# Synthesis and Analgesic Effects of N-[3-[(Hydroxyamino)carbonyl]-1-oxo-2(R)-benzylpropyl]-L-isoleucyl-L-leucine, a New Potent Inhibitor of Multiple Neurotensin/Neuromedin N Degrading Enzymes

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The synthesis of N-[3-[(hydroxyamino)carbonyl]-1-oxo-2(R)-benzylpropyl]-L-isoleucyl-L-leucine (JMV-390-1, **6a**), a multipeptidase inhibitor based on the C-terminal sequence common to neurotensin (NT) and neuromedin N (NN), is described. This compound behaves as a full inhibitor of the major NT/NN degrading enzymes in vitro, e.g. endopeptidase 24.16, endopeptidase 24.15, endopeptidase 24.11, and leucine aminopeptidase (type IV-S), in the nanomolar range ($IC_{50}$'s from 30 to 60 nM). Compound **6a** was found to increase endogenous recovery of NT and NN from slices of mice hypothalamus depolarized with potassium. In various assays commonly used to select analgesics, e.g. hot-plate test, tail-flick test, acetic acid-induced writhing test, in mice, compound **6a** proved to be potent when intracerebroventricularly (icv) injected. The analgesic effects observed were totally (hot-plate test) or largely (tail-flick test) reversed by the opioid antagonist naltrexone. Furthermore, icv injection of compound **6a** (10 μg/mouse) was found to significantly potentiate the hypothermic effects of NT or NN.

## Introduction

Neurotensin (NT) (<Glu[1]-Leu[2]-Tyr[3]-Glu[4]-Asn[5]-Lys[6]-Pro[7]-Arg[8]-Arg[9]-Pro[10]-Tyr[11]-Ile[12]-Leu[13]-OH) and neuromedin N (NN) (Lys[1]-Ile[2]-Pro[3]-Tyr[4]-Ile[5]-Leu[6]-OH) are two related neuropeptides which are synthesized in a common polypeptide precursor distributed throughout the central nervous system and the small intestine of mammals.[1] In the central nervous system, both peptides appear to act through the same receptor[2] and exert a variety of biological effects including hypothermia,[3] analgesia,[4] and modulation of dopamine transmission in the nigro-striatal and meso-corticolimbic systems.[5] Neurotensin also acts as a paracrine and endocrine modulator of digestive functions,[6] being a potent inhibitor of gastric acid secretion,[7] and exerts numerous actions on the cardiovascular system of mammals[8] including the induction of hypotension. Neurotensin is mainly inactivated by three metalloendopeptidases. Endopeptidase 24.15 (EC 3.4.24.15) hydrolyses at the Arg[8]–Arg[9] bond,[9] endopeptidase 24.16 (EC 3.4.24.16) at the Pro[10]–Tyr[11] bond,[10] and endopeptidase 24.11 (EC 3.4.24.11) designated enkephalinase cleaves at both the Pro[10]–Tyr[11] and Tyr[11]–Ile[12] bonds.[11] In addition, neuromedin N is primarily degraded by bestatin-sensitive aminopeptidase(s) that also belong to the family of metallopeptidases.[2] These enzymes are putative candidates for the physiological inactivation of neurotensin and neuromedin N, and therefore their inhibition will be useful for investigating the physiology of neurotensinergic systems. Moreover, inhibition of neurotensin and neuromedin N metabolism represents a new approach in the search for analgesics. Potent and specific inhibitors of metallopeptidases such as angiotensin converting enzyme (ACE, EC 3.4.15.1)[12] or enkephalinase have been rationally designed.[13] On the basis of the work of B. P. Roques and his collaborators for the synthesis of kelatorphan,[14] we applied the concept of multipeptidase inhibitors[15] to the synthesis of potent inhibitors of the metallopeptidases that degrade neurotensin and neuromedin N. For this purpose, an hydroxamate group, able to behave as a bidentate ligand for the metal atom, was introduced on a peptide backbone bearing the subsite specificity for neurotensin. Thus, the synthesis of N-[3-[(hydroxyamino)carbonyl]-1-oxo-2(R,S)-benzylpropyl]-L-isoleucyl-L-leucine was performed and the two diastereoisomers N-[3-[(hydroxyamino)carbonyl]-1-oxo-2(R)-benzylpropyl]-L-isoleucyl-L-leucine (JMV-390-1, **6a**) and of N-[3-[(hydroxyamino)carbonyl]-1-oxo-2(S)-benzylpropyl]-L-isoleucyl-L-leucine (JMV-390-2, **6b**) were separated by HPLC. The absolute configuration of these analogues was deduced from $^1$H-NMR measurements. When tested on neurotensin degradation by endopeptidase 24.16, it appears that compound **6a** was far more potent than its diastereoisomer **6b**. Consequently, compound **6a** was further characterized with respect to its biochemical properties and was studied in mice in various assays commonly used to select analgesics, hot-plate test, tail-flick test, and acetic acid-induced writhing test, as well as for its ability either to intrinsically modify body temperature or to potentiate the NT- or NN-induced hypothermia. Compound **6a** was also evaluated for its ability to protect from degradation endogenously released NT and NN in mouse hypothalamic slices.

## Results and Discussion

**Synthesis.** N-[3-[(Hydroxyamino)carbonyl]-1-oxo-2(R,S)-benzylpropyl]-L-isoleucyl-L-leucine was synthesized by two ways which are summarized in Scheme I (method A) and Scheme II (method B). In the first synthetic scheme (A), the key intermediate, the benzalsuccinic acid 1, was synthesized by Stobbe condensation of benzaldehyde with diethyl succinate to lead to *cis*- and *trans*-diethyl α-benzalsuccinate which was saponified to

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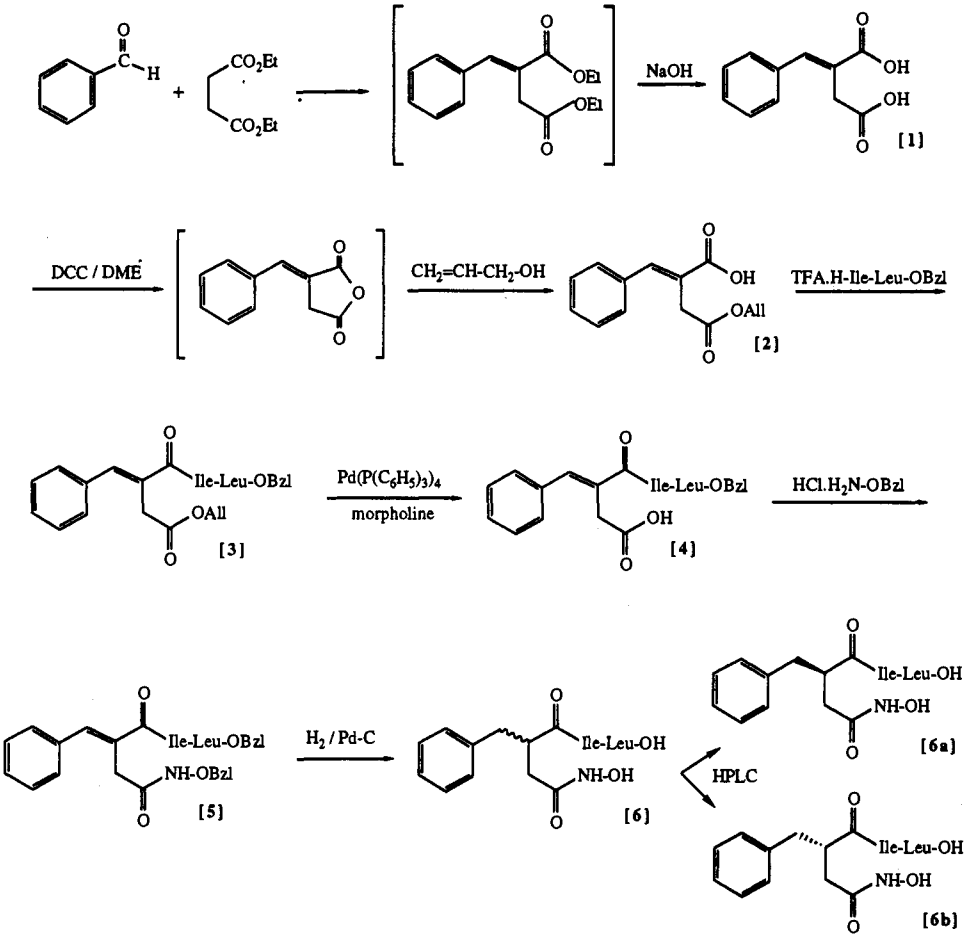
**Scheme I.** Synthesis of Compound **6a** (Route A)




