# Endomorphin-1 Analogues Containing β-Proline Are μ-Opioid Receptor Agonists and Display Enhanced Enzymatic Hydrolysis Resistance

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In this paper we describe the synthesis and affinity toward the μ-opioid receptor of some tetrapeptides obtained from endomorphin-1, H-Tyr-Pro-Trp-Phe-$NH_2$ (**1**), by substituting each amino acid in turn with its homologue. The ability to bind μ-opioid receptors depends on the β-amino acid, and in particular **4**, which contains β-L-Pro, has a $K_I$ in the nanomolar range. The peptides **4** and **5** are significantly more resistant to enzymatic hydrolysis than **1**. The same compounds, as well as the μ-opioid receptor agonist DAMGO, produced a concentration-dependent inhibition of forskolin-stimulated cyclic AMP formation, thus behaving as μ-opioid agonists. These features suggest that this novel class of endomorphin-1 analogues may represent suitable candidates for the in vivo investigation as potential μ-opioid receptor agonists.

## Introduction

The discovery of the endogenous peptides endomorphin-1, H-Tyr-Pro-Trp-Phe-$NH_2$, and endomorphin-2, H-Tyr-Pro-Phe-Phe-$NH_2$,[1−3] in the mammalian brain encouraged the application of natural or synthetic peptides as analgesics instead of morphine.[4−8] Indeed, both morphine and endomorphins mainly act as agonists at the same μ-opioid receptors, but the latter are thought to inhibit pain without some of the undesirable side effects of morphine.[6−10] Quite often, however, endomorphins, as well as opioid peptides in general, have a limited in vivo efficacy, since they are easily degraded by different proteases.[11−14] To increase their efficacy, the concomitant use of some peptidase inhibitors can be considered.[15] On the other hand, several peptidomimetics or more stable peptide analogues, in which the original structures of endogenous peptides have been modified,[15,16] including endomorphin-1[17] and endomorphin-2[18] diastereoisomers, have been synthesized and investigated. In several cases, the peptidomimetics exhibit long-term toxicity and have difficulty in penetrating the blood−brain barrier.[19,20] For this reason, the search for new peptidomimetics or peptide analogues is of current interest.[15,16,21]

In addition, the structure−activity relationships of endomorphins which have been systematically modified through the introduction of modified residues within the sequence are of particular interest, since the basis of ligand recognition and the selectivity of opioid receptors are only partially known. Indeed, there is still no conclusive evidence of the presence of a μ-opioid address in ligands, even though it seems plausible that the selectivity could depend on the presence of spatially distinct hydrophobic pockets in different receptors.[22,23]

The comparison of several endogenous and synthetic tetrapeptide opioid ligands showed that μ-selectivity and affinity strongly depend on the amino acid sequence. In particular, the presence of a Tyr in the first position, a Pro or D-Ala in the second position, lipophilic residues in the third and fourth positions, and amidation at the C-terminus seem to be very important features.[1,15−17,22−24]

A more subtle requirement is the spatial disposition of each residue.[23] Indeed, conformational differences may be responsible for large variation in affinity and selectivity. Several attempts have recently been made to determine the bioactive conformation of endomorphin-1. These studies, using molecular modeling and 2-dimensional NMR in different solvents and environments, have indicated that proline is a key residue, on the basis of its role as a spacer that fixes the peptide shape and induces the other residues to assume the proper spatial orientation for ligand−receptor interaction.[17,18,22,24,25]

In a recent study of peptides containing β-amino acids, we examined modified endomorphins with a β-amino acid in their sequence. This change could give tetrapeptides which are degraded or inactivated by enzymes less rapidly than endogenous peptides. We previously reported the preparation of some tetrapeptides in which each residue at a time in the series H-Tyr-Pro-Trp-Phe$NH_2$ was replaced by the corresponding β-isomer, to obtain new tetrapeptides the same size as the parent endomorphin, and tested their affinities for μ-opioid receptors through binding assays in rat brain membranes.[26] These tetrapeptides showed different affinities for μ-opioid receptors. Our results stress that proline is important for fixing the conformation of the other residues.

