74. Anal. ($C_{20}H_{28}N_2O_4$) C, H, N.

***N*-(3-Carboxy-2-benzylidene-1-oxopropyl)glycine Isopentylamide.** To a solution of 0.49 g (1.36 mmol) of the preceding compound in EtOH (10 mL) was added at 0 °C 1.5 mL of 1 N NaOH. The reaction was treated following procedure B. The white precipitate obtained (0.35 g 76%) was filtered, washed with water, and dried: mp 134 °C; R_f (B) 0.50.

***N*-[3-[[(Benzyloxy)amino]carbonyl]-2-benzylidene-1-oxopropyl]glycine Isopentylamide.** This compound was obtained following procedure A. From 0.3 g (0.93 mmol) of the preceding compound and 0.15 g (0.93 mmol) of *O*-benzylhydroxylamine hydrochloride, an oily compound was obtained (0.33 g, 81%): R_f (B) 0.61. Anal. ($C_{25}H_{31}N_3O_4$) C, H, N.

***N*-[3(*RS*)-[(Hydroxyamino)carbonyl]-2-benzyl-1-oxopropyl]glycine Isopentylamide (12).** This compound was obtained following the procedure described for compound 1. From 0.23 g (0.53 mmol) of the preceding compound was obtained 0.18 g (97%) of the title compound: R_f (B) 0.30; ^{1}H NMR δ 1.83 and 2.15 (CH_2), 2.47 and 2.77 (CH_2Ph), 2.92 (CH), 3.58 (Gly CH_2), 7.13 (Ph), 8.17 (Gly NH), 8.60 (NOH), 10.28 (HNO). Anal. ($C_{18}H_{27}N_3O_4$) C, H, N.

***N*-[3-(Ethoxycarbonyl)-2-benzylidene-1-oxopropyl]-L-alanine *tert*-Butyl Ester.** This compound was obtained following procedure A. From 3.13 g (13.36 mmol) of 3-(ethoxycarbonyl)-2-benzylidenepropanoic acid and 2.42 g (13.36 mmol) of L-alanine *tert*-butyl ester was obtained an oily product (4.8 g, 100%): R_f (A) 0.83. Anal. ($C_{20}H_{29}NO_5$) C, H, N.

***N*-(3-Carboxy-2-benzylidene-1-oxopropyl)-L-alanine *tert*-Butyl Ester.** To a solution of 4.7 g (12.9 mmol) of the preceding compound in EtOH (40 mL) was added at 0 °C 14.2 mL of 1 N NaOH. The reaction was treated following procedure B. An oily compound was obtained (3.84 g, 89%): R_f (A) 0.30.

***N*-[3-[[(Benzyloxy)amino]carbonyl]-2-benzylidene-1-oxopropyl]-L-alanine *tert*-Butyl Ester.** This compound was obtained following procedure A. From 3.2 g (9.6 mmol) of the preceding compound and 1.53 g (9.6 mmol) of *O*-benzylhydroxylamine hydrochloride, an oily compound was obtained (4.04 g, 96%): R_f (A) 0.76. Anal. ($C_{25}H_{30}N_2O_5$) C, H, N.

***N*-[3-[[(Benzyloxy)amino]carbonyl]-2-benzylidene-1-oxopropyl]-L-alanine.** This analogue was obtained following the procedure described for N-[3-benzyloxyaminocarbonyl-2-benzylidene-1-oxopropyl]glycine. From 4 g (9.12 mmol) of the preceding compound, a white precipitate was obtained (1.74 g, 50%): mp 174 °C; Rf (B) 0.48. Anal. ($C_{21}H_{22}N_2O_5$), C, H, N.

***N*-[3(*RS*)-[(Hydroxyamino)carbonyl]-2-benzyl-1-oxopropyl]-L-alanine (13).** This compound was obtained following the procedure described for compound 1. From 0.29 g (0.76 mmol) of the preceding compound, 0.22 g (100%) of the title compound was obtained: R_f (C) 0.73; ^{1}H NMR δ 1.00 and 1.18 (Ala CH_3), 1.70–3.00 (CH_2CHCH_2Ph), 7.13 (Ph), 8.07 and 8.17 (Ala NH), 8.60 and 8.68 (NOH), 10.28 and 10.36 (HNO). Anal. ($C_{14}H_{18}N_2O_5$) C, H, N.