the diacid **1**.[16] The diacid was converted to the anhydride with *N,N'*-dicyclohexylcarbodiimide, and treatment with allyl alcohol resulted in the monoallyl ester **2** which by condensation with TFA·H-Ile-Leu-OBzl resulted in compound **3**. Deprotection of the allyl ester was performed with tetrakis(triphenylphosphine)palladium(0)[17] to produce compound **4**, which was reacted with *O*-benzylhydroxylamine hydrochloride in the presence of BOP[18] as coupling reagent to lead to compound **5**. Hydrogenation of **5** resulted in a mixture of the two diastereoisomers of compound **6**. In the second synthetic scheme (B), the benzalsuccinic acid **1** was synthesized from triphenylphosphine and succinic anhydride according to Hudson and Chopard,[19] followed by alkaline hydrolysis. The diacid was converted to the anhydride with *N,N'*-dicyclohexylcarbodiimide, and treatment with ethanol led to the $\beta$-ethyl $\alpha$-benzalsuccinate **9**. Condensation of compound **9** with HCl·H-Ile-Leu-OtBu **10** resulted in compound **11**. Basic hydrolysis of compound **11** and condensation with *O*-benzylhydroxylamine hydrochloride in the presence of BOP as coupling reagent, followed by treatment with trifluoroacetic acid, led to a mixture of the two diastereoisomers of compound **6**. The diastereoisomers of compound **6** were separated by HPLC and produced two compounds named **6a** and **6b**. After HPLC separation, the configuration of the benzylsuccinyl moiety was tentatively established by $^1$H-NMR spectroscopy according to Fournié-Zaluski et al.[20] Indeed, $^1$H-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 on the relative configuration of each asymmetric carbon. Comparison of the spectra of H-L-Phe-L-Ile-L-Leu-OH and H-D-Phe-L-Ile-L-Leu-OH as well as H-L-Phe-L-Ile-OH and H-D-Phe-L-Ile-OH revealed that the side-chain protons of L-Ile were more shielded (e.g. $\delta(CH_3)$ 0.70–0.77 ppm, $\delta(H\gamma,\gamma')$ 0.86–1.25 ppm, $\delta(H\beta,\beta'$ 1.62–1.63 ppm)) in the D-Phe isomers than in the natural dipeptides containing L-Phe (e.g. $\delta(CH_3)$ 0.83–0.89 ppm, $\delta(H\gamma,\gamma')$ 1.09–1.53 ppm, $\delta(H\beta,\beta')$ 1.73–1.81 ppm) (Table I). These effects are probably due to a difference in the mean orientation of the side chains in the two diastereoisomers. In H-D-Phe-L-Ile-OH and H-D-Phe-L-Ile-L-Leu-OH the isoleucine protons of the side chain are shielded by the ring current of phenylalanine, whereas in the natural H-L-Phe-L-Ile-OH and H-L-Phe-L-Ile-L-Leu-OH peptides, the isoleucine protons of the side chain have the same chemical shifts as in any other peptide containing isoleucine.[21] The spectra of the two separate diastereoisomers **6a** and **6b** (Table I) show large chemical shift differences for the isoleucine side chain protons. The isoleucine side chain protons of compound **6a** (e.g. $\delta(CH_3)$ 0.79–0.83 ppm, $\delta(H\gamma,\gamma')$ 1.06–1.44 ppm, $\delta(H\beta,\beta')$ 1.69 ppm) being more deshielded than in the second diastereoisomer **6b** (e.g. $\delta(CH_3)$ 0.61–0.64 ppm, $\delta(H\gamma,\gamma')$ 0.83–1.16 ppm, $\delta(H\beta,\beta')$ 1.62 ppm). Consequently, the proposed structure for diastereoisomer **6a** is *N*-[3-[(hydroxyamino)carbonyl]-1-oxo-2(*R*)-benzylpropyl]-L-isoleucyl-L-leucine, which is analogous to the natural di- and tripeptides H-L-Phe-L-Ile-OH and H-L-Phe-L-Ile-L-Leu-OH. Obviously, diastereoisomer **6b**, which correspond to H-D-Phe-L-Ile-OH and H-D-Phe-L-Ile-L-Leu-OH is assumed to be *N*-[3-[(hydroxyamino)carbonyl]-1-oxo-2(*S*)-benzylpropyl]-L-isoleucyl-L-leucine.

**Scheme II.** Synthesis of Compound **6a** (Route B)

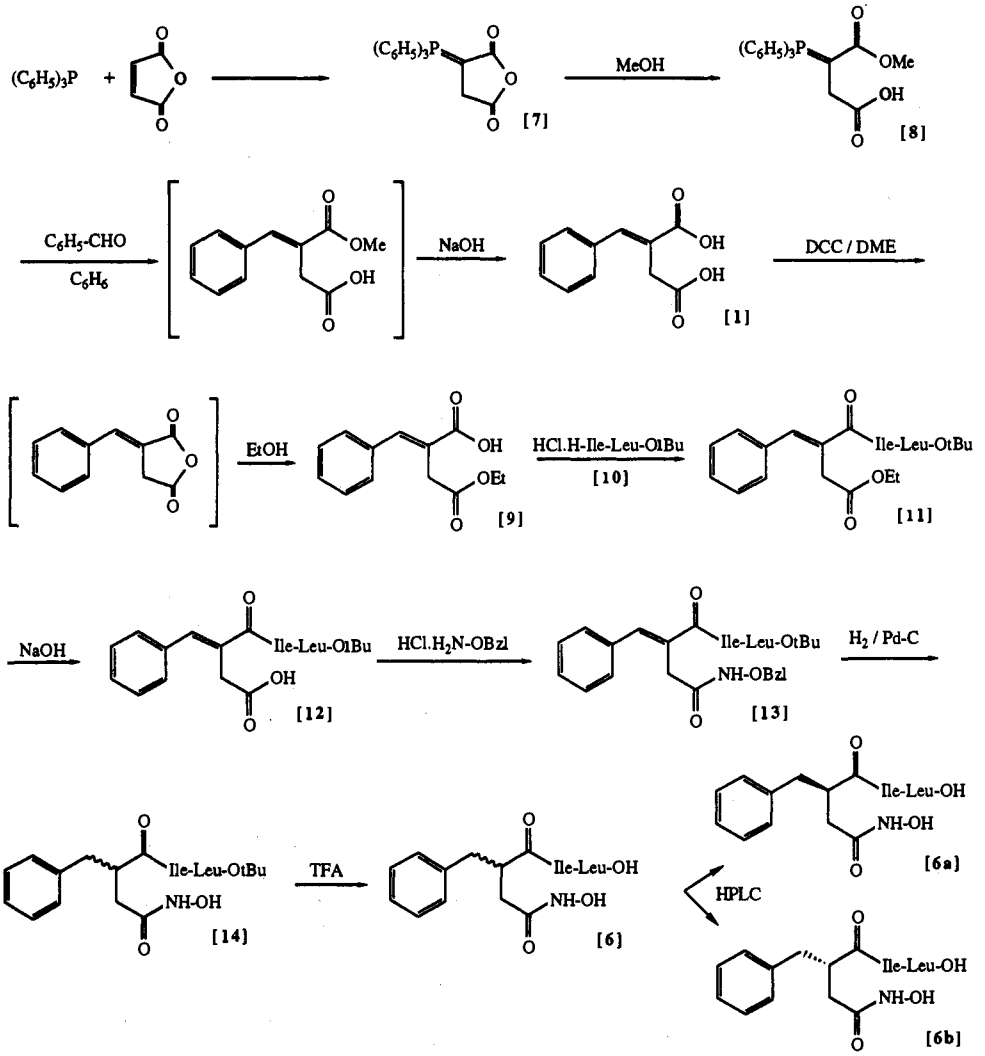



**Table I.** $^1$H NMR Data of the Two Diastereoisomers **6a** and **6b** and of the Model peptides H-Phe-Ile-Leu-OH, H-D-Phe-Ile-Leu-OH, H-Phe-Ile-OH, and H-D-Phe-Ile-OH

| | | **6a** | | **6b** | | Phe-Ile-Leu | | D-Phe-Ile-Leu | | Phe-Ile | | D-Phe-Ile | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| residues | | δ (ppm) | $^3J$ (Hz) | δ (ppm) | $^3J$ (Hz) | δ (ppm) | $^3J$ (Hz) | δ (ppm) | $^3J$ (Hz) | δ (ppm) | $^3J$ (Hz) | δ (ppm) | $^3J$ (Hz) |
| -CONHOH | | | | | | | | | | | | | |
| $-CH_2-$ | | 2.24 | 8.4 | 2.24 | 6.9 | | | | | | | | |
| | | 1.93 | 6.1 | 2.04 | 7.5 | | | | | | | | |
| | | ($^2J$: 14.9) | | ($^2J$: 14.7) | | | | | | | | | |
| (Phe) | ($NH_3^+$) | | | | | 8.05 | | 8.11 | | 8.09 | | 8.17 | |
| | Hα | 3.07 | | 3.14 | | 4.14 | | 4.19 | | 4.13 | | 4.19 | |
| | Hβ | 2.87 | 6.9 | 2.75 | 9.2 | 3.06 | 5.3 | 3.06 | 6.4 | 3.11 | 5.4 | 3.04 | 6.7 |
| | Hβ′ | 2.34 | 7.0 | 2.59 | 5.7 | 2.91 | 7.4 | 2.94 | 8.0 | 2.93 | 8.0 | 2.97 | 7.8 |
| | | ($^2J$: 13.4) | | ($^2J$: 13.3) | | ($^2J$: 14.1) | | ($^2J$: 13.6) | | ($^2J$: 14.1) | | ($^2J$: 13.8) | |
| | Ar | 7.3-7.1 | | 7.3-7.1 | | 7.4-7.1 | | 7.4-7.2 | | 7.4-7.2 | | 7.4-7.2 | |
| Ile | NH | 7.81 | 8.9 | 7.77 | 9.1 | 8.51 | 8.9 | 8.44 | 8.7 | 8.57 | 8.4 | 8.55 | 8.5 |
| | Hα | 4.17 | | 4.07 | | 4.28 | | 4.29 | | 4.24 | | 4.20 | |
| | Hβ | 1.69 | | 1.62 | | 1.73 | | 1.63 | | 1.81 | | 1.62 | |
| | Hγγ′ | 1.44/1.06 | | 1.16/0.83 | | 1.53/1.09 | | 1.25/0.86 | | 1.45/1.20 | | 1.22/0.99 | |
| | $CH_3$d | 0.83 | 6.9 | 0.61 | 7.0 | 0.89 | 6.6 | 0.73 | | 0.89 | 6.9 | 0.70 | 6.7 |
| | $CH_3$t | 0.79 | 7.5 | 0.64 | 7.3 | 0.83 | | 0.73 | | 0.86 | 7.4 | 0.77 | 7.4 |
| Leu | NH | 7.95 | 7.7 | 7.96 | 7.6 | 8.26 | 7.8 | 8.26 | 7.5 | | | | |
| | Hα | 4.22 | | 4.17 | | 4.24 | | 4.19 | | | | | |
| | Hββ′ | 1.60/1.50 | | 1.62/1.47 | | 1.64/1.53 | | 1.63/1.51 | | | | | |
| | Hγ | 1.30 | | 1.62 | | 1.53 | | 1.51 | | | | | |
| | $CH_3$ | 0.88 | 6.5 | 0.87 | 6.4 | 0.90 | 6.6 | 0.88 | 6.5 | | | | |
| | $CH_3$ | 0.82 | 6.4 | 0.80 | 6.4 | 0.84 | 6.4 | 0.84 | 6.5 | | | | |

**Effects of Compounds 6a and 6b on Metallopeptidase Activities.** Compounds **6a** and **6b** were all able to inhibit various metallopeptidase activities, including endopeptidases EC 24.16, EC 24.11, and EC 24.15 and leucine-aminopeptidase (type IV-S), compound **6a** being far more potent than its diastereoisomer **6b** (Table II). This is in accordance with the fact that in general, enzymes interact preferentially with compounds exhibiting ster-

**Table II.** $K_i$ Value (nM) for the Ability of Compounds **6a** and **6b** To Inhibit Metallopeptidase Activities[a]

| compound | peptidase | $K_i$ (nM) |
|---|---|---|
| JMV-390-1 (**6a**) | endopeptidase 24.16 | 58 |
| | endopeptidase 24.11 | 40 |
| | endopeptidase 24.15 | 31 |
| | Leu-aminopeptidase | 52 |
| | angiotensin converting enzyme | 70 000 |
| | post-prolyl dipeptidyl aminopeptidase | NE ($10^{-4}$ M) |
| JMV-390-2 (**6b**) | endopeptidase 24.16 | 1320 |

[a] Values are the means from two independent determinations. NE = no effect.

