# New Kelatorphan-Related Inhibitors of Enkephalin Metabolism: Improved Antinociceptive Properties

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In order to improve the in vivo protection of enkephalins from enzymatic degradation, a new series of inhibitors derived from kelatorphan [$HONHCOCH_2CH(CH_2Ph)CONHCH(CH_3)COOH$], the first-described complete inhibitor of enkephalin metabolism, were designed by modification of the C-terminal amino acid. The progressive lengthening of the chain of this residue shows that a β-alanine seems to be the best basic model for the conception of such types of compounds. On the other hand, the methylation of the amide bond, which is well accepted by aminopeptidase N (EC 3.4.11.2) and dipeptidylaminopeptidase, induced a significant loss of affinity for neutral endopeptidase -24.11. Starting from these data, compounds containing a variously substituted β-alanine residue and corresponding to the general formula $HONHCOCH_2CH(CH_2Ph)CONHCH(R_1)CH(R_2)COOH$ were synthesized. All these molecules inhibit neutral endopeptidase -24.11 and dipeptidylaminopeptidase in the nanomolar range, and those containing an aromatic chain (compound **7A**, $R_1 = CH_2Ph, R_2 = H$, and compound **8A**, $R^1 = Ph$, $R_2 = H$) inhibit the biologically relevant aminopeptidase N, with $IC_{50}$'s around $10^{-8}$ M. Intracerebroventricular injection in mice of these multienzyme inhibitors produced an efficient and naloxone-reversible analgesic response (hot plate test): compounds **7A** and **8A** were shown to be more potent than kelatorphan in increasing the jump latency time, in agreement with their in vitro properties, and these new compounds were found to increase the forepaw lick latency, a reflex considered as a typical morphine response.

The amplitude of the pharmacological responses[1-4] elicited by kelatorphan [$HONHCOCH_2CH(CH_2Ph)CONHCH[CH_3)COOH$], which protects the endogenous enkephalins (Tyr-Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe-Leu) from enzymatic inactivation, clearly demonstrates the large interest in designing multipeptidase inhibitors.[5] Kelatorphan inhibits the three metallopeptidases involved in the in vitro enkephalin metabolism through interaction with the $S'_1$ and $S'_2$ subsites of the active sites of neutral endopeptidase -24.11 (NEP), dipeptidylaminopeptidase (DAP), and aminopeptidase N (EC 3.4.11.2) (APN), which cleave the $Gly^3$–$Phe^4$, $Gly^2$–$Gly^3$, and $Tyr^1$–$Gly^2$ bonds of enkephalins, respectively.[6] As expected from a complete in vivo inhibition of enkephalin metabolism, kelatorphan was shown to produce stronger antinociceptive effects than those induced by the NEP inhibitor thiorphan,[7] by the nonselective aminopeptidase blocker bestatin,[8] or by the association of these two molecules.[5]

Kelatorphan displays inhibitory potencies for NEP and DAP in the nanomolar range, while its efficiency is about 3 orders of magnitude lower on aminopeptidase N. However, its selectivity for this latter enzyme is more than 100 times higher than that of bestatin.[9] Therefore, in order to improve the affinity for APN without loss of inhibitory potencies for NEP and DAP, we have investigated the influence of various chemical modifications of kelatorphan on the recognition of the active sites of the three enzymes. Three modifications were introduced into the C-terminal moiety of kelatorphan: (i) replacement of the α-amino acid by an unsubstituted β-, γ-, or δ-amino acid in order to induce an increased flexibility of the C-terminal residue and a displacement of the carboxylate group, (ii) methylation of the amide bond, and (iii) increase in the size and the nature of the P-'2 moiety in the series of inhibitors containing a C-terminal β-amino acid.

Highly efficient inhibitors were obtained, and their ability to protect endogenous enkephalins was evaluated in vivo by using two different analgesic tests.

## Results

**Chemistry.** The various inhibitors were synthesized by using a strategy similar to that previously described for the preparation of kelatorphan and analogues, consisting of two main steps with benzylidenesuccinic acid as the initial precursor (Figure 1). This compound was chosen in order to prevent isomerization between the α and β positions observed with the saturated analogue, caused by the nonregioselective opening of the succinimide intermediate formed during the synthesis.[11]

Initially, the simplest method appeared to be a condensation of a *O*-benzylhydroxylamine with the β-carboxylate of the benzylidenesuccinyl moiety, in order to obtain the 4-[(benzyloxy)amino]-4-oxo-2-benzylidenebutanoic acid as a common precursor for the synthesis of all the inhibitors. However, this approach was unsuccessful as the latter derivative spontaneously gave the corresponding highly stable *N*-(benzyloxy)succinimide which cannot undergo any further condensation, whatever the

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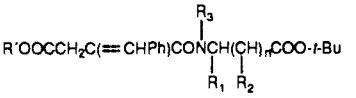
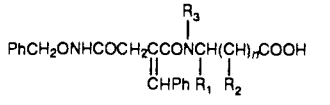
Table I. Physical Properties of Intermediate Compounds

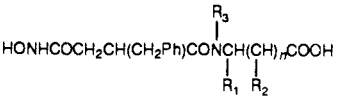


| | | | | R′ = $C_2H_5$ | | | R′ = H | | | R′ = $NHOCH_2Ph$ | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $R_1$ | $R_2$ | $R_3$ | $n$ | no. | yield, %[a] | $R_f$[a] | no. | yield, % | $R_f$ | no. | yield, % | $R_f$ | mp, °C |
| H | H | H | 1 | **1a** | 99.6 | 0.66 (A) | **2a** | 46.5 | 0.58 (A) | **3a** | 18.4 | 0.46 (C) | 121 |
| $CH_3$ (*RS*) | H | H | 1 | **1b** | 94 | 0.70 (C) | **2b** | 55.6 | 0.62 (A) | **3b** | 61.4 | 0.57 (C) | 105 |
| $CH_2Ph$ (*S*) | H | H | 1 | **1c** | 87.8 | 0.80 (A) | **2c** | 51.5 | 0.41 (C) | **3c** | 61.4 | 0.75 (C) | 60 |
| Ph (*S*) | H | H | 1 | **1d** | 95 | 0.71 (C) | **2d** | 79 | 0.67 (A) | **3d** | 73 | 0.66 (C) | oil |
| H | $CH_3$(*RS*) | H | 1 | **1e** | 98 | 0.76 (C) | **2e** | 92.4 | 0.67 (A) | **3e** | 22.4 | 0.68 (C) | 110 |
| H | $CH_2Ph$ (*RS*) | H | 1 | **1f** | 100 | 0.80 (C) | **2f** | 87 | 0.66 (A) | **3f** | 61.6 | 0.71 (C) | 65–67 |
| $CH_3$ | $CH_3$ | H | 1 | **1g** | 68.5 | 0.69 (C) | **2g** | 62.2 | 0.66 (A) | **3g** | 82.7 | 0.56 (C) | 85 |
| H | H | H | 2 | **1h** | 99.8 | 0.75 (C) | **2h** | 76.4 | 0.33 (C) | **3h** | 35.4 | 0.64 (C) | 146–148 |
| H | H | H | 3 | **1i** | 98.7 | 0.68 (C) | **2i** | 58.1 | 0.71 (A) | **3i** | 55.3 | 0.61 (C) | 133 |
| $CH_2Ph$ (*S*) | | $CH_3$ | 0 | **1j** | 100 | 0.84 (C) | **2j** | 49.5 | 0.40 (C) | **3j** | 31.0 | 0.56 (B) | oil |