Another possible modification is the introduction of homo-β-amino acids to the sequence. β-Peptides formed by homologated β-amino acids have been studied for years to discover stable secondary structures.[27−34] In several cases, the substitution of α-amino acids by their

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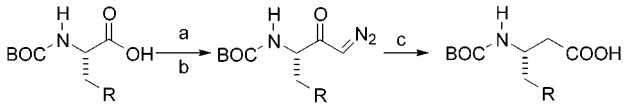
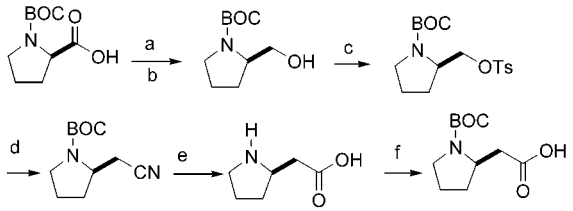
**Scheme 1.** Synthesis of Homo-Amino Acids[a]




R = $C_6H_4OH$, 60%; Ind, 72%; Ph, 75%

[a] Reagents and conditions: (a) EtOCOCl (1.1 equiv), triethylamine (TEA) (2.1 equiv), THF, −15 °C, 15 min; (b) $CH_2N_2$, $Et_2O$, −5 °C, 1 h, 85−92%; (c) $CF_3COOAg$ (0.11 equiv)/TEA (2.8 equiv), $THF/H_2O$ (9:1), −25 °C, 4 h, 70−82%.

**Scheme 2.** Synthesis of D-Homo-Proline[a]




[a] Reagents and conditions: (a) *N*-methylmorpholine (1.2 equiv), isobutylchloroformate (1.2 equiv), THF, −5 °C, 10 min (not isolated); (b) $NaBH_4$ (1.2 equiv), $I_2$ (0.48 equiv), THF, 0 °C, 2 h, 75%; (c) TsCl (1.2 equiv), TEA (1.2 equiv), catalytic dimethylaminopyridine (DMAP), $CH_2Cl_2$, 0 °C, 3 h, 78%; (d) KCN (3 equiv), DMSO, 90 °C, 4 h, 60%; (e) 12 N HCl/AcOH (5:1), reflux, 4 h, 80%; (f) 2 N NaOH/acetone, $BOC_2O$ (1.5 equiv), 0 °C, 2 h, 98%.

*β*-isomers in biologically active peptides resulted in increased activity and enzymatic stability.[35−37]

In this paper we report the synthesis of tetrapeptides based on endomorphin-1, in which one residue at a time was replaced by its *β*-homologue. Most of these homo-amino acids are commercially available, or can be readily prepared in optically pure form. We report the affinities displayed by the modified endomorphins for *μ*-opioid receptors as tested by displacement assays performed on *μ*-opioid receptors in rat brain membranes labeled with [$^3$H]DAMGO. In conjunction with this, we also present the effects of these peptides displaying the higher affinities to *μ*-opioid receptors on cyclic AMP formation in whole SH-SY5Y human neuroblastoma cells to better understand if they act as *μ*-receptor agonist or antagonist. Finally, we examined the degradation rates of some tetrapeptides in the presence of commercially available proteolytic enzymes to determine if the structural and conformational changes gave improved resistance in comparison to native endomorphin-1.