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Registry No. 1, 86962-93-8; 2, 96866-03-4; 3, 96866-04-5; 4, 96896-83-2; 5, 87438-06-0; 6, 87438-04-8; 7, 92175-55-8; 8, 96896-84-3; 9, 96866-05-6; 10, 96896-85-4; 11, 92175-56-9; 12, 96896-86-5; 13 (isomer 1), 92175-64-9; 13 (isomer 2), 92175-57-0; 16, 92175-59-2; *O*-benzylhydroxylamine hydrochloride, 2687-43-6; 2-(ethoxycarbonyl)-3-phenylpropanoic acid, 67682-05-7; 2(*RS*)-[[(benzyloxy)amino]carbonyl]-3-phenylpropanoic acid ethyl ester, 67681-98-5; 2(*RS*)-[[(benzyloxy)amino]carbonyl]-3-phenylpropanoic acid, 67681-99-6; glycine *tert*-butyl ester tosylate, 16652-58-7; *N*-[2(*RS*)-[[(benzyloxy)amino]carbonyl]-1-oxo-3-phenylpropyl]glycine benzyl ester, 96866-06-7; glycine methyl ester hydrochloride, 5680-79-5; *N*-[2(*RS*)-[[(benzyloxy)amino]-carbonyl]-1-oxo-3-phenylpropyl]glycine methyl ester, 96866-07-8; *N*-(*tert*-butyloxycarbonyl)glycine, 4530-20-5; isopentylamine, 107-85-7; *N*-(*tert*-butyloxycarbonyl)glycine isopentylamide, 84851-16-1; glycine isopentylamide trifluoroacetate, 87429-14-9; *N*-[2(*RS*)-[[(benzyloxy)amino]carbonyl]-1-oxo-3-phenylpropyl]-glycine isopentylamide, 96866-08-9; benzylamine, 100-46-9; *N*-(*tert*-butyloxycarbonyl)glycine benzylamide, 19811-52-0; glycine benzylamide trifluoroacetate, 81110-67-0; *N*-[2(*RS*)-[[(benzyl-oxy)amino]carbonyl]-1-oxo-3-phenylpropyl]glycine benzylamide, 96866-09-0; (R)-2-bromo-3-phenylpropanoic acid, 42990-55-6; *O*-benzylhydroxylamine, 622-33-3; *N*-(benzyloxy)-L-phenylalanine, 96866-10-3; formic acid, 64-18-6; *N*-(benzyloxy)-*N*-formyl-L-phenylalanine, 96866-11-4; glycine benzyl ester *p*-tosylate, 1738-76-7; *N*-(benzyloxy)-*N*-formyl-L-phenylalanylglycine benzyl ester, 96866-12-5; *N*-(benzyloxy)-*N*-formyl-L-phenylalanylglycine isopentylamide, 96866-13-6; benzylacrylic acid, 5669-19-2; 3-(*RS*)-[(benzyloxy)amino]-2-benzylpropanoic acid, 96866-14-7; 3(*RS*)-[*N*-(benzyloxy)-*N*-formylamino]-2-benzylpropanoic acid, 96866-15-8; *N*-[3(*RS*)-[*N*-(benzyloxy)-*N*-formylamino]-1-oxo-2-benzylpropyl]glycine benzyl ester, 96866-16-9; *N*-[3(*RS*)-[*N*-(benzyloxy)-*N*-formylamino]-1-oxo-2-benzylpropyl]glycine methyl

ester, 96866-17-0; 2-amino-*N*-(3-methylbutyl)acetamide, 87429-13-8; *N*-[3(*RS*)-[*N*-(benzyloxy)-*N*-formylamino]-1-oxo-2-benzylpropyl]glycine isopentylamide, 96866-18-1; *N*-[3(*RS*)-[*N*-(benzyloxy)-*N*-formylamino]-1-oxo-2-benzylpropyl]glycine benzylamide, 96866-19-2; 3-(ethoxycarbonyl)-2-benzylidenepropanoic acid, 87439-00-7; glycine *tert*-butyl ester, 6456-74-2; *N*-[3-(ethoxycarbonyl)-2-benzylidene-1-oxopropyl]glycine *tert*-butyl ester, 87439-01-8; *N*-(3-carboxy-2-benzylidene-1-oxopropyl)glycine *tert*-butyl ester, 87439-02-9; *N*-[3-[[(benzyloxy)amino]carbonyl]-2-benzylidene-1-oxopropyl]glycine *tert*-butyl ester, 96866-20-5; *N*-[3-[[(benzyloxy)amino]carbonyl]-2-benzylidene-1-oxopropyl]glycine, 87438-57-1; *N*-[3-(ethoxycarbonyl)-2-benzylidene-1-oxopropyl]glycine isopentylamide, 96866-21-6; *N*-(3-carboxy-2-benzylidene-1-oxopropyl)glycine isopentylamide, 96866-22-7; *N*-[3-[[(benzyloxy)amino]carbonyl]-2-benzylidene-1-oxopropyl]glycine isopentylamide, 96866-23-8; L-alanine *tert*-butyl ester, 21691-50-9; *N*-[3-(ethoxycarbonyl)-2-benzylidene-1-oxopropyl]-L-alanine *tert*-butyl ester, 96896-87-6; *N*-(3-carboxy-2-benzylidene-1-oxopropyl)-L-alanine *tert*-butyl ester, 96896-88-7; *N*-[3-[[(benzyloxy)amino]carbonyl]-2-benzylidene-1-oxopropyl]-L-alanine *tert*-butyl ester, 96896-89-8; *N*-[3-[[(benzyloxy)amino]carbonyl]-2-benzylidene-1-oxopropyl]-L-alanine, 96896-90-1; EC 3.4.24.11, 82707-54-8; aminopeptidase, 9031-94-1; dipeptidylaminopeptidase, 9032-67-1.