[a] See Experimental Section for reaction procedures and chromatographic solvent systems.

Table II. Physical Properties of Intermediate and Final Compounds





| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $R_1$ | $R_2$ | $R_3$ | $n$ | no. | yield, %[a] | $R_f$[a] | mp, °C | no. | yield, % | $R_f$ | mp, °C | $t_R$(HPLC)[b] |
| H | H | H | 1 | **4a** | 63 | 0.51 (A) | 122 | **5** | 58 | 0.33 (D) | 148–150 | 5′32″ |
| $CH_3$ (*RS*) | H | H | 1 | **4b** | 62.2 | 0.65 (A) | 134 | **6** | 90 | 0.20 (A) | 114–116 | 5′56″, 8′5″ |
| $CH_2Ph$ (*S*) | H | H | 1 | **4c** | 46 | 0.62 (A) | 110 | **7** | 94.5 | A: 0.33 (A)<br>B: 0.27 (A) | **A**: 196<br>**B**: 190 | **A**: 19′35″<br>**B**: 48′18″ |
| Ph (*S*) | H | H | 1 | **4d** | 40.4 | 0.66 (A) | 105–107 | **8** | 76 | **A**: 0.33 (A)<br>B: 0.22 (A) | **A**: 200<br>**B**: 206 | **A**: 14′39″<br>**B**: 33′10″ |
| H | $CH_3$ (*RS*) | H | 1 | **4e** | 43.4 | 0.62 (A) | 110–112 | **9** | 53.6 | 0.30 (E) | 88 | 6′53″, 7′29″ |
| H | $CH_2Ph$ (*RS*) | H | 1 | **4f** | 74 | 0.65 (A) | 144 | **10** | 97 | 0.30 (A) | 84–86 | 36′35″, 41′32″ |
| $CH_3$ | $CH_3$ | H | 1 | **4g** | 61.2 | 0.21 (C) | 155–156 | **11** | 100 | **A**: 0.31 (G)<br>**B**: 0.20 (G) | A: 98<br>**B**: 199–200 | **A**: 8′38″<br>**B**: 10′38″ |
| H | H | H | 2 | **4h** | 81 | 0.63 (A) | 145–147 | **12** | 93 | 0.36 (D) | 218–220 | 6′7″ |
| H | H | H | 3 | **4i** | 80.7 | 0.2 (C) | 133 | **13** | 87 | 0.52 (A) | <50 | 7′25″ |
| $CH_2Ph$ (*S*) | | $CH_3$ | 0 | | | | | **14** | 63.7 | 0.27 (A) | 140–143 | 13′24″ |

[a] See Experimental Section for reaction procedures and chromatographic solvent systems. [b] Retention times corresponding to HPLC experiments performed with a Nucleosil $C_8$ column using as the solvents the mixture TFA (0.07%)/$CH_3CN$ (80:20).

method used (DCC/HOBt, DCC/TEA,[12] active ester, acyl chloride, etc). Consequently, the synthesis was performed by amidification of the carboxylate group by various β-amino esters, followed by introduction of the hydroxyamino group on the free β-carboxyl group. The reduction of the benzylidene moiety was carried out during the last deprotecting step of the synthesis (Figure 1). For the *N*-methyl compound, the *tert*-butyl ester was removed by boron tris(trifluoroacetate) (BTFA)[13] with a simultaneous deprotection of the hydroxyamino group. The yields and the physical properties of these different intermediates and final compounds are summarized in Tables I and II

The β-amino acids that were not commercially available were synthesized following two general procedures: the β-substituted β-amino acids ($R_1 \neq H$, $R_2 = H$) were obtained from the corresponding α-amino acids with a full retention of configuration using the Arndt–Eistert method.[14] The α-substituted β-amino acids ($R_1 = H$, $R_2 \neq H$) and the α,β-disubstituted β-amino acid ($R_1 = R_2 = CH_3$) were prepared by nucleophilic addition of an excess of hydroxylamine on the corresponding α,β-unsaturated acid.[15] In this latter case, the β-amino acids were obtained as racemates. The *tert*-butyl esters could be prepared by the classical acid-catalyzed isobutylene addition method.

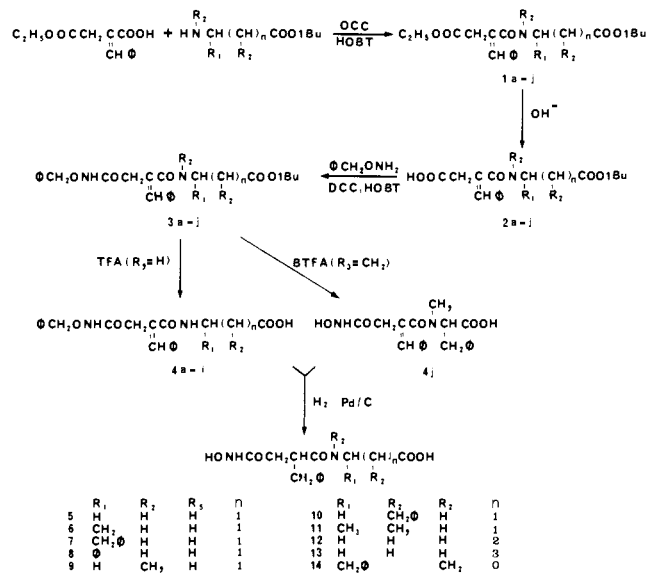


Figure 1. Scheme for the synthesis of hydroxamate inhibitors.