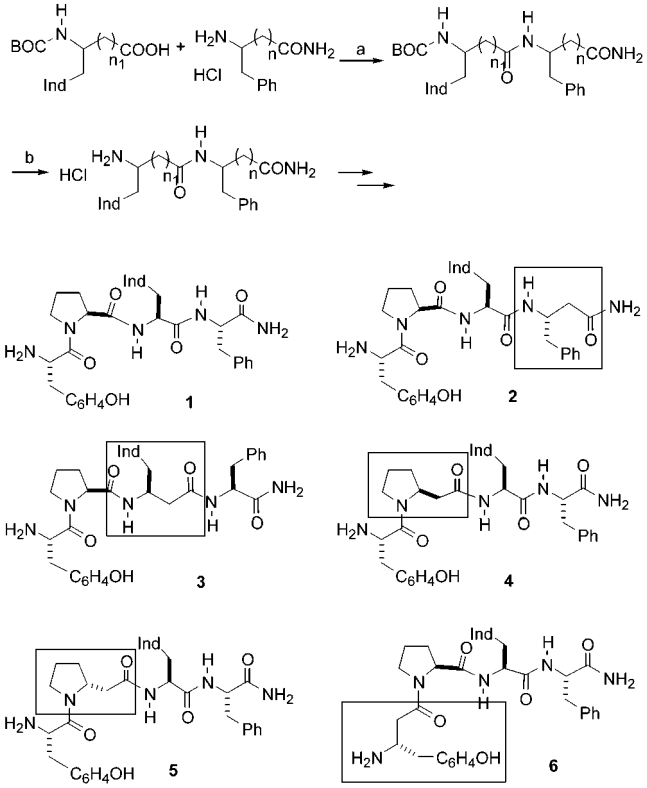
## Results and Discussion

**Chemistry: Synthesis and Characterization of Tetrapeptides.** Most of the optically pure (*S*)-*N*-*tert*-butyloxycarbonyl-*β*-amino acids were purchased, or readily prepared from *N*-*tert*-butyloxycarbonyl-α-amino acid by means of the Arndt−Eistert homologation, with overall yields of 60−75% (Scheme 1).[38]

(*R*)-*N*-*tert*-Butyloxycarbonyl-homo-Pro was obtained starting from D-proline using a multistep homologation procedure which was slightly modified with respect to the literature,[39] since in this case the Arndt−Eistert[40] reaction gave us poor yields (Scheme 2).

The *N*-Boc-(*R*)-Pro mixed anhydride was reduced with sodium borohydride[41] to Boc-prolinol, which was tosylated and treated with KCN to give the *N*-Boc-pyrrolidylacetonitrile.[42] Acid hydrolysis simultaneously gave Boc deprotection and cyano group hydrolysis to afford (*R*)-homo-Pro in good yield. Final treatment with di-*tert*-butyl dicarbonate gave *N*-Boc-(*R*)-homo-Pro. In a similar way, *N*-Boc-(*S*)-homo-Pro could be obtained starting from *N*-Boc-(*S*)-Pro.

**Scheme 3.** Synthesis of Homo-Tetrapeptides **1**−**6**[a]




[a] Reagents and conditions: (a) TEA (3 equiv), hydroxybenzotriazole (HOBt) (1.5 equiv), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (EDCI)/HCl (1.5 equiv), $CHCl_3$/DMF (9:1), 0 °C, $N_2$, 8 h, 60−90%; (b) saturated HCl/dioxane, 0 °C, 45 min, 100%.

Peptides **1**−**6** were prepared in solution using a convergent approach, by coupling the amino amide and the *N*-*tert*-butyloxycarbonyl-amino acid in the presence of EDCI/HOBt (Scheme 3).[38] The resulting Boc-protected peptides were purified by flash chromatography over silica gel, with yields of 60−90%. Deprotection was achieved by treatment with saturated HCl in dioxane,[38] and the resulting HCl peptide salts were used without purification for the next coupling. Finally, HCl tetrapeptide salts were purified by reversed-phase preparative HPLC, to give **1**−**6** as TFA salts. Purities were determined to be 94−97% by analytical reversed-phase HPLC (Table 2).

The final tetrapeptide TFA salts were characterized by MALDI-MS and/or FAB-MS analysis (Table 2).