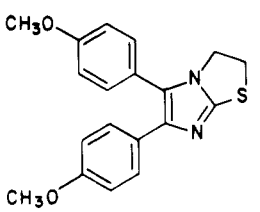
5,6-Diaryl-2,3-dihydroimidazo[2,1-*b*]thiazoles: A New Class of Immunoregulatory Antiinflammatory Agents

Paul E. Bender,*† David T. Hill,† Priscilla H. Offen,† Kazys Razgaitis,† Patricia Lavanchy,† Orum D. Stringer,† Blaine M. Sutton,† Don E. Griswold,‡ Michael DiMartino,‡ Donald T. Walz,‡ Ivan Lantos,§ and Christine B. Ladd§

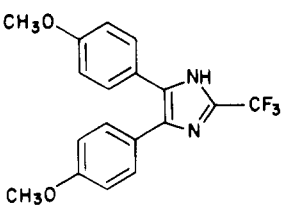
Departments of Medicinal Chemistry, Immunology, and Synthetic Chemistry, Research and Development Division, Smith Kline and French Laboratories, Philadelphia, Pennsylvania 19101. *Received December 26, 1984*

A series of substituted 5,6-diaryl-2,3-dihydroimidazo[2,1-*b*]thiazoles were synthesized and evaluated in the rat adjuvant-induced arthritis and mouse oxazolone-induced contact sensitivity assays to determine the potential of these compounds for use as immunoregulatory antiinflammatory agents. This class of compounds was derived by combining salient structural features of the antiinflammatory agent flumizole and the immunoregulatory drug levamisole. Unlike the latter two, a number of compounds in the target series were found to possess the desired combination of activities. Exploration of structure–activity relationships in the adjuvant-induced arthritic rat assay revealed that optimal potency was exhibited by symmetrically substituted 5,6-diaryl compounds having one of the following alkyl heteroatom or halogen functions at the para position: methoxy, ethoxy, methylthio, *N*-ethyl-*N*-methylamino, fluoro, or chloro. Scrambling of these two substituent classes to yield the unsymmetrically substituted 5,6-diaryl compounds resulted in potent activity only with the 5-alkyl heteroatom, 6-halo-substituted regioisomers. However in the oxazolone-induced contact sensitivity assay, no consistent relationship of variation in activity with structural change was apparent. The initial target compound 5,6-bis(4-methoxyphenyl)-2,3-dihydroimidazo[2,1-*b*]thiazole (1) was compared with its progenitors in additional models of inflammation and immunoregulation.

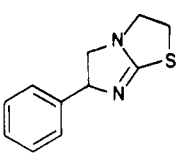
Rheumatoid arthritis, a systemic disease characterized by inflammation and progressive joint destruction, continues to be treated primarily symptomatically.[1] Although of unknown etiology, this disease is characterized by a variety of immune abnormalities, including excess immunoglobulin production, the presence of autoantibodies, and an impairment in thymic derived lymphocyte function.[2a–d] The search for an improved agent to treat rheumatoid arthritis and other inflammatory diseases with immunological abnormalities has gone in two major directions: the investigation of nonsteroidal antiinflammatory drugs (NSAIDs) possessing diminished ulcerogenic properties and the study of immunomodulating agents.[3] Fusion of both sets of properties into a single drug that could reduce the arthritic inflammation, restore immune function to normal, and provide protection from tissue destruction would be therapeutically advantageous. By combining closely related pharmacophoric structures of the potent antiinflammatory agent flumizole[4a,b] with the biological response modifier levamisole,[4c] compound 1 was designed and synthesized as our first such hybrid.[4d] Although many antiinflammatory flumizole analogues and immunomodulatory levamisole analogues have been reported, [3,4a,5–7] hybrid molecules possessing such a combined profile are not well-known.



1



flumizole



levamisole

Compound 1 stimulated mouse subliminal oxazolone-induced contact sensitivity in a manner analogous to le-

† Department of Medicinal Chemistry.
‡ Department of Immunology.
§ Department of Synthetic Chemistry.

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