However, in order to improve the yield and to avoid problems of solubility, they were synthesized through the action of *N*,*N*-dimethylformamide di-*tert*-butyl acetal on the various *N*-(benzyloxycarbonyl)-protected amino acids,[16] followed by hydrogenolysis.

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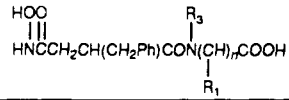
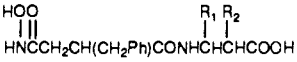
**Table III.** Inhibitory Potencies of Hydroxamate Peptide Analogues on the Three Enkephalin-Degrading Enzyme Activities



| no. | $R_3$ | $R_1$ | $n$ | stereochemistry | $IC_{50}$, nM[a] NEP[b] | APN[c] | DAP[d] |
|---|---|---|---|---|---|---|---|
| A | H | H | 1 | (*R*) + (*S*) | 1.4 ± 0.4 | 2000 ± 200 | 21 ± 5 |
| 5 | H | H | 2 | (*R*) + (*S*) | 2.4 ± 0.4 | 670 ± 70 | 36 ± 4 |
| 12 | H | H | 3 | (*R*) + (*S*) | 27.5 ± 2.5 | 1500 ± 500 | 150 ± 50 |
| 13 | H | H | 4 | (*R*) + (*S*) | 51.5 ± 3.5 | 500 ± 50 | 250 ± 50 |
| B | H | $CH_2Ph$ | 1 | (*RS*) | 2.5 ± 0.7 | 130 ± 10 | 2.5 ± 0.6 |
| 14 | $CH_3$ | $CH_2Ph$ | 1 | (*RS*) + (*SS*) | 150 ± 50 | 120 ± 10 | 2.3 ± 0.3 |



| no. | $R_1$ | $R_2$ | stereochemistry | $IC_{50}$, nM[a] NEP[b] | APN[c] | DAP[d] |
|---|---|---|---|---|---|---|
| C | $CH_3$ | | (*RS*) | 1.7 ± 0.6 | 350 ± 50 | 0.9 ± 0.1 |
| 5 | H | H | (*R*) + (*S*) | 2.4 ± 0.4 | 670 ± 70 | 36 ± 4 |
| 6 | $CH_3$ | H | (*RS*) (*SS*) (*RS*) (*RR*) | 2.0 ± 0.1 | 445 ± 50 | 10 ± 1 |
| 7A | $CH_2Ph$ | H | (*RS*) | 3.0 ± 0.5 | 22.5 ± 5 | 0.46 ± 0.06 |
| 7B | $CH_2Ph$ | H | (*SS*) | 7.6 ± 1.5 | 1430 ± 70 | 28.5 ± 3.5 |
| 8A | Ph | H | (*RS*) | 2.9 ± 0.9 | 32.5 ± 4.5 | 2.6 ± 0.1 |
| 8B | Ph | H | (*SS*) | 3.0 ± 0.3 | 400 ± 30 | 7.2 ± 0.4 |
| 9 | H | $CH_3$ | (*RS*) (*SS*) (*SR*) (*RR*) | 2.3 ± 0.3 | 360 ± 30 | 23 ± 5 |
| 10 | H | $CH_2Ph$ | (*RS*) (*SS*) (*SR*) (*RR*) | 1.8 ± 0.2 | 71.5 ± 6.5 | 2.5 ± 0.6 |
| 11A | $CH_3$ | $CH_3$ | (*RRS* + *SSR*) or (*SRS* + *RSR*)[e] | 26.5 ± 4.5 | nd[f] | 10.5 ± 1.5 |
| 11B | $CH_3$ | $CH_3$ | or (*RRR* + *SSS*) or (*SRR* + *RSS*) | 13 ± 3 | nd | 17.5 ± 3.5 |

[a] Values are the mean ± SEM from three independent experiments computed by log profit of five inhibitor concentrations. [b] Concentration inhibiting 50% of NEP activity with 20 nM [$^3$H]-D-Ala$^2$-Leu-enkephalin as substrate. [c] Concentration inhibiting 50% of APN activity with 10 nM [$^3$H]Leu-enkephaltin as substrate. [d] Concentration inhibiting 50% of DAP activity with 10 nM [$^3$H]Leu-enkephalin as substrate. [e] Mixture of two enantiomers, which was confirmed by NMR, but the relative configuration was not determined. [f] nd = not determined.

The various inhibitors synthesized contained from one to three asymmetric centers. Compounds **5**, **12**, and **13**, having only one asymmetric carbon at the level of the benzylsuccinic moiety, were obtained as racemates and were not resolved. Compounds **7** and **8**, which were synthesized with a β-amino acid of well-defined stereochemistry (*S* configuration), were obtained as a mixture of two diastereoisomers, which were separated on a silica gel column. The absolute configuration of each isomer was tentatively determined by HPLC taking into account their retention time on a $C_{18}$ reverse-phase column.[17] As previously shown, it may be assumed that a dipeptide analogue that has the same spatial orientation of the lateral chains as a natural dipeptide (LL isomer) has a shorter retention time than its diastereoisomer (LD or DL). Consequently, for compounds **7** and **8**, the first-eluted isomers, designated **7A** and **8A**, were assumed to have the *RS* configuration (corresponding to a natural dipeptide) and the second isomers, **7B** and **8B**, to have the *SS* configuration.

The β-amino acids used for the synthesis of compounds **6** and **9–11** being racemates, these inhibitors were prepared as a mixture of the four stereoisomers. Only compound **11** was separated by chromatography into two compounds (**11A** and **11B**), whose configuration was not determined. The N-methylated compound **14** was obtained as a mixture of diastereoisomers and cis–trans isomers, and these various forms were not separated.

**Inhibitory Potencies of Hydroxamates on the Three Enkephalin-Degrading Enzyme Activities.** The inhibitory potencies of the various hydroxamates were measured as described under Experimental Section by using the rabbit kidney NEP, the pig kidney APN, and the rat brain DAP. The results are summarized in Table III.