In this manner, we prepared a series of tetrapeptides based on the endomorphin-1 sequence, H-Tyr-Pro-Trp-Phe-$NH_2$, containing a *β*-homo-amino acid. Since Pro is thought to be responsible for the spatial disposition of the other residues, we prepared tetrapeptides containing both homo-L-Pro and homo-D-Pro.[43]

**Radioligand Binding Studies and Discussion.** The tetrapeptides were incubated with rat brain membrane homogenates containing *μ*-opioid receptors and using [$^3$H]DAMGO as a *μ*-specific radioligand. All experiments were performed in triplicate. From the

**Table 1.** Affinities and Hill Slopes of DAMGO and Ligands **1**−**6** for [$^3$H]DAMGO Binding Sites in Rat Brain Membranes[a]

| compd | sequence | $K_I$ (nM) | $IC_{50}$ (nM) | $n_H$ |
|---|---|---|---|---|
| | DAMGO | 1.6(±0.3) | 9.9(±0.6) | 0.90(±0.05) |
| **1** | Tyr-Pro-Trp-Phe-$NH_2$ (endomorphin-1)[b] | 0.16(±0.02) | 0.5(±0.2) | 0.74(±0.03) |
| **2** | Tyr-Pro-Trp-β-Phe-$NH_2$[b] | 79.1(±0.3) | 80.1(±0.8) | 0.8(±0.1) |
| **3** | Tyr-Pro-β-Trp-Phe-$NH_2$[b] | 73.0(±0.5) | 23.3(±0.6) | 0.7(±0.1) |
| **4** | Tyr-L-β-Pro-Trp-Phe-$NH_2$[b] | 2.1(±0.3) | 4(±2) | 0.69(±0.03) |
| **5** | Tyr-D-β-Pro-Trp-Phe-$NH_2$[b] | 67(±6) | 79(±2) | 0.6 (±0.2) |
| **6** | β-Tyr-Pro-Trp-Phe-$NH_2$[b] | 26(±4) | 990(±40) | 0.8(±0.1) |

[a] Means ± SE of three experiments performed in triplicate. [b] TFA salts.

**Table 2.** Purities and Masses of Tetrapeptides **1**−**6**

| compd | purity (%)[a] | peptide mass [M + H][b] | compd | purity (%)[a] | peptide mass [M + H][b] |
|---|---|---|---|---|---|
| **1** | 96 | 611.2[c] | **4** | 95 | 625.0,[c] 625.4[d] |
| **2** | 94 | 625.0[c] | **5** | 97 | 625.3,[c] 625.6[d] |
| **3** | 96 | 625.2[c] | **6** | 94 | 625.1[c] |

[a] Average value from HPLC of methods 1 and 2. [b] Calculated values: **1**, 610.3; **2**−**6**, 624.3. [c] FAB-MS. [d] MALDI-MS.

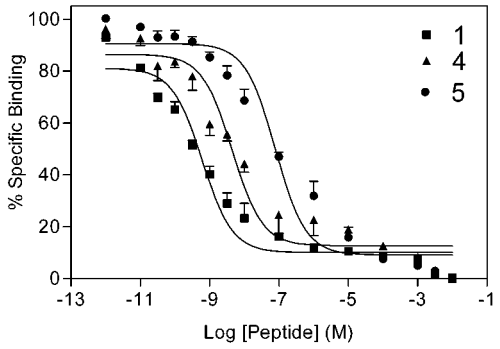



**Figure 1.** Competition curves of **1**, **4**, and **5** with [$^3$H]DAMGO for its specific binding sites in rat brain membranes. The membranes were incubated with various concentrations of ligands ($10^{-12}$ to $5 \times 10^{-2}$) and 1 nM DAMGO for 60 min at room temperature.

displacement binding assays, $K_I$, $IC_{50}$, and Hill factors were obtained for DAMGO, endomorphin-1 (**1**), and tetrapeptides **2**−**6**, and are reported in Table 1. The competition curves of the most active peptides **4** and **5** with [$^3$H]DAMGO are reported in Figure 1 and compared with that of **1**.