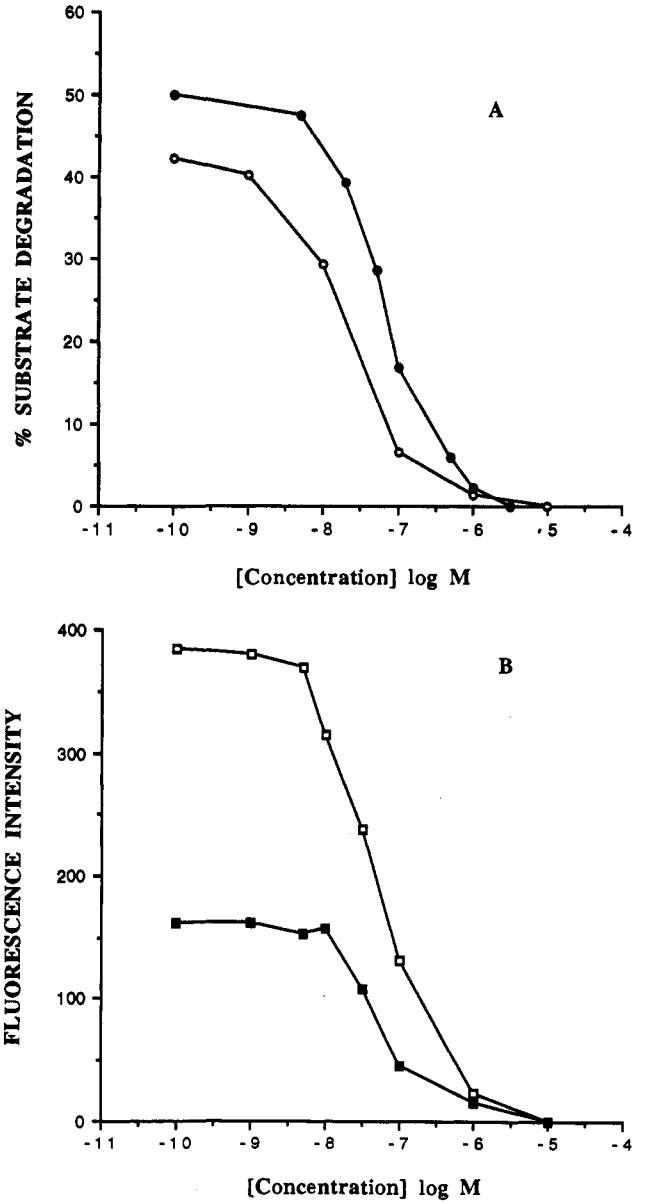



**Figure 1.** Inhibition of metallopeptidases by compound **6a**: (A) endopeptidase 24.11 (○), endopeptidase 24.16 (●); (B) endopeptidase 24.15 (■), Leu-aminopeptidase (□). Enzyme activities were measured as described in Materials and Methods. The data are the mean of two separate experiments performed in duplicate.

eochemical orientation of side chains similar to that of the natural substrates. For this reason, compound **6a** was selected for further experimental investigation. Figure 1 shows the inhibition curves of compound **6a** on endopeptidases 24.16, 24.15, and 24.11 and leucine-aminopeptidase. As it can be seen, compound **6a** completely inhibited these metallopeptidases in a concentration-dependent manner. From these inhibition curves, $K_i$ values for compound **6a** were derived for each peptidase (Table II). For compound **6a**, $K_i$ values ranged from 30 to 60 nM, indicating that this compound was a potent, and nonspecific inhibitor of the metallopeptidases tested here. Compound **6a** is far more potent than the specific dipeptide inhibitors of EC 24.16 described by Dauch et al., the more potent of them, H-Pro-Ile-OH, having a half-maximal effect at a dose of 90 μM.[22] However, not all metallopeptidases were highly sensitive to compound **6a** since on angiotensin converting enzyme activity, compound **6a** has an $IC_{50}$ of about 70 μM (not shown). Finally, compound **6a** did not affect the activity of the serine post-prolyl dipeptidyl aminopeptidase (not shown).

**Effects of Compound 6a on the Recovery of Endogenously Released Neurotensin and Neuromedin N.** The potency of compound **6a** on various metallopeptidases degrading NT and NN prompted us to study its effects on the recovery of endogenous NT and NN from mice hypothalamic slices depolarized by a high potassium concentration. The effects of compound **6a** on the $K^+$-evoked release of neurotensin and neuromedin N, respectively, were studied by means of already described techniques.[23] As previously observed, basal peptide release was undetectable.[23] In control conditions, i.e. in the absence of compound **6a**, $K^+$ induced a transient release of peptides. A first depolarization with 50 mM $K^+$ (S1) evoked a transient release of iNT (immunoreactive neurotensin) and iNN (immunoreactive neuromedin N), the amount of released iNT being higher than that of iNN. A second depolarization (S2) in the absence of peptidase inhibitor also evoked a transient release of iNT and iNN which, however, was usually smaller than that observed upon S1. Superfusion of the tissues with 1 μM of compound **6a** during the S2 phase markedly increased the recovery of both neurotensin and neuromedin N by a factor of about 2–3 (Figure 2). This effect was even more pronounced at 10 μM of compound **6a**. Neurotensin and neuromedin N increased by a factor of approximately 5 (Figure 2). The ratio of the total amount of peptide recovered during the S2 phase to that recovered during the S1 phase was calculated for control experiments and for experiments in the presence of 1 and 10 μM of compound **6a** (Figure 3). Ratio values (S2/S1) were 0.8 and 0.7 for neurotensin and neuromedin N, respectively, in the absence of compound **6a** and increased 4–5-fold in the presence of 10 μM inhibitor. The effects of compound **6a** and of other peptidase inhibitors such as thiorphan and bestatin on the recovery of neurotensin and neuromedin N released from mouse hypothalamus were compared.[24] They showed that compound **6a** was the most efficient peptidase inhibitor to increase the recovery of endogenously released neurotensin and neuromedin N.

**Effect of Compound 6a on Nociception. Hot-Plate Test.** This simple assay permits the measurement of the individual antinociceptive effect of a peptidase inhibitor. The icv administration of compound **6a** over the dose range 5–50 μg/mouse induced a dose-related increase in jump latency, the maximum effect being obtained with 50 μg/animal (Figure 4). At the highest dose (50 μg/mouse) most of the animals treated with compound **6a** reached the cutoff time whereas the paw licking latency was not modified. The time course of the induced analgesia was also

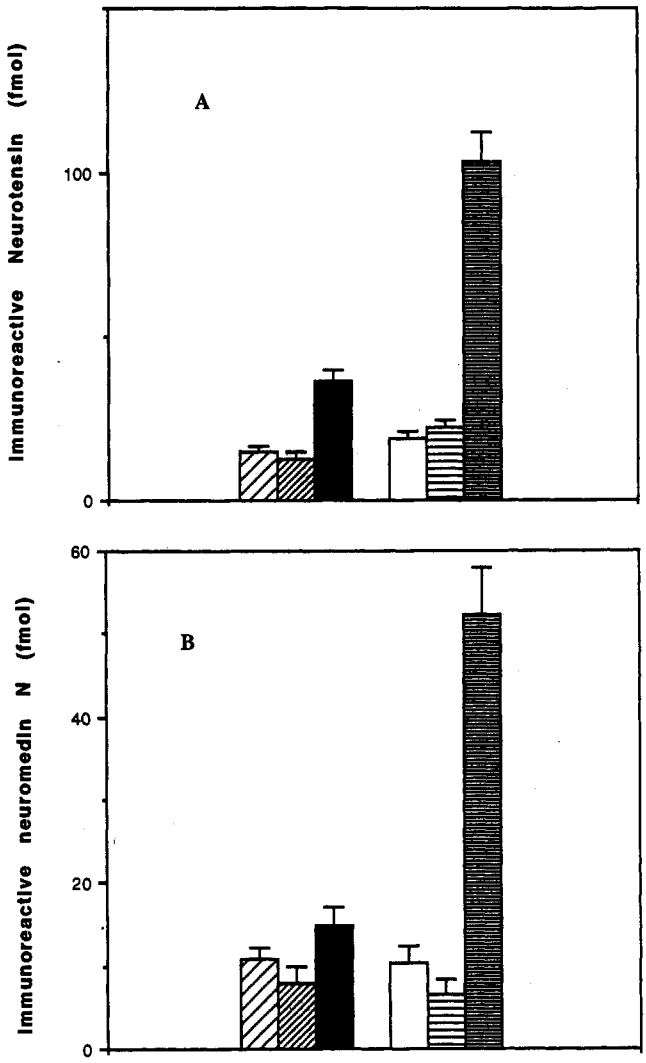



**Figure 2.** Effects of compound **6a** on the $K^+$-evoked release of neurotensin (A) and neuromedin N (B) from mouse hypothalamus. (▨) (□) S1 phase, absence of peptidase inhibitor, control; (▨) (▤) S2 phase, absence of inhibitor, control; (■) S2 phase, + 1 μM compound **6a**; (▤) S2 phase, + 10 μM compound **6a**. Values represent the means ± SEM from three separate experiments. The mean neurotensin content of the hypothalamic tissues incubated in this series of experiments was 1.72 ± 0.15 pmol ($n$ = 12) per superfusion chamber, the mean neuromedin N content was 0.69 ± 0.06 pmol ($n$ = 12).