As compared to compound A, designated HACBO-Gly,[10] the replacement of the glycine residue by a β-alanine (compound **5**) does not significantly change the inhibitory potency toward NEP and DAP. The lengthening of the chain in compounds **12** and **13** induced a progressive increase of $IC_{50}$'s on both enzymes. This effect was more marked on NEP than on DAP since the $IC_{50}$ ratio between compounds **13** and **5** is about 40 for the neutral endopeptidase and only 10 for DAP. Side-chain elongation had varying effects on APN inhibition. The replacement of the glycine moiety in compound A by a C-terminal β-alanine (compound **5**) or a β-aminovaleric acid (compound **13**) produced respectively a 3- and 4-fold increased potency on APN. Conversely, compound **12** with three methylene groups inhibited APN with approximately the same $IC_{50}$ as compound **A**.

The comparative activities of compound B and its corresponding N-methylated analogue (compound **14**) show that, as previously reported with thiol inhibitors,[18] introduction of a substituent in the peptide bond induces a large decrease in NEP recognition. In contrast, this structural modification does not influence the inhibitory potency on APN and DAP. The activities of compounds **5–11**, containing diversely substituted β-amino acids, are reported in Table III. The inhibition of NEP by compounds **5–10** was not significantly different from that observed with kelatorphan (compound C). All the $IC_{50}$'s were around 2 nM whatever the position and the size of the substituent introduced on the β-amino acid. Furthermore, inhibition

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Table IV. Analgesic Effects of Various Inhibitors in the Hot Plate Test in Mice

| compd[a] | latency time, s[b] | % analgesia |
|---|---|---|
| control | 94.4 ± 6.3 | |
| 5 | 125.3 ± 14.0* | 21.5 ± 9.6 |
| 6 | 124.2 ± 13.4* | 20.5 ± 9.2 |
| 7A | 193.7 ± 15.3*** | 68.2 ± 10.5 |
| 8A | 184.8 ± 17.5** | 62.1 ± 12 |
| 9 | 162.3 ± 17.3*** | 46.6 ± 11.9 |
| 10 | 151.8 ± 18.3* | 39.4 ± 12.6 |
| kelatorphan | 167.2 ± 8.9*** | 50.0 ± 6.1 |

[a] All the compounds were injected icv at a dose of 50 μg in 10 μL of saline ($n$ = 10), temperature = 55 ± 0.5 °C. [b] Statistical comparison with control: (*) $p < 5\%$; (**) $p < 2\%$; (***) $p < 0.5\%$.

of NEP by **7A**, as compared to **7B**, or **8A** as compared to **8B**, indicates that the stereochemistry of the $P'_1$ residue does not influence their activity. Similarly, taking into account that compounds **6**, **9**, and **10** are mixtures of the four possible stereoisomers and that their inhibitory potencies are in the same range as that of kelatorphan, which is optically pure, it may be assumed that the absolute configuration of the C-terminal-substituted β-amino acid is not a critical parameter for NEP recognition. Compounds **11A** and **11B**, which contain a α,β-dimethyl-β-alanine, were somewhat less active, with $IC_{50}$'s in the 10–20 nM range.

Unlike NEP, the inhibition of DAP by compounds **5–11** emphasizes the importance of the size and the position of the substituent for the recognition of this peptidase. The presence of a β-alanine in the $P'_2$ position decreases significantly the inhibitory potencies of **5** and **6** as compared to compounds **C** and **A**, respectively. However, the introduction of various substituents on the β-alanine moiety improves the affinity (compounds **7–10**). Furthermore, it can be observed that in all cases a benzyl group is preferred to a methyl group (compound **10** versus **9** or compound **7** versus **6**) and a substituent in the α position relative to the amide bond is preferred to a substituent in the β position (compound **6** versus **9** or compound **7** versus **10**). the absolute configuration of the $P'_1$ residue modulates the DAP affinity of the inhibitors as exemplified with compounds **7A** and **7B**. The data reported in Table III show that the $S'_1$ and $S'_2$ subsites of APN could have the same structural characteristics as those present in the corresponding subsites of DAP. The two most potent APN inhibitors (compounds **7A** and **8A**) contain an aromatic side chain in the α position relative to the amide bond ($IC_{50}$'s in the 20–30 nM range). As in the case of DAP, the absolute configuration of the $P'_1$ residue (compound **7A** versus **7B** or **8A** versus **8B**) leads to decreased affinity for APN.

**Analgesic Activity of the Hydroxamate-Containing Inhibitors.** The antinociceptive properties of the most potent inhibitors were investigated on the hot plate test in mice. These compounds were administered by an intracerebroventricular route in order to minimize possible differences in their pharmacokinetic properties. In a preliminary screening their effects on the jump latency time were compared at a single dose (50 μg per mouse). As observed in Table IV, the six compounds can be divided into three groups, the least efficient (compounds **5** and **6**) giving about 20% analgesia, the second corresponding to compounds **9** and **10**, characterized by antinociceptive responses of around 40%, and finally compounds **7A** and **8A**, which gave more than 60% analgesia.

Following these preliminary results, compounds **7A** and **8A** were studied in more detail and dose–response curves were carried out by using the jump and the forepaw lick latency tests (Figures 2 and 3). Both compounds produced

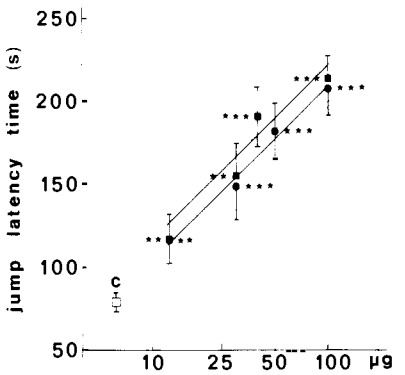


**Figure 2.** Analgesic effects of compounds **7A** (circles) and **8A** (squares) in the hot plate jump test: dose–response curve. The compounds were injected icv at the indicated doses in a constant volume (10 μL) of saline ($n$ = 10). Temperature = 55 ± 0.50 °C. Cutoff time 240 s. (**) $p < 2\%$; (***) $p < 0.5\%$.