The affinity values for DAMGO and **1** agree with the literature ($K_I$ = 1.9 and 0.36 nM, respectively).[1,15,22,44] All the compounds displayed a concentration-dependent displacement of [$^3$H]DAMGO. In all cases the slope factor ($n_H$) was less than unity. By comparing the values for peptides **2**−**6**, it is clear that the peptides **2**, **3**, **5**, and **6** display a low affinity for μ-opioid receptors, while **4** was the most potent in displacing [$^3$H]DAMGO, (Table 1). In particular, **4** has a $K_I$ value in the nanomolar range, although it is slightly less potent than the reference compound **1** (Table 1, Figure 1). These results indicate that substituting Phe, Trp, and Tyr with homo-Phe, homo-Trp, and homo-Tyr, respectively, in the tetrapeptide sequence caused a significant loss of affinity with respect to the reference compound **1**. These results can be explained by considering that these residues are responsible for specific hydrophobic ligand−receptor interactions, and thus a change in their spatial disposition is detrimental for a good ligand−receptor fit. On the other hand, substituting Pro with homo-L-Pro gives the new homo-peptide **4**, which retains a good μ-receptor affinity. Since Pro is thought to act as a

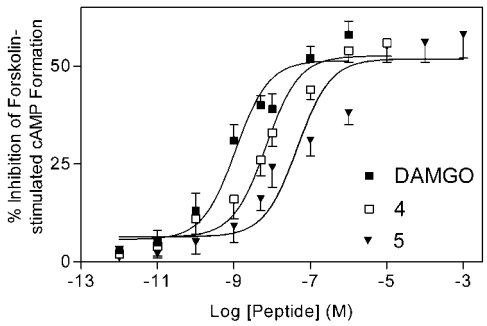



**Figure 2.** Effects of DAMGO and endomorphin-1 analogues **4** and **5** on forskolin-stimulated cyclic AMP production in SH-SY5Y cells.

spacer that keeps the other residues in the correct spatial disposition, our results seem to indicate that homo-Pro still permits **4** to assume a geometry similar to that of **1**, probably due to the enhanced conformational freedom given by the extra carbon. Although it is possible that the different tetrapeptides interact with the receptor in distinct ways, it seems likely that they would fit in a similar way, due to flexibility of the tetrapeptide chain. In addition, a certain degree of "induced fit" should also be considered for receptor conformation, which would enable molecules with different shapes to bind to the same part of a receptor.[45] The significantly lower affinity observed by substituing Pro with homo-D-Pro in **5** confirms that the absolute configuration remains a very important aspect of the role of Pro as a spacer, despite the greater flexibility gained by the peptide.[17,26]

Compound **4**, which was the most effective in displacing [$^3$H]DAMGO from rat brain membranes, and compound **5** were further investigated to evaluate any effect on cyclic AMP production in intact SH-SY5Y cells which constitutively express abundant μ-opioid receptors and fewer δ-opioid receptors.[44,46] The potent μ-opioid agonist DAMGO, as well as **4** and **5**, suppressed forskolin (10 μM)-stimulated cyclic AMP production in a concentration-dependent manner (Figure 2). The $IC_{50}$ values of DAMGO, **4**, and **5** were 1.10 ± 0.27, 6.53 ± 0.25, and 45.0 ± 9.2 nM, respectively. The potency of DAMGO was in the nanomolar range as well as previously reported by Toll et al. for the same cell line.[46] Harrison et al.,[44] adopting the same assay system, have reported that endomorphin-1 produced concentration-dependent inhibition of foskolin-stimulated cyclic AMP production and the $pIC_{50}$ was 7.72 ± 0.13. Further, naloxone (100 nM) significantly reversed the effect elicited by the DAMGO, **4** (1 μM), and **5** (10 μM) (data not shown). These findings indicate that **4** and **5** act in the μ-opioid receptor as agonists, and their $IC_{50}$ values for the inhibitory effects in cyclic AMP production are well