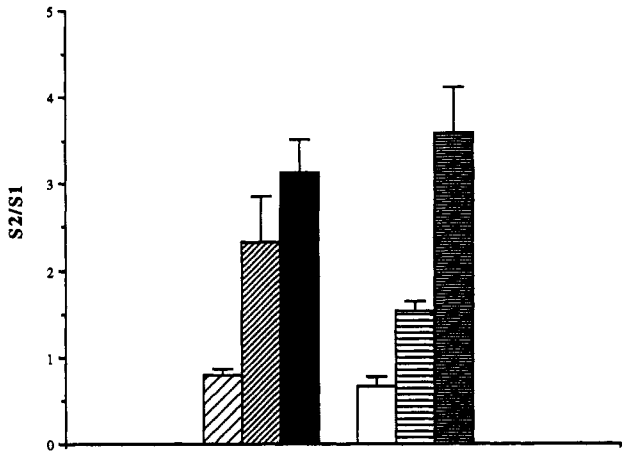



**Figure 3.** Effects of compound **6a** on the ratio of peptides released during the S2 phase to that released during the S1 phase (S2/S1). The amount of peptide released during a depolarization phase was calculated by summing up the peptide contents of all the fractions that showed measurable peptide levels following $K^+$ application. Values represent the means ± SEM from six separate experiments in the absence of compound **6a** (control) and from three separate experiments in the presence of compound **6a** at the indicated concentrations: (▨) control neurotensin; (□) control neuromedin N; (▤) neuromedin N + 1 μM compound **6a**; (▨) neuromedin N + 10 μM compound **6a**; (▤) neurotensin + 1 μM compound **6a**; (■) neurotensin + 10 μM compound **6a**.

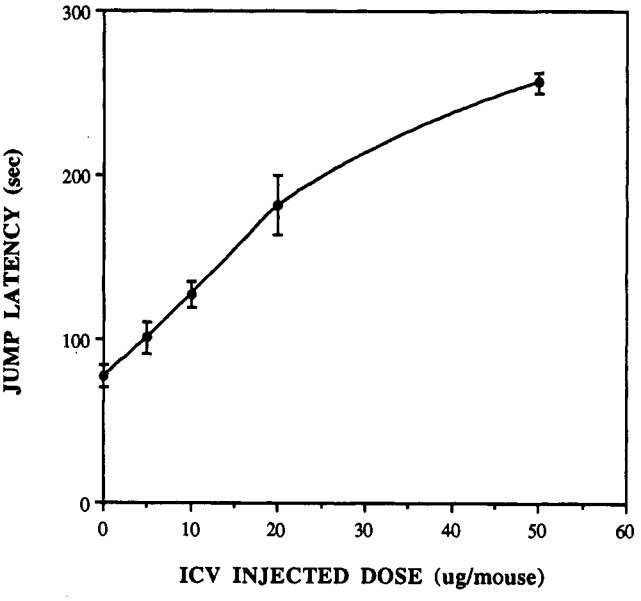



**Figure 4.** Analgesic effect of compound **6a** in the hot-plate test: dose–response curve. Compound **6a** was injected icv 15 min before testing at the indicated doses, in a constant volume (10 μL) ($n$ = 10–12). ANOVA indicates a difference ($P < 0.0001$) whereas effects elicited by the 10–50 μg/mouse of compound **6a** differ from the controls, in the Dunnett test ($P < 0.01$).

evaluated. Duration of the analgesic effects of compound **6a** after icv injection in mice as compared to untreated animals was evaluated. The jump latency of controls was higher shortly after saline icv injection than at later times, likely on account of the stress of the icv injection. Despite this effect, the jump latency was significantly higher in **6a** injected mice (10 μg/mouse) at each considered time (5–60 min) (Figure 5).

**Writhing Test.** In the writhing test, compound **6a** decreased significantly the number of abdominal writhes 30 min after the intracerebroventricular injection. In control mice, the number of stretches counted was 20.4 ± 2.9, 30 min after icv injection of saline. In treated mice, intracerebroventricular injection of 10 μg of compound **6a** significantly decreased the number of stretches to 4.5 ± 1.6, 30 min after administration (M ± SEM of six to seven mice per group, the difference with respective controls being significant ($P < 0.001$), indicating again the analgesic effects induced by compound **6a**.

**Tail-Flick Test.** It has been often underlined that enkephalinase inhibitors such as thiorphan are inactive on mouse tail-flick test and on mouse tail-withdrawal tests.[25] These features might likely be due either to a lack of or insufficient release of enkephalin induced by these intense but short nociceptive stimuli, or to the fact that in these tests, opioid peptides such as β-endorphin, dynorphin, or other neuropeptides less sensitive to enkephalinase should be preferentially involved in pain regulation. However, it has recently been reported that mixed peptidase inhibitors such as kelatorphan or RB 38A are active on mouse tail-flick and on mouse tail-

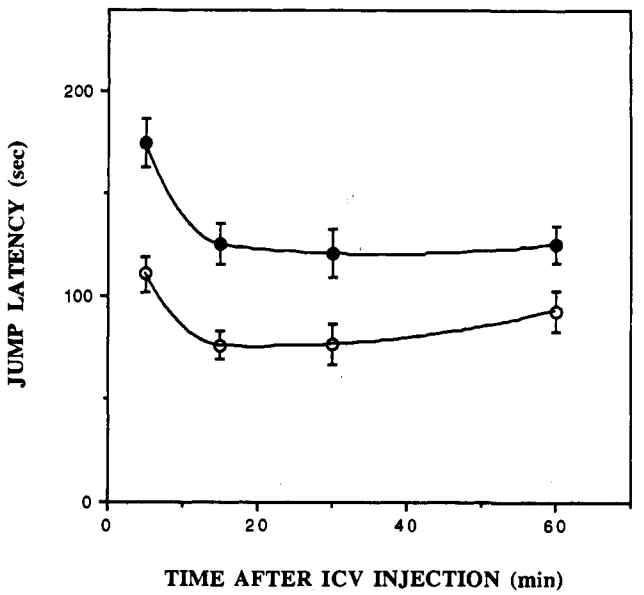



**Figure 5.** Analgesic effect of 10 μg of compound **6a** in the hot-plate test: time–response curve. Compound **6a** was injected icv at the indicated doses in a constant volume (10 μL) of saline ($n$ = 7–12); (○) controls; (●) 10-μg-treated animals. Data analyzed by ANOVA indicate a difference ($P < 0.0001$) between two groups, suggesting a parallelism between their two curves (no interactions between the two curves) and revealing an obvious time–effect relationship with $P < 0.001$. The Newman–Keuls test analyzing this last point indicated that only the latency at the first time (5 min after icv injection) was different from those at the other three considered times.

withdrawal tests[26] suggesting a significant involvement of enkephalins in pain sensation related to these tests. In the tail flick test, compound **6a** (10 μg) was effective 30 min following the intracerebroventricular injection. In control mice the withdrawal latency was 3.4 ± 0.2 s, 30 min after intracerebroventricular injection of saline. In mice treated with compound **6a** (10 μg) the withdrawal latency was 5.4 ± 0.7 s, 30 min after injection (M ± SEM of six mice per group, the difference with respective controls being significant ($P < 0.05$). These results indicate that the multipeptidase inhibitor **6a** is active in simultaneously inhibiting various enzymes concerned in the degradation of neuropeptides involved in the pain sensation.

**Effects of Naltrexone on Antinociception Induced by Compound 6a.** The antinociceptive activity of compound **6a** both in the hot-plate test (jump latency) and in the tail flick test was significantly reduced by subcutaneous administration of the opioid receptor antagonist naltrexone (1 mg/kg) 15 min prior to icv injection of the peptidase inhibitor (Table III). Naltrexone completely abolished the antinociceptive effects of the peptidase inhibitor **6a** on the hot-plate test and largely reduced the effects on the tail-flick test. On the contrary, naltrexone (1 mg/kg) 15 min prior to intracerebroventricular injection of compound **6a** completely failed to antagonize the antinociceptive activity of the peptidase inhibitor in the writhing test (Table III). Since on the hot-plate test the analgesic effect of compound **6a** was completely reversed by the opioid antagonist naltrexone, one may consider that it depends only on the involvement of opioid peptides. On the other hand, the partial antagonism by naltrexone of the effect of compound **6a** on the tail-flick test, and moreover the lack of antagonism on the writhing test, suggest that other peptides than opioid peptides could be

**Table III.** Antagonism by Naltrexone of Induced Analgesia Induced by Compound **6a** in the Hot-Place Test, Tail-Flick Test, and Writhing Test[a]

| | nociceptive tests | | |
|---|---|---|---|
| treatments sc/icv | hot-plate test (jump latency) (s, ± SEM) | tail-flick test (tail withdrawal latency) (s, ± SEM) | writhing test (stretches/ 5 min) |
| saline/saline | 91 ± 11 | 3.6 ± 0.5 | 18.4 ± 1.5 |
| naltrexone/saline | 63 ± 9 | 4 ± 0.8 | 30.6 ± 2.1 |
| saline/ compound 6a | 144 ± 17 | 5.9 ± 0.4 | 4.4 ± 2.8 |
| naltrexone/ compound 6a | 64 ± 11 | 4.6 ± 0.3 | 3.8 ± 2.5 |

[a] Saline (0.20 mL/20 g) or naltrexone (1 mg/kg) were coadministered subcutaneously (sc) 15 min before the intracerebroventricular (icv) injection of either saline (10 μL/mouse) or compound **6a** (10 μg/mouse). The nociceptive tests were performed 15 min later. M ± SEM of six to seven mice per group. Whatever the considered test, the ANOVA reveals a significant difference between groups in the hot-plate test ($P < 0.0002$), in the tail-flick-test ($P < 0.03$), and in the writhing test ($P < 0.0001$). In the hot-plate test, the Newman–Keuls test indicates that only the group saline/compound **6a** differs from the other three assays. In the tail-flick test, the Newman–Keuls test indicates that the group saline/compound **6a** differs from saline/saline as well as naltrexone/saline, but not from the naltrexone/compound **6a** group whereas this later group does not differ from any of the other three groups. In the writhing test, the Newman–Keuls test indicates that there is no difference between the groups saline/compound **6a** and naltrexone/compound **6a**. The group saline/saline and the group naltrexone/saline differ one from the other as well as from each other group.