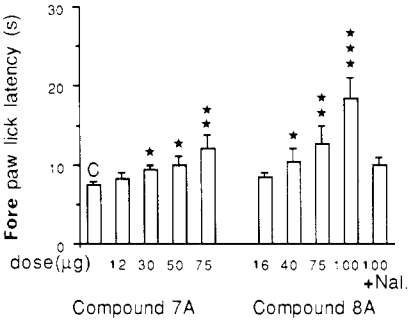


**Figure 3.** Analgesic effects of compounds **7A** and **8A** in the hot plate licking test. The compounds were injected icv at the indicated doses in a constant volume (10 μL) of saline ($n$ = 10). Naloxone (Nal, 1 mg/kg, sc) was injected 10 min prior injection of compound **8A**. Temperature = 55 ± 0.5 °C. Cutoff time 30 s. (C) control; (*) $p < 5\%$; (**) $p < 2\%$; (***) $p < 0.5\%$.

significant antinociceptive responses on the jump escape, even with the lowest concentration used, 12.5 μg, and at doses of 75 μg, more than 80% analgesia was obtained. A pain-suppressive effect was also observed with the paw lick test, but this was significant only for doses greater than 30 μg. The jump escape and the forepaw lick effects were prevented by prior administration of the opioid receptor antagonist naloxone (1 mg/kg, sc).

## Discussion

The chemical modifications of the C-terminal moiety of inhibitors belonging to the kelatorphan series yield interesting structural data on the active site of the three metallopeptidases involved in enkephalin metabolism.

The progressive lengthening of the C-terminal residue was carried out to study the putative stabilizing interaction involving the carboxylate group of the inhibitor and a positively charged or H bond donor residue located in the active site of the three metallopeptidases. As observed in Table III, the introduction of an additional methylene group in compound A, leading to compound **5**, did not change the inhibitory potency for NEP or DAP and slightly increased APN recognition. Further elongation produced a loss of affinity for NEP and DAP by factors of 25 and 10, respectively, while a small improvement of APN inhibition was obtained with compounds **5** and **13** characterized by a two- and four-methylene-containing C-terminal moiety, respectively. In all cases, the variations in the $IC_{50}$'s were relatively slight and therefore are unlikely to correspond to the loss of the critical ionic interaction that would occur if the enzyme was a true carboxypeptidase.[19] This is not unexpected since none of the

enzymes studied belong to this well-characterized group of metallopeptidases. Interestingly, similar slight modification of activity was observed with thermolysin, a bacterial endopeptidase used as a model of the active site of NEP.[18,20] Indeed the $IC_{50}$'s on this enzyme were as follows: compound A, $2.08 \times 10^{-5}$ M; compound **5** $3.66 \times 10^{-4}$ M; compound **12**, $1.28 \times 10^{-4}$; compound **13**, $3.76 \times 10^{-6}$ M. Nevertheless, these data indicate that a $\beta$-alanine seems to be the better basic model for the conception of highly potent and complete inhibitors of enkephalin metabolism. A similar result was obtained with the series of NEP inhibitors containing a carboxylic group as the Zn-coordinating agent.[21]

The second modification involved the amide bond of the inhibitor. The comparison of the inhibitory potency of compound B and its N-methylated analogue **14** shows a 60-fold decrease in the recognition of NEP. This effect confirms the previously reported importance[18] of the hydrogen bond involving the NH of the inhibitor amide group and an appropriate acceptor group present in the NEP active site. If the large analogies between the active sites of NEP and thermolysin, a peptidase whose tridimensional structure has been determined by crystallographic analysis,[22] are taken into account, then this acceptor group could correspond to the carbonyl of the amide group of the lateral chain of an Asn residue. Interestingly, the presence of an *N*-methyl group in **14** is well accepted by both the APN and DAP, showing a significant difference between the active sites of these two peptidases and that of NEP. Another metallopeptidase, angiotensin converting enzyme (ACE), was also shown to strongly interact with peptides and related inhibitors containing an N-substituted amino acid, or an imino acid such as a proline, as the $P'_2$ residue.[23] The N-methylation may be therefore a useful means of obtaining inhibitors selective for DAP and APN, as compared to NEP, in the series of hydroxamate-containing peptides.

The third modification carried out was the introduction of various substituents on the C-terminal $\beta$-alanine moiety of compound **5**. Various studies have shown that both APN and DAP have hydrophobic $S'_2$ subsites,[24,25] and the presence of well-positioned lipophilic chains should lead to a better inhibition of these peptidases. The $IC_{50}$ values reported in Table III for compounds **5–10** are in the nanomolar range and indicate that NEP is insensitive to the presence, the nature, and the position of the substituent. These data are in agreement with those reported for various retroinhibitors[26] containing the same type of side chains and confirm that NEP possesses a hydrophobic but not structurally stringent $S'_2$ subsite. Nevertheless, the 10 times lower affinity for NEP elicited by compound **11** shows that the NEP $S'_2$ subsite is not able to perfectly accommodate the two methyl groups together. Consequently, it may be hypothesized that the greater potency of the monosubstituted derivatives is due to the flexibility of the methylene group, which improves the fit of the side chain in the $S'_2$ subsite. In contrast to NEP, inhibition of DAP is significantly modified by the position and the nature of the substituent borne by the $\beta$-alanine moiety. The highest inhibitory potency ($IC_{50}$ = 0.46 nM) was obtained with compound **7A**, which contains an (*S*)-$\beta$-phenylalanine as the C-terminal residue. This confirms the presence of a hydrophobic $S'_2$ subsite in this peptidase, which seems to interact preferentially with aromatic residues.

Another interesting result concerns the significantly increased inhibition of APN, achieved by introducing an aromatic chain (a benzyl or phenyl group) on the $C_3$ carbon of the $\beta$-alanine. Indeed, the inhibitory potency of compounds **7A** and **8A** ($IC_{50}$ = 22.5 and 32.5 nM, respectively) is in the same range as that of leucinethiol ($IC_{50}$ = 20 nM), the best inhibitor of aminopeptidase N described so far.[27]

The analgesic potency of the inhibitors **7A** and **8A** has been assessed with the hot plate test by measurement of two parameters: the jump latency time and the paw lick. For the jump escape, the great majority of mice reached the cutoff time chosen for the experiment (240 s) at a dose of 75 $\mu$g. This effect is significantly greater than that observed with kelatorphan, for which the maximum effect was shown to culminate around 200 s (75% analgesia).[10] It can be noticed that on this test similar analgesic responses were obtained for morphine and compounds **7A** and **8A** with concentrations of the inhibitors only about 50 times higher than that of the alkaloid (C. Schmidt et al., in preparation). Given the similar inhibitory potencies of kelatorphan and compounds **7A** and **8A** for NEP, the better antinociceptive properties of the two latter inhibitors is likely correlated with their better affinity for APN.

Even more interesting is the observation of a significant effect of **7A** and **8A** on the hind paw lick, which is considered as a typical index of opioid-induced analgesia.[28] Conflicting data are reported in the literature about the efficiency of enkephalin-degrading enzyme inhibitors on this test. When a highly pain-sensitive strain of mice was used, a significant increase in the lick latency was described by Scott et al.[29] and Mendelsohn et al.[30] with NEP inhibitors such as thiorphan and analogues. Conversely, thiorphan, bestatin, or an association of these two has generally been found to be inefficient on this test as well as on the tail withdrawal and tail flick latency,[31-33] with nonselected series of mice. From these data, it has been proposed[33] that inhibitors would be active only on tests where naloxone has pronociceptive effects and that the enkephalins are involved in the control of only some nociceptive responses. Under the conditions used in this study (male Swiss mice, hot plate 55 ± 0.5 °C), thiorphan and *retro*-thiorphan were found to be efficient on the escape latency but inactive on the forepaw lick, in agreement

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with previous reports.[7,18] As already shown with morphine, a larger concentration was required to obtain antinociceptive responses on the lick time. Therefore, it may be assumed that there is a significant difference in the sensitivity of both tests to the stimulation of opioid receptors by endogenous enkephalins (when inhibitors were used) or by synthetic opiates (when morphine is used). This may explain why inhibitors such as thiorphan and bestatin, which induce relatively modest analgesic responses on the jump latency time, were unable to produce a significant effect on the lick time, even when coadministered. In contrast, kelatorphan at high dose (>80 μg)[34] and compounds **7A** and **8A**, which produce very efficient antinociceptive responses on the jump escape, were found active in the forepaw lick.

Finally, even if the neutral endopeptidase and aminopeptidase N are implicated in the breakdown of other neuropeptides, the pharmacological effects obtained after icv administration in mice of compounds **7A** and **8A** indicate that these inhibitors protect very efficiently endogenous enkephalins from their in vivo degradation. Indeed, the analgesic responses are obtained with the inhibitor alone and do not require a coadministration of exogenous enkephalins. Moreover, the dose-dependent antinociceptive responses observed indicate that the amplitude of the effect is correlated with the concentration of the inhibitor and consequently with the enkephalin level in the brain. Finally, the antinociceptive effect is completely prevented by naloxone an antagonist of opioid receptors.

Our results clearly indicate that the endogenous opioid peptides, enkephalins, when efficiently protected from degrading enzymes, are able to produce almost all the analgesic effects elicited by morphine (C. Schmidt et al., in preparation). If efficient inhibitors are capable of mimicking morphine activity, it now has to be determined whether they are devoid of its addictive properties. Experiments are currently been performed to answer this crucial question.

## Experimental Section

[$^3$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 Nucléaire (CEN, Saclay, France).

**Purification of Enkephalin-Degrading Enzymes.** Neutral endopeptidase -24.11 was purified to homogeneity from rabbit kidney in one step as previously described.[35] The membrane-bound dipeptidylaminopeptidase was purified from rat brain by a slight modification of reported methods.[36] Aminopeptidase N was from Boehringer (Meylan, France) and used without further purification.

**Determination of Inhibitory Potency.** $IC_{50}$ values were determined as previously described in detail.[37] Briefly, NEP (at a final concentration of 1 pmol/100 μL) was preincubated for 15 min at 25 °C with or without increasing concentrations of inhibitor in a total volume of 100 μL in 50 mM Tris-HCl buffer. [$^3$H]-D-Ala$^2$-Leu-enkephalin ($K_m$ = 30 μM) was added to a final concentration of 20 nM, and the reaction was stopped after 30 min by adding 10 μL of 0.5 M HCl. The dipeptidylaminopeptidase and the aminopeptidase activities were measured by the same procedure using [$^3$H]Leu-enkephalin, at a final concentration of 10 nM, as substrate ($K_m$ = 25 μM for dipeptidylaminopeptidase, and $K_m$ = 50 μM for aminopeptidase). The tritiated metabolites formed were separated on polystyrene beads.

**Analgesic Tests.** The analgesic activity of the various inhibitors was assessed by using the mouse hot plate test at 55 ± 0.5 °C.[38] Drugs were administered intracerebroventricularly to male Swiss mice (23–25 g, Deprê, France) in a volume of 10 μL according to the method of Haley and McCormick.[39] The jump latency and forepaw lick times were measured 15 min after drug administration (cutoff time 240 s for jump escape and 30 s for forepaw lick). The results were compared with those of vehicle-treated mice (controls) by analysis of variance and by Bonferoni test. The antinociceptive responses were also evaluated as a percentage of analgesia. The 100% of analgesia is represented by the difference between the cutoff time chosen (240 s) and the latency time of the control mice. A percentage of analgesia is calculated for each dose of inhibitor injected by the ratio:

$$\%\ \text{analgesia} = \frac{\text{latency time (experimental)} - \text{latency time (control)}}{100\%\ \text{analgesia}}$$

**Chemistry.** The protected α-amino acids were from Bachem (Bubendorf, Switzerland); *N*,*N*-dimethylformamide di-*tert*-butyl acetal was from Fluka (Buchs, Switzerland). *trans*-2-Methyl-2-butenoic acid, 4-aminobutyric acid, 5-aminovaleric acid, DL-3-amino-3-phenylpropionic acid, DL-3-aminobutyric acid, DL-3-aminoisobutyric acid monohydrate, (*S*)-2-phenylglycine, and *O*-benzylhydroxylamine hydrochloride were from Janssen (Beerse, Belgium). Benzylacrylic acid was prepared following Mannich and Ritsert.[40] Ethyl 2-benzylidene-3-carboxypropanoate was obtained according to Cohen and Milovanovic.[41] *N*-(Benzyloxycarbonyl)-3(*S*)-benzyl-β-alanine and *N*-(benzyloxycarbonyl)-3(*S*)-phenyl-β-alanine were prepared as described.[42,43] All the solvents (Normapur label) were from Prolabo (Paris, France). 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) $CH_2Cl_2$/MeOH/AcOH (9:1:0.5); (B) $CH_2Cl_2$/MeOH (10:0.5); (C) $CH_2Cl_2$/MeOH (9:1); (D) $CH_2Cl_2$/MeOH/AcOH (7:1:0.5); (E) AcOEt/Py/AcOH/$H_2O$ (160:20:6:11); (F) 1-BuOH/$H_2O$/AcOH (4:1:1); (G) AcOEt/Py/AcOH/$H_2O$ (260:20:6:11). The purity of the final compounds was checked also by HPLC on a reverse-phase Nucleosil $C_8$ column (Waters) with $CH_3CN$/TFA (0.07%) buffer (pH 4.00) as solvent. The eluted peaks were monitored at 210 nm.