**Table IV.** Potentiation by Compound **6a** of the Analgesic Effect of Endogenous Inactive Doses of Met-enkephalin or Neuromedin N on the Hot-Plate Test[a]

| treatment icv | jump latency ± SEM (s) | treatment icv | jump latency ± SEM (s) |
|---|---|---|---|
| saline | 87 ± 5 | compound **6a** (10 μg) | 133 ± 18 |
| Met-enkephalin (30 μg) | 98 ± 15 | Met-enkephalin (30 μg) + compound **6a** (10 μg) | >240 |
| neuromedin N (1 μg) | 79 ± 6 | neuromedin N (1 μg) + compound **6a** (10 μg) | 201 ± 16 |

[a] Compound **6a** and Met-enkephalin or neuromedin N were injected icv simultaneously. The test was performed 30 min later. M ± SEM of eight mice.

involved in the analgesic effects related to compound **6a** according to the test. The multiplicity of the peptide candidates to such a function and the low number of available specific antagonists render impossible their identification.

**Effect of Compound 6a on Neuromedin N and Met-enkephalin-Induced Antinociception.** In order to evaluate the participation of both Met-enkephalin and neuromedin N on analgesia and the effects of compound **6a**, inactive doses of Met-enkephalin (30 μg) and of neuromedin N (1 μg) were simultaneously icv injected in association with compound **6a** (10 μg) in mice. The analgesic effects were measured by the hot-plate test. As can be seen in Table IV, compound **6a** showed pronounced analgesic effects by itself (jump latency = 133 ± 18 s as compared to the controls 86.5 ± 5.2 s) (Table IV). Compound **6a** strongly increased the jump latency time of the coadministered peptidase-sensitive Met-enkaphalin and neuromedin N (jump latency ≥ 240 and = 201 ± 16 s, respectively). In the presence of 30 μg of Met-enkephalin, each animal reached the cutoff time, and only two animals on 12 tested licked their paws. The potentiation of the analgesia induced by exogenous subanalgesic

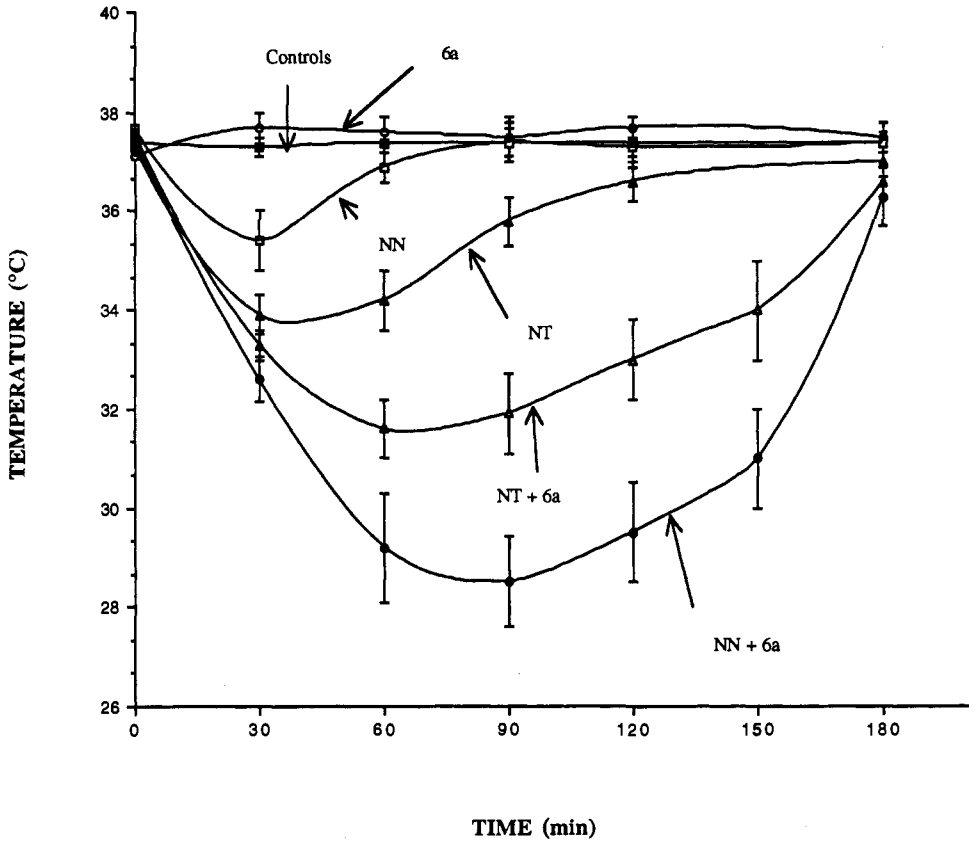



**Figure 6.** Effects of compound **6a** (10 μg) on neurotensin (NT)- and neuromedin N (NN)-induced hypothermia. Compound **6a** and NT or NN were injected icv simultaneously. The colonic temperature was measured immediately before injection at the indicated times. Values are means ± SEM of 6–10 mice per group: (■) controls; (○) compound **6a** (10 μg); (□) NN, 0.25 μg per mouse; (▲) NT 0.1 μg per mouse; (△) NT 0.1 μg + compound **6a** 10 μg per mouse; (●) NN 0.25 μg + compound **6a** 10 μg per mouse.

doses of peptidase-sensitive Met-enkaphalin or neuromedin N by compound **6a** confirms the antinociceptive potency of compound **6a** and its protecting effects on Met-enkephalin and neuromedin N against peptidases. The ability of compound **6a** to inhibit endopeptidases 24.11, 24.15, and 24.16 and Leu-aminopeptidase, four NT/NN degrading enzymes, two of which were also involved in the degradation of opioid peptides, correlated with its antinociceptive activity.

**Effect of Compound 6a on the Neurotensin- and Neuromedin N-Induced Hypothermia.** As can be seen in Figure 6, neurotensin (0.1 μg) and neuromedin N (0.25 μg) induce hypothermia. Compound **6a** (10 μg) itself is without effects on hypothermia. However, when compound **6a** (10 μg) was simultaneously icv injected with either neuromedin N or neurotensin, it significantly potentiated their hypothermic effect (Figure 6). This potentiation was observed both on the amplitude and duration of the effects. Although neuromedin N (0.25 μg) was less potent in inducing hypothermia than neurotensin (0.1 μg), unexpectedly, compound **6a** was more effective in potentiating neuromedin N-induced hypothermia than neurotensin-induced hypothermia. The potentiating activity of compound **6a** on neurotensin- or neuromedin N-induced hypothermia confirms the protecting effects of the peptidase inhibitor on the metabolism of the two neuropeptides in vivo.

## Conclusions

This paper presents the synthesis and pharmacological evaluations of a new nonspecific mixed hydroxamate metallopeptidases inhibitor based on the common C-terminal NT/NN sequence. This compound, *N*-[3-[(hydroxyamino)carbonyl]-1-oxo-2(*R*)-benzylpropyl]-L-isoleucyl-L-leucine, **6a**, turned out to be a potent multipeptidase inhibitor, highly active on in vitro neurotensin and neuromedin N recovery, exhibiting significant analgesic and hypothermic properties. Owing to the inhibitory potency against endopeptidases 24.11, 24.15, and 24.16 and leucine-aminopeptidase, and to its analgesic properties, these results suggest that the *R* isomer of compound JMV-390 can be considered as a complete inhibitor of the major peptidases degrading NT/NN, but also probably of other peptides sensitive to these peptidases, including some opioid peptides.

## Material and Methods

**Experimental Procedures.** Melting points were taken on a Buchi apparatus in open capillary tubes. Optical rotation were determined with a Perkin-Elmer 141 polarimeter. Ascending TLC was performed on precoated plates of silica gel 60 $F_{254}$ (Merck) with the following solvent systems (by volume): AcOEt, A; AcOEt/hexane, B (7:3), C (5:5), D (3:7), E (1:5); AcOEt/AcOH, F (100:0.5); AcOEt/hexane/AcOH, G (70:30:0.5), H (50:50:0.5), I (30:70:0.5); $CHCl_3$/MeOH/AcOH, J (85:10:5), K (120:10:5), L (180:10:5); AcOEt/py/AcOH/$H_2O$ M, (40:20:5:10), N (60:20:5:10). Peptide derivatives were located with charring reagent or ninhydrin. Column chromatography were performed with silica gel 60, 60–229 mesh, ASTM (Merck). HPLC purifications were run on a Merck-Hitachi instrument on a S.F.C.C. C8 UltraBase (10 μm) 20- × 250-mm column, with an UV detection at 230 nm, at a flow rate of 7 mL/min of a mixture of (A) ammonium acetate 0.05 M, pH 6.5, and B, methanol. $^1$H-NMR spectra were recorded as DMSO-$d_6$ solutions at 305 K on an AMX 360 Bruker spectrometer with chemical shifts reported in δ relative to the solvent signal set to 2.50 ppm. Proton assignments were made on the basis of 2D COSY and TOCSY experiments. Mass spectra

were recorded on a JEOL JMS DX 100 and DX 300 spectrometer in a FAB positive mode. L amino acids and derivatives were from Bachem, Novabiochem, or Propeptide. All reagents were of analytical grade. The following abbreviations were used: BOP, [(benzotriazolyl)oxy]tris((dimethylamino)phosphonium) hexafluorophosphate; DCC, *N,N'*-dicyclohexylcarbodiimide; DCU, *N,N'*-dicyclohexylurea; DME, ethylene glycol dimethyl ether; DMF, dimethylformamide; NMM, *N*-methylmorpholin. Other abbreviations used were those recommended by the IUPAC-IUB Commission [*Eur. J. Biochem.* 1984, *138*, 9–37].

**Animals.** Male Swiss albino mice (CD1, Charles River, 20–22 g) were used. They had free access to standard semisynthetic laboratory diet and tap water, under controlled experimental conditions (temperature 22 ± 1 °C, 7 a.m.–7 p.m. light dark cycle). Experiments were carried out between 10 a.m. and 6 p.m.

**Drugs and Solutions.** *N*-[3-[(Hydroxyamino)carbonyl]-1-oxo-2(*R*)-benzylpropyl]-L-isoleucyl-L-leucine (**6a**) was dissolved in a minimal amount of NaOH (0.1 N), and the saline solution was neutralized with HCl (0.1 N). Met-enkephalin, NT, and NN were dissolved in saline. The icv injections were performed according the method of Haley and Mc Cormick[27] in a volume of 10 μL/mouse.

**Iodinated Peptides and Fluorogenic Substrates.** Monoiodo[$^{125}$I-Tyr$^3$]neurotensin (specific activity, 2000 Ci/mmol) was prepared and purified as reported.[28] [$^{125}$I-Tyr$^1$,D-Ala$^2$]Leu-enkephalin (specific activity 2000 Ci/mmol) was prepared and purified as previously described for Leu-enkephalin.[29] Leu-(4-methylcoumarinyl)-7-amide (Leu-7AMC) was from Sigma (St. Louis, MO), 7-methoxycoumarin-3-carboxyl-Pro-Leu-Gly-Pro-D-Lys-2,4-dinitrophenyl [Mec-Pro-Leu-Gly-Pro-DLys(Dnp)-OH] was from Novabiochem (Laufelfingen, Switzerland), and *N*-(3-[2-furyl]acryloyl)-Phe-Gly-Gly (FAPGG) was from Sigma (St. Louis, MO).

**Enzyme Preparations.** Purified rabbit kidney endopeptidase 24.11 was a generous gift from Professor B. Roques (Université R. Descartes, Paris). It generated [$^3$H]Tyr-Gly-Gly from [$^3$H]-Leu-enkephalin with a specific activity of 0.2 nmol·min$^{-1}$·mg$^{-1}$ (25 °C, pH 7.5). Endopeptidase 24.16 and endopeptidase 24.15 were purified from rat brain as reported.[30,31] Endopeptidase 24.16 and endopeptidase 24.15 hydrolyzed neurotensin to yield neurotensin (1–10) and neurotensin (1–8), respectively, with specific activities of 0.5 nmol·min$^{-1}$·mg$^{-1}$ (endopeptidase 24.16, 37 °C, pH 7.4) and 0.8 nmol·min$^{-1}$·mg$^{-1}$ (endopeptidase 24.15, 37 °C, pH 7.4). Purified Leu-aminopeptidase (type IV-S) from porcine kidney microsomes (19 units/mg as defined by the manufacturer) was from Sigma (St. Louis, MO). ACE purified from rabbit lung was from Sigma (St. Louis, MO).

**Enzymatic Assays.** Endopeptidase 24.16 was assayed by incubating 0.3 μg of enzyme preparation with 50 000 cpm of [$^{125}$I-Tyr$^3$]neurotensin and with varying concentrations of compound **6a** (or **6b**) in a final volume of 100 μL of 50 mM Tris buffer, pH 7.4, for 10 min at 37 °C. The reaction was stopped by adding 10 μL of 5 N HCl. The samples were analyzed by reverse-phase HPLC on a RP $C_{18}$ Lichrosorb column (Merck). Elution was performed in 0.1% trifluoroacetic acid (TFA), 0.05% triethylamine (TEA) with a linear gradient running from 10% to 40% acetonitrile in 42 min at a flow rate of 1 mL/min as previously described.[32] One-milliliter fractions were collected and counted for radioactivity. In this system, [$^{125}$I-Tyr$^3$]neurotensin eluted at 23 min, and its radioactive degradation product [$^{125}$I-Tyr$^3$]-neurotensin (1–10) eluted at 18 min. The percent of intact peptide was calculated as the ratio of radioactivity recovered from the column in the 23-min peak to that recovered in the 18-min + 23-min peaks.

Endopeptidase 24.11 activity was assayed by incubating 0.1 μg of enzyme preparation with 50 000 cpm of [$^{125}$I-Tyr$^1$,D-Ala$^2$]-Leu-enkephalin and with varying concentrations of compound **6a** in a final volume of 100 μL of 50 mM Tris buffer, pH 7.4, for 15 min at 25 °C. The reaction was stopped by adding 10 μL of 5 N HCl. The samples were analyzed by reverse-phase HPLC on a RP C18 Lichrosorb column (Merck). Elution was performed in 0.1% trifluoroacetic acid (TFA), 0.05% triethylamine (TEA) with a linear gradient running from 10% to 60% acetonitrile in 35 min at a flow rate of 1 mL/min. One-milliliter fractions were collected and counted for radioactivity. In this system, [$^{125}$I-Tyr$^1$,D-Ala$^2$]Leu-enkephalin eluted at 23 min and its radioactive degradation product [$^{125}$I-Tyr]-D-Ala-Gly eluted at 13 min. The percent of intact peptide was calculated as the ratio of radioactivity recovered from the column in the 28-min peak to that recovered in the 13-min + 28-min peaks.

Endopeptidase 24.15 activity was assayed with the fluorogenic substrate Mcc-Pro-Leu-Gly-Pro-D-Lys(Dnp)-OH[33] (QFS). Briefly, 20 μg of enzyme preparation was incubated with 10 μg of the fluorogenic substrate (QFS), 50 μM dithiothreitol, and varying concentrations of compound **6a** in a final volume of 100 μL of 50 mM Tris buffer, pH 7.4, for 60 min at 37 °C. The reaction was stopped by adding 2 mL of 1 M sodium formate, pH 4. Fluorescence was read with $\lambda_{ex}$ = 345 nm and $\lambda_{em}$ = 405 nm.

Leu-aminopeptidase activity was assayed with the fluorogenic substrate Leu-7AMC. The enzyme preparation (4 ng) was incubated with 0.1 mM Leu-7AMC, 1 μM phosphoramidon (to inhibit contaminating endopeptidase 24.11 activity), and varying concentrations of compound **6a** in a final volume of 100 μL of 50 mM Tris buffer, pH 7.4, for 30 min at 37 °C. The reaction was stopped by adding 2 mL of 1 M sodium formate, pH 4. Fluorescence was read with $\lambda_{ex}$ = 380 nm and $\lambda_{em}$ = 460 nm.

ACE was assayed by incubating 0.025 unit/mL of enzyme preparation with *N*-(3-[2-furyl]acryloyl)-Phe-Gly-Gly (FAPGG) (10 μM) and with varying concentrations of compound **6a** (or **6b**) in a final volume of 200 μL of 50 mM Tris-HCl buffer (NaCl 300 mM), pH 7.4, for 30 min at 37 °C.

**Superfusion Experiments.** Mice were sacrificed, their brains were removed, and hypothalami were rapidly dissected and sliced (1 × 1 × 0.2 mm) with a McIlwain tissue chopper. The slices were placed in Krebs-bicarbonate buffer (NaCl 127 mM, KCl 3.83 mM, $KH_2PO_4$ 1.18 mM; $MgSO_4$ 1.18 mM, $CaCl_2$ 1.8 mM, $NaHCO_3$ 20 mM, glucose 11 mM), pH 7.4. The buffer was constantly gassed with 95% $O_2$, 5% $CO_2$. After rinsing, the slices were distributed in 500-μL superfusion chambers (Millipore) in fresh oxygenated buffer at 37 °C. Each chamber contained the equivalent of two hypothalami. The slices were superfused at a flow rate of 0.25 mL/min with a peristaltic pump (Ismatec MP 13). After a 60-min equilibration period, 1-mL fractions were collected on ice. Starting at fraction 5 and during 5 min, the superfusion buffer was replaced by a medium in which 50 mM $K^+$ was substituted for the equivalent concentration of $Na^+$ (first depolarization S1). This was repeated at fraction 13 for another 5-min period (second depolarization S2). In each experiment, six chambers were simultaneously perfused. Three chambers served as controls while the other three chambers were superfused with compound **6a** (1 or 10 μM) starting at fraction 13 and for the rest of the experiment (up to fraction 22). At the end of the experiment the tissues in each chamber were sonicated in 300 μL of 0.1 N HCl and centrifuged. The supernatants and the collected fractions were boiled for 5 min and kept frozen until assays; 200 μL from each superfusion fraction or 100 μL of varying dilutions of the hypothalamic extracts were assayed for their immunoreactive neurotensin and neuromedin N contents by means of highly specific and sensitive radioimmunoassays as previously described.[34]

**Analgesic Tests. Hot-Plate Test.** This test was derived from that described by Eddy and Leimbach.[35] A plastic cylinder (height = 20 cm, diameter = 14 cm) was used to confine the mouse to the heat surface of a hot plate. The plate was heated to a temperature of 55 ± 0.5 °C, using a thermoregulated water circulating pump. The latency period until the mouse jumped was registered using a stopwatch. To avoid injury, animals not responding within 240 s were removed from the hot plate, and a latency time of 240 s was recorded (cutoff time). The latencies of both forepaw licking and jump were determined for each animal.

**Tail-Flick Test.** The antinociceptive responses were determined by measuring the time required to respond to a painful radiating thermal stimulus, according to the method of D'Amour and Smith[36] modified for mice. The light from a projection bulb was focused at about 40 mm from the start of the tail. The movement of the tip exposed a photocell which turned off the electric stimulus and stopped the digital clock measuring the latency. The intensity of the thermal stimulus was adjusted to obtain a control latency between 4 and 6 s. A cutoff time of 10 s was used to prevent blistering. Each value resulted from a triplicate determination performed at 15-s intervals.