The structure of all the synthesized compounds was confirmed by $^1$H NMR spectroscopy (Brüker WH 270 MHz) in DMSO-$d_6$ solution ($5 \times 10^{-3}$ M). Complete assignement of $^1$H NMR signals was performed by classical double-resonance experiments. Melting points of the crystallized compounds were determined on a Kofler apparatus (±2 °C) and are reported uncorrected. The structure of the final compounds was also verified by mass spectroscopy (Nernag R 10 C, DIC $NH_3$ mode). Satisfactory analyses (<±0,4%) were obtained (C, H, N) for all compounds.

The following abbreviations are used: THF, tetrahydrofuran; TEA, triethylamine; TFA, trifluoroacetic acid; Py, pyridine; HOBT, 1-hydroxybenzotriazole; DCC, dicyclohexylcarbodiimide; $Me_2SO$-$d_6$, hexadeuterodimethyl sulfoxide; HMDS, hexamethyldisiloxane; Ph, phenyl.

**Synthesis of α-Substituted β-Amino Acid and α,β-Disubstituted β-Amino Acid (Procedure A).** A solution of 20 mM hydroxylamine hydrochloride in 1 mL of hot water was added to a refluxing solution of sodium ethoxide (from 20 mM of sodium and 16 mL of absolute ethanol). The resulting suspension was

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cooled quickly in an ice–water mixture, filtered, and washed with small portion (total 2 mL) of absolute ethanol. Ten millimolar of the corresponding α, β-unsaturated acid was added to the filtrate, and the mixture was refluxed overnight. The suspension was allowed to remain at room temperature, and the precipitate was collected, washed with ice-cold water (3 mL) and absolute ethanol (3 mL), and dried.

2(*RS*)-Benzyl-β-alanine: $R$ = 25.4%; mp >260 °C; $R_f$(F) 0.31 (lit.[42] chlorohydrate mp 154–150 °C). 2,3-Dimethyl-β-alanine: $R$ = 30.4%; mp 258 °C; $R_f$(F) 0.14 (lit.[44] 202 °C).

**Preparation of *tert*-Butyl Esters (Procedure B).** Ten millimolar of *N*-(benzyloxycarbonyl) amino acid was dissolved in dry benzene (15 mL), and *N,N*-dimethylformamide di-*tert*-butyl acetal (40 mM) was added dropwise to the refluxing mixture within 30 min. The solution was refluxed for a further 30 min, cooled, and washed with $H_2O$ (15 mL), $NaHCO_3$ (10%) (2 × 15 mL), and saturated NaCl solution (15 mL). The organic layer was dried and evaporated and the compound obtained hydrogenated in MeOH with 10% Pd on charcoal (30 mg/mM) for 5 h at room temperature. After filtration, the solution was evaporated in vacuo.

β-Alanine *tert*-butyl ester,[45] oil, $R_f$(A) 0.53; 3(*R,S*)-methyl-β-alanine *tert*-butyl ester, yellow oil, $R_f$(A) 0.45; 3(*S*)-benzyl-β-alanine *tert*-butyl ester, yellow oil, $R_f$(A) 0.51; 3(*S*)-phenyl-β-alanine *tert*-butyl ester, yellow oil, $R_f$ (A)0.47; 2(*R,S*)-methyl-β-alanine *tert*-butyl ester, oil, $R_f$(C) 0.20; 2(*RS*)-benzyl-β-alanine *tert*-butyl ester, oil, $R_f$(A) 0.49; 2,3-dimethyl-β-alanine *tert*-butyl ester, mp 82–84 °C, $R_f$(A) 0.42; 4-aminobutyric acid *tert*-butyl ester, oil, $R_f$(A) 0.43 (lit.[46] phosphite mp 163–166 °C); 5-aminovaleric acid *tert*-butyl ester,[47] oil, $R_f$(A) 0.32; *N*-methyl-L-phenylalanine *tert*-butyl ester, yellow oil, $R_f$(C) 0.50.

**General Procedure for the Coupling Step (Procedure C). Preparation of Compounds 1a–j.** To a solution of 10 mM 3-(ethoxycarbonyl)-2-benzylidenepropionic acid in THF (30 mL), cooled to 0 °C, were added successively 10 mM of the corresponding amino acid *tert*-butyl ester in $CHCl_3$(20 mL), 10 mM HOBT in THF (15 mL), and 11 mM DCC in $CHCl_3$ (10 mL). After 1 h at 0 °C, the mixture was stirred at room temperature overnight. After filtration of dicyclohexylurea (DCU) and evaporation of the solvents the residue was dissolved in EtOAc and washed successively with $H_2O$ (2 × 100 mL), citric acid (10%) (3 × 100 mL), $H_2O$ (1 × 100 mL), $NaHCO_3$ (10%) (3 × 100 mL), $H_2O$ (1 × 100 mL), and finally, saturated NaCl solution (100 mL). The organic layer was dried over $Na_2SO_4$ and evaporated in vacuo.

**General Procedure for the Alkaline Hydrolysis of Esters (Procedure D). Preparation of Compounds 2a–j.** To a solution of 10 mM of the ester compound (**1a–j**) in EtOH (40 mL) and water (10 ml) was added at 0 °C 11 mM of 1 M NaOH. The mixture was stirred at 0 °C for 1 h and at room temperature for 5 h. The solution was concentrated in vacuo, dilued with 40 mL of water, washed with EtOAc (2 × 20 mL), acidified to pH 2 with 1 M HCl, and extracted with EtOAc (3 × 20 mL). The organic layer was washed with $H_2O$ (2 × 30 mL) and saturated NaCl solution (30 mL), dried, and evaporated in vacuo.