**Writhing Test.** This test was derived from that of Sigmund et al.[37] Mice received intraperitoneally in a volume of 10 mL/kg a 0.5% acetic acid solution. They were then placed individually in large beakers. The stretches were counted over a 5-min period from the fifth minute after the acetic acid solution injection. A stretch was characterized by an elongation of the body, the development of tension in the abdominal muscles, and the extension of the forelimbs.

**Body Temperature.** Colonic temperature was measured with a thermistor probe (Ellab TE3, probe RM 6, Copenhagen, Denmark) inserted to a depth of 4 cm into the rectum.

**Statistical Analysis.** Statistics were conducted using ANOVA completed when significant by either the Dunnet test or the Newman-Keuls test (program "SOLO", BMDP statistical software).

**Chemistry: Method A: $C_6H_5CH{=}C(COOH)CH_2COOH$ (1).** Benzaldehyde (42.4 g, 400 mmol), beforehand distilled under reduced pressure and stored under nitrogen atmosphere, was added to a solution of diethyl succinate (69.6 g, 400 mmol) in sodium ethylate (Na: 11.5 g, 500 mmol/EtOH: 240 mL). The reaction mixture was refluxed at 80 °C and allowed to stir under argon for 3 h. It was then cooled down to room temperature, and 1.8 L of water were added. The mixture was extracted with $Et_2O$ (200 mL), ethyl acetate (200 mL), and again with $Et_2O$ (200 mL), and the aqueous layers were acidified with 50 mL of HCl (11 N). The diethyl ester $C_6H_5CH{=}C(CO_2Et)CH_2CO_2Et$ was extracted with $Et_2O$ (2 × 200 mL), washed with $H_2O$ (200 mL), dried over sodium sulfate, and concentrated in vacuo to give an yellow oil: yield 72 g (77%).

The crude product (72 g, 307 mmol) was saponified in EtOH (600 mL) with 1 N NaOH (600 mL) for 24 h at room temperature. The reaction mixture was extracted with $Et_2O$ (300 mL) and acidified with 11 N HCl (60 mL). The organic layer was washed with $H_2O$ (200 mL), dried over sodium sulfate, and concentrated in vacuo. The expected compound was crystallized from acetone/hexane, yield 11.20 g, and after recrystallization, 9.49 g of diacid was obtained (15%): $R_f$(F) 0.51, $R_f$(G) 0.14; mp 174–176 °C; $[\alpha]_D$ +0.7 (c 0.83, DMF). Anal. ($C_{11}H_{10}O_4$) C, H.

**$C_6H_5CH{=}C(COOH)CH_2COOAll$ (2).** Compound 1 (4 g, 19.4 mmol) was dissolved in DME (30 mL), and the reaction mixture was cooled down to 0 °C before DCC (4 g, 19.4 mmol) was added. After 45 min, DCU was eliminated by filtration and the solvent evaporated. Allyl alcohol (25 mL) was then added and the reaction mixture refluxed at 110 °C and stirred over night. The excess of alcohol was evaporated under reduced pressure, 100 mL of $Et_2O$ was added, and the expected compound was extracted with a saturated sodium bicarbonate solution (5 × 100 mL). The aqueous layers were acidified with 11 N HCl (40 mL), and 100 mL of ethyl acetate was added; the organic layer was washed with 1 M potassium hydrogen sulfate (2 × 100 mL), dried over sodium sulfate, and concentrated in vacuo to give a clear oil: yield 3.6 g (75%); $R_f$(H) 0.59, $R_f$(I) 0.48; $[\alpha]_D$ +0.2 (c 1.01, DMF). Anal. ($C_{14}H_{14}O_4$) C, H.

**$C_6H_5CH{=}C(CO\text{-}Ile\text{-}Leu\text{-}OBzl)CH_2COOAll$ (3).** Boc-Ile-Leu-OBzl[38] (3.04 g, 7 mmol) was partially deprotected with trifluoroacetic acid (10 mL). After 30 min at room temperature, the trifluoroacetic acid was removed in vacuo and coevaporated with hexane, and the expected TFA salt was precipitated by addition of ether–hexane (1:5). The obtained powder was collected, washed with hexane, and dried in vacuo over potassium hydroxide: yield 2.95 g (94%). It was dissolved in DMF (20 mL) containing compound 2 (1.72 g, 7 mmol) and BOP (3.1 g, 7 mmol), and NMM (1.5 mL) was last added to this solution. After 2 h, a saturated sodium bicarbonate solution was added with stirring, followed by ethyl acetate (50 mL). The organic layer was washed with a saturated sodium bicarbonate solution (2 × 50 mL), water, 1 M potassium hydrogen sulfate (2 × 50 mL), and water, dried over sodium sulfate, and then concentrated in vacuo. The residue was purified by silica gel column chromatography, with ethyl acetate–hexane (3–7) as solvent, and gave a white powder after trituration with $Et_2O$–hexane: yield 2.48 g (67%); $R_f$(C) 0.77, $R_f$(D) 0.46; mp 79–81 °C; $[\alpha]_D$ +25.5 (c 1.00, DMF). Anal. ($C_{33}H_{42}N_2O_6$) C, H, N.

**$C_6H_5CH{=}C(CO\text{-}Ile\text{-}Leu\text{-}OBzl)CH_2COOH$[17] (4).** The allyl ester 3 (2.48 g, 4.41 mmol) was dissolved in THF (50 mL) in an argon atmosphere before tetrakis(triphenylphosphine)palladium-(0) [$(Ph_3P)_4Pd$ (508 mg, 0.44 mmol)] and morpholine (1.22 mL, 14 mmol) were added in the dark. After 20 min, the solvent was evaporated. The residue was purified by silica gel column chromatography using ethyl acetate–hexane–acetic acid (7-3-0.05) as solvent, to give after trituration with $Et_2O$–hexane a white powder: yield 2.05 g (89%); $R_f$(F) 0.83, $R_f$(G) 0.35; mp 78–80 °C; $[\alpha]_D$ +19.6 (c 0.85, DMF). Anal. ($C_{30}H_{38}N_2O_6$) C, H, N.

**$C_6H_5CH{=}C(CO\text{-}Ile\text{-}Leu\text{-}OBzl)CH_2CONHOBzl$ (5).** Compound 4 (2 g, 3.7 mmol) was dissolved in DMF (10 mL) containing $HCl{\cdot}H_2N{\cdot}OBzl$ (560 mg, 3.5 mmol) and BOP (1.64 g, 3.7 mmol), and NMM (0.8 mL) was last added to this solution. After 2 h, a saturated sodium bicarbonate solution (50 mL) was added, and the precipitate was collected by filtration, washed with a saturated sodium bicarbonate solution (2 × 50 mL), water, 1 M potassium hydrogen sulfate (2 × 50 mL), and water, and dried over phosphorus pentoxide. After crystallization from ethyl acetate–hexane, a white pure powder was obtained: yield 1.24 g (56%); $R_f$(B) 0.74, $R_f$(C) 0.46; mp 164–166 °C; $[\alpha]_D$ +10.5 (c 1.00, DMF). Anal. ($C_{37}H_{45}N_3O_6$) C, H, N.

**$C_6H_5CH_2CH(CO\text{-}Ile\text{-}Leu\text{-}OH)CH_2CONHOH$ (6, JMV-390).** Compound 5 (1.24 g, 1.97 mmol) was dissolved in 95% ethanol (50 mL) and hydrogenated in the presence of a 10% Pd/C catalyst. After 3 h, no more starting material could be detected by TLC. The catalyst was removed by filtration, and the solvent was concentrated in vacuo. The residue gave a white powder by trituration with $Et_2O$. It was collected by filtration, rinsed several times with $Et_2O$, and dried in vacuo over phosphorus pentoxide: yield 806 mg (91%); $R_f$(J) 0.48–0.62, $R_f$(K) 0.13–0.17. The two diastereoisomers were separated by HPLC on a WL620 SFCC $C_{18}$ column (10 μm, 150 × 22.5) using $NH_4OAc$/MeOH 40:60 as solvent, at a flow of 7 mL/min and detection at 220 nm. Compound **6a** (mp 118–120 °C; $[\alpha]_D$ 1.2 (c 0.75, DMF); m/z 450) eluted first at 9.86 min and compound **6b** (mp 144–146 °C; $[\alpha]_D$ −6.5 (c 0.7, DMF); m/z 450) at 18.56 min. Anal. ($C_{23}H_{35}N_3O_6$) C, H, N for the two diastereoisomers.

**Method B: 2-(Triphenylphosphoranylidene)succinic Anhydride (7).** Compound 7 was prepared as described in the literature.[19] To a solution of $Ph_3P$ (52.46 g, 0.2 mol) in acetone (200 mL) was added dropwise, with stirring, maleic anhydride (19.5 g, 0.2 mol) in acetone (100 mL). The phosphorane began to precipitate during the addition. After filtration, it was washed with cold acetone so that it became colorless, and with $Et_2O$, yield 60 g (83%): $R_f$(B) 0.45, $R_f$(C) 0.23; mp 185–187 °C; $[\alpha]_D$ +0.4 (c 0.96, DMF). Anal. ($C_{22}H_{17}O_3P$) C, H.

**$Ph_3P{=}C(COOCH_3)CH_2COOH$ (8).** The addition product 7 (60 g, 167 mmol) was suspended in methanol (300 mL). After 24 h, the clear solution was concentrated in vacuo and gave, after addition of ethyl acetate (200 mL), a white powder. It was then crystallized from benzene–ethyl acetate, yield 49.2 g (75%): $R_f$(J) 0.65, $R_f$(K) 0.43; mp 150–152 °C; $[\alpha]_D$ +0.2 (c 0.97, DMF). Anal. ($C_{23}H_{21}O_4P$) C, H.