**Preparation of Compounds 3a–j.** These compounds were obtained following procedure C, from 1 equiv of the preceding compounds (**2a–j**), 1 equiv of *O*-benzylhydroxylamine hydrochloride, and 1 equiv of triethylamine.

**General Procedure for the Acidic Hydrolysis of *tert*-Butyl Esters (Procedure E). (1) Preparation of Compounds 4a–i.** TFA (10 mM) was added dropwise to a solution of *tert*-butyl ester **3a–i** (1 mM) dissolved in $CH_2Cl_2$ (1 mL) at 0 °C. The mixture was stirred at 0 °C for 1 h and 4 °C overnight. After evaporation of the excess TFA, the residue was washed with $Et_2O$ and dried.

**(2) Preparation of *N*-[3-[(Hydroxyamino)carbonyl]-2-benzylidene-1-oxopropyl]-*N*-methyl-L-phenylalanine (4j).** Boron tris(trifluoroacetate): (3 mM) (1.0 M solution in TFA) was added to a solution of *N*-[3-[[benzyloxy)amino]carbonyl]-2-benzylidene-1-oxopropyl]-*N*-methyl-L-phenylalanine *tert*-butyl ester (**3j**) (1 mM) dissolved in TFA (2 mL) cooled to 60 °C under $N_2$ atmosphere. The mixture was stirred at 0 °C for 40 min. After evaporation of the solvent, the residue was precipitated in $Et_2O$ and dried: $R$ = 63.7%, mp 140–143 °C, $R_f$(A) 0.27.

**General Procedure for the Hydrogenolysis (Procedure F). Preparation of Compounds 5–14.** To a 10% Pd on charcoal (30 mg/mM) suspension in MeOH, saturated by hydrogen, was added the compound to be hydrogenolyzed (**4a–j**) in MeOH. The mixture was stirred for 5 h at room temperature. After filtration, the solution was evaporated in vacuo. The physical characteristics of all the synthesized compounds are reported in Tables I and II.

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**Registry No.** **1a**, 120397-45-7; **1b**, 120686-21-7; **1c**, 120686-22-8; **1d**, 120686-23-9; **1e**, 120686-24-0; **1f**, 120686-25-1; **1g**, 120686-26-2; **1h**, 120686-27-3; **1i**, 120686-28-4; **1j**, 120711-13-9; **2a**, 120378-08-7; **2b**, 120686-29-5; **2c**, 120686-30-8; **2d**, 120686-31-9; **2e**, 120686-32-0; **2f**, 120686-33-1; **2g**, 120686-34-2; **2h**, 120686-35-3; **2i**, 120686-36-4; **2j**, 120711-14-0; **3a**, 120686-37-5; **3b**, 120686-38-6; **3c**, 120686-39-7; **3d**, 120686-40-0; **3e**, 120686-41-1; **3f**, 120686-42-2; **3g**, 120686-43-3; **3h**, 120686-44-4; **3i**, 120686-45-5; **3j**, 120711-15-1; **4a**, 120377-74-4; **4b**, 120686-46-6; **4c**, 120686-47-7; **4d**, 120686-48-8; **4e**, 120686-49-9; **4f**, 120686-50-2; **4g**, 120686-51-3; **4h**, 120686-52-4; **4i**, 120686-53-5; **5**, 120377-47-1; **6**, 120377-48-2; **7**, 120377-51-7; **8**, 120377-52-8; **9**, 120377-49-3; **10**, 120377-50-6; 11, 120686-10-4; **12**, 120686-11-5; **13**, 120686-12-6; **14**, 120686-13-7; $HONHCOCH_2CH(CH_2Ph)CONHCH(CH_2Ph)CH_2COOH$, 120377-51-7; $HONHCOCH_2CH(CH_2Ph)CONHCH(Ph)CH_2COOH$, 120377-52-8; $CH_2{=}C(CH_2Ph)COOH$, 5669-19-2; $CH_3CH{=}C(CH_3)COOH$, 80-59-1; $HONH_2$, 7803-49-8; $NH_2CH_2CH(CH_2Ph)COOH$, 114746-81-5; $NH_2CH(CH_3)CH(CH_3)COOH$, 32723-74-3; $NH_2CH_2CH(CH_2Ph)COOH{\cdot}HCl$, 26250-89-5; $CH_3CH_2OCOCH_2C(=CHPh)COOH$, 87439-00-7; $CbzNH(CH_2)_2COOH$, 2304-94-1; $CbzNHCH(CH_3)CH_2COOH$, 51440-81-4; $CbzNHCH(CH_2Ph)CH_2COOH$, 26250-86-2; $CbzNHCH(Ph)CH_2COOH$, 14441-08-8; $CbzNHCH_2CH(CH_3)COOH$, 67799-89-7; $CbzNHCH_2CH(CH_2Ph)COOH$, 120686-14-8; $CbzNHCH(CH_3)CH(CH_3)COOH$, 120686-15-9; $CbzNH(CH_2)_3COOH$, 5105-78-2; $CbzNH(CH_2)_4COOH$, 23135-50-4; $CbzN(CH_3)CH(CH_2Ph)COOH$, 2899-07-2; $CH_3CH(OBu\text{-}t)_2$, 6123-62-2; $NH_2(CH_2)_2COOBu\text{-}t$, 15231-41-1; $NH_2CH(CH_3)CH_2COOBu\text{-}t$, 120686-16-0; $NH_2CH(CH_2Ph)CH_2COOBu\text{-}t$, 120686-17-1; $NH_2CH(Ph)CH_2COOBu\text{-}t$, 120686-18-2; $NH_2CH_2CH(CH_3)COOBu\text{-}t$, 120786-19-8; $NH_2CH_2CH(CH_2Ph)COOBu\text{-}t$, 120686-19-3; $NH_2CH(CH_3)CH(CH_3)COOBu\text{-}t$, 120686-20-6; $NH_2(CH_2)_3COOBu\text{-}t$, 50479-22-6; $NH_2(CH_2)_4COOBu\text{-}t$, 63984-03-2; $CH_3NHCH(CH_2Ph)COOBu\text{-}t$, 114525-94-9; $PhCH_2ONH_2{\cdot}HCl$, 2687-43-6; phosphite, 14901-63-4; endopeptidase, 9001-92-7; dipeptidylaminopeptidase, 9032-67-1; aminopeptidase N, 9054-63-1.

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