**$C_6H_5CH{=}C(COOH)CH_2COOH$ (1).** Benzaldehyde (13.7 mL, 135 mmol) was added to a benzene solution (100 mL) containing compound 8 (49.2 g, 125 mmol). After 24 h, the mixture was washed with water, the benzylidene compound was extracted with a 1 M NaOH solution (300 mL), and the organic layer was removed. The reaction was stirred at room temperature for 48 h. The mixture was washed with $Et_2O$, and the expected diacid was precipitated on a 1 M potassium hydrogen sulfate solution (>300 mL). It was collected by filtration, washed with water and hexane, and dried over phosphorus pentoxide: yield 21.2 g (76%): $R_f$(J) 0.80, $R_f$(K) 0.32; mp 182–184 °C; $[\alpha]_D$ +0.7 (c 1.05, DMF). Anal. ($C_{11}H_{10}O_4$) C, H.

**$C_6H_5CH{=}C(COOH)CH_2COOEt$ (9).** Compound 1 (21 g, 102 mmol) was dissolved in DME (70 mL), and the reaction mixture cooled down to 0 °C before DCC (21 g, 102 mmol) was added. After 45 min, DCU was eliminated by filtration and the solvent evaporated. Anhydride was dissolved in ethanol (150 mL) and the reaction refluxed at 80 °C for 20 h. The solvent was then evaporated, and 100 mL of ethyl acetate was added. The expected compound was extracted with a saturated sodium bicarbonate solution (7 × 100 mL), the aqueous layers were acidified with 11 N HCl (60 mL), and compound 9 was extracted with 200 mL of ethyl acetate. The organic layer was washed with water, dried over sodium sulfate, and concentrated in vacuo to give a white

powder: yield 10.5 g (44%); $R_f$(B) 0.49, $R_f$(C) 0.40; mp 66–68 °C; $[\alpha]_D$ +0.4 (c 1.06, DMF). Anal. ($C_{13}H_{14}O_4$) C, H.

**HCl·H-Ile-Leu-OtBu (10).** To a solution of HCl·H-Leu-OtBu (447 mg, 2 mmol) in DMF (10 mL) were added Z-Ile-OH (544 mg, 2.05 mmol) and BOP (907 mg, 2.05 mmol) followed by NMM (0.24 mL). After 2 h, the reaction mixture was treated as described for compound **3** to give after precipitation with $Et_2O$–hexane a white powder: yield 765 mg (88%); $R_f$(D) 0.79, $R_f$(E) 0.30; mp 81–83 °C; $[\alpha]_D$ −18.6 (c 0.59, DMF). Anal. ($C_{24}H_{38}N_2O_5$) C, H, N.

It was then dissolved in 95% ethanol (50 mL) containing 0.1 N HCl (2 mmol) and hydrogenated in the presence of a 10% Pd/C catalyst. After 2 h, no more starting material could be detected by TLC. The workup was identical to that of compound **6**, yield 674 mg (100%).

**$C_6H_5CH$=C(CO-Ile-Leu-OtBu)$CH_2COOEt$ (11).** Compound **11** was obtained as described for compound **3** starting from 470 mg of compound **9** (2 mmol), 657 mg of amine **10** (1.95 mmol), 885 mg of BOP (2 mmol), and 0.45 mL of NMM: yield 887 mg (88%); $R_f$(C) 0.80, $R_f$(D) 0.55; mp 104–106 °C; $[\alpha]_D$ +19.6 (c 9.40, DMF). Anal. ($C_{29}H_{44}N_2O_6$) C, H, N.

**$C_6H_5CH$=C(CO-Ile-Leu-OtBu)$CH_2COOH$ (12).** Ethyl ester **11** (887 mg, 1.72 mmol) was saponified in ethanol with 0.86 mL of a 2 N NaOH solution. Potassium hydrogen sulfate (1 M) (10 mL) and ethyl acetate (30 mL) were added after 2 h of stirring. The organic layer was washed with 1 M potassium hydrogen sulfate (30 mL), dried over sodium sulfate, and concentrated in vacuo to give a white powder: yield 840 mg (100%); $R_f$(L) 0.63, $R_f$(C) 0.88, $R_f$(D) 0.46; mp 110–112 °C; $[\alpha]_D$ +13.5 (c 1.10, DMF). Anal. ($C_{27}H_{40}N_2O_6$) C, H, N.

**$C_6H_5CH$=C(CO-Ile-Leu-OtBu)$CH_2CONHOBzl$ (13).** Introduction of the hydroxylamine function was realized as for compound **5** starting from 271 mg of HCl·$H_2$N-OBzl (1.70 mmol), 840 mg of compound **12** (1.72 mmol), 761 mg of BOP (1.72 mmol), and 0.39 mL of NMM: yield 848 mg (84%); $R_f$(C) 0.56, $R_f$(D) 0.22; mp 171–174 °C; $[\alpha]_D$ +4.7 (c 0.97, DMF). Anal. ($C_{34}H_{47}N_3O_6$) C, H, N.

**$C_6H_5CH_2CH$(CO-Ile-Leu-OtBu)$CH_2CONHOH$ (14).** Compound **13** (840 mg, 1.41 mmol) was hydrogenated in the same conditions as for compound **5** and gave after precipitation with $Et_2O$ a white powder: yield 600 mg (84%); $R_f$(K) 0.52, $R_f$(A) 0.52, $R_f$(B) 0.21; mp 123–125 °C; $[\alpha]_D$ −24.5 (c 1.01, DMF). Anal. ($C_{27}H_{43}N_3O_6$) C, H, N.

**$C_6H_5CH_2CH$(CO-Ile-Leu-OH)$CH_2CONHOH$ (6, JMV-390).** Compound **14** (600 mg, 1.18 mmol) was deprotected with trifluoroacetic acid (10 mL). After 60 min at room temperature, the trifluoroacetic acid was removed in vacuo, coevaporated with hexane, and the expected TFA salt precipitated by addition of $Et_2O$. The obtained powder was collected, washed with $Et_2O$, and dried in vacuo over potassium hydroxide: yield 488 mg (92%); $R_f$(J) 0.48–0.62, $R_f$(K) 0.13–0.17. The two diastereoisomers were separated by HPLC on a WL620 SFCC $C_{18}$ column (10 μm, 150 × 22.5) using $NH_4OAc$/MeOH (40:60) as solvent, at a flow of 7 mL/min and detection at 220 nm. Compound **6a** ($m/z$ 450) eluted first at 9.86 min and compound **6b** ($m/z$ 450) at 18.56 min. They have physical characteristics identical with the same compounds prepared according to the scheme A. Anal. ($C_{23}H_{35}N_3O_6$) C, H, N for the two diastereoisomers.

**"Dipeptide and Tripeptide Models". H-Phe-Ile-OH (15).** Boc-Phe-Ile-OtBu was prepared as described for compound **3** starting from 172 mg of Boc-Phe-OH (0.65 mmol), 134 mg of HCl·H-Ile-OtBu (0.60 mmol), 287 mg of BOP (0.65 mmol), and 0.145 mL of NMM: yield 260 mg (100%); $R_f$(D) 0.69, $R_f$(E) 0.43; mp 108–109 °C; $[\alpha]_D$ −6.8 (c 0.44, DMF). Anal. ($C_{24}H_{38}N_2O_5$) C, H, N.

The protected dipeptide (240 mg, 0.55 mmol) was treated with trifluoroacetic acid as described for compound **6**: yield 200 mg (93%); $R_f$(M) 0.72, $R_f$(N) 0.59; mp 67–70 °C; $[\alpha]_D$ +18.0 (c 0.5, DMF).

**H-D-Phe-Ile-OH (16).** Boc-D-Phe-Ile-OtBu was prepared as described for compound **3** starting from 172 mg of Boc-D-Phe-OH (0.65 mmol), 134 mg of HCl·H-Ile-OtBu (0.60 mmol), 287 mg of BOP (0.65 mmol), and 0.145 mL of NMM: yield 250 mg of Boc-D-Phe-Ile-OtBu (96%); $R_f$(D) 0.61, $R_f$(E) 0.46; mp 85–87 °C; $[\alpha]_D$ +23.5 (c 0.51, DMF). Anal. ($C_{24}H_{38}N_2O_5$) C, H, N. After deprotection of **16** by TFA as described previously, 170 mg of **18** (79%) were obtained: $R_f$(M) 0.44, $R_f$(N) 0.19; mp 194–196 °C; $[\alpha]_D$ −17.1 (c 0.48, DMF).

**H-Phe-Ile-Leu-OH (17).** Z-Phe-Ile-Leu-OBzl was prepared as described for compound **3** starting from 195 mg of Z-Phe-OH (0.65 mmol), 269 mg of TFA H-Ile-Leu-OBzl (described for the synthesis of compound **3**) (0.60 mmol), 287 mg of BOP (0.65 mmol), and 0.145 mL of NMM: yield 370 mg (100%); $R_f$(C) 0.64, $R_f$(D) 0.22; mp 162–164 °C; $[\alpha]_D$ −16.7 (c 0.33, DMF). Anal. ($C_{36}H_{45}N_3O_6$) C, H, N.

The protected tripeptide (340 mg, 0.55 mmol) was hydrogenated in the same conditions as for compound **6**: yield 200 mg (93%); $R_f$(M) 0.58, $R_f$(N) 0.49; mp 176–178 °C; $[\alpha]_D$ −31.3 (c 0.40, DMF).

**H-D-Phe-Ile-Leu-OH (18).** Z-D-Phe-Ile-Leu-OBzl was prepared as described for compound **3** starting from 195 mg of Z-D-Phe-OH (0.65 mmol), 269 mg of TFA·H-Ile-Leu-OBzl (0.60 mmol), 287 mg of BOP (0.65 mmol), and 0.145 mL of NMM: yield 360 mg of Z-D-Phe-Ile-Leu-OBzl (97%); $R_f$(C) 0.65, $R_f$(D) 0.28; mp 173–175 °C; $[\alpha]_D$ −3.0 (c 0.34, DMF). Anal. ($C_{36}H_{45}N_3O_6$) C, H, N. Deprotection of **18** by hydrogenolysis in the presence of 10% Pd/C as catalyst yielded 210 mg of **18** (100%): $R_f$(M) 0.54, $R_f$(N) 0.39; mp 178–180 °C; $[\alpha]_D$ −18.6 (c 0.59, DMF).

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