**Table 3.** HPLC Data of Peptides Derived from Enzymatic Hydrolysis vs Authentic Samples in Two Different Solvent Systems

| compd | $R_f$ (peptide vs authentic, method 1) | $R_f$ (peptide vs authentic, method 2) | compd | $R_f$ (peptide vs authentic, method 1) | $R_f$ (peptide vs authentic, method 2) |
|---|---|---|---|---|---|
| **1** | 19.38/19.40 | 35.11/35.05 | Tyr-L-$\beta$-Pro-Trp-Phe | 20.40/20.40 | 36.72/36.76 |
| Tyr-Pro-Trp | 16.88/16.89 | 28.92/28.89 | **5** | 20.12/20.10 | 36.22/36.20 |
| Phe-$NH_2$ | 13.83/13.83 | 14.10/14.09 | Tyr-D-$\beta$-Pro-Trp | 17.25/17.28 | 27.60/27.63 |
| Tyr-Pro-Trp-Phe | 20.47/20.46 | 34.80/34.86 | **6** | 19.87/19.92 | 36.19/36.22 |
| Tyr-Pro | 10.71/10.68 | 12.85/12.83 | $\beta$-Tyr-Pro-Trp | 17.28/17.30 | 29.18/29.21 |
| Trp-Phe-$NH_2$ | 13.43/13.39 | 16.11/16.13 | $\beta$-Tyr-Pro | 12.01/12.00 | 15.61/15.58 |
| **4** | 19.54/19.53 | 35.17/35.12 | $\beta$-Tyr-Pro-Trp-Phe | 20.99/21.01 | 37.57/37.54 |
| Tyr-L-$\beta$-Pro-Trp | 16.50/16.45 | 24.75/24.70 | | | |

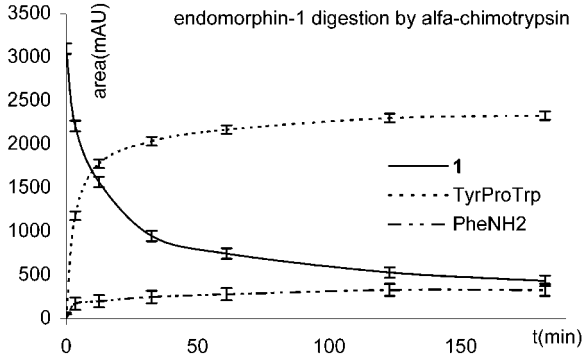



**Figure 3.** Kinetic curve of **1** digestion and digestion products upon incubation with α-chymotrypsin.

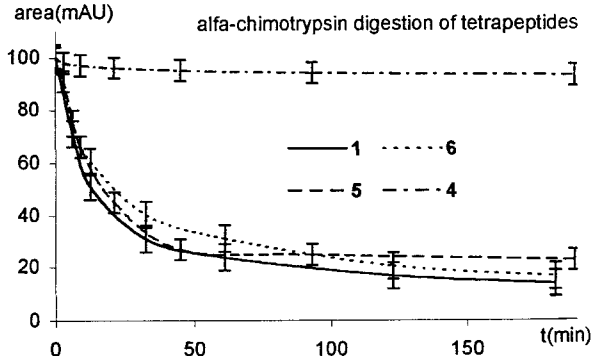



**Figure 4.** Kinetic curve of **1** and **4**−**6** digestion upon incubation with α-chymotrypsin.

correlated to their $K_I$ values for the $\mu$-opioid receptor obtained in binding studies.

**Tetrapeptide Enzymatic Digestion.** A very important topic to examine in the field of biologically active peptides is their resistance to enzymatic degradation. Thus, we tested the resistance of the modified tetrapeptide **4**, which showed the higher $\mu$-opioid receptor affinity (Table 1), to some common, commercially available enzymes.[47] To verify the effect of residue substitution, we also examined the hydrolysis of **5** and **6** under the same conditions. The peptides were incubated in a buffered solution (pH 7.4) with α-chymotrypsin, aminopeptidase-M, and carboxypeptidase-Y at 37 °C.[47] At designated intervals, we quenched an aliquot of the incubated mixture, and the mixture was analyzed by reversed-phase HPLC. Sampling times were chosen so that a representative kinetic curve could be constructed. Peak attributions were determined by comparison with authentic fragments prepared by taking into account the more plausible[47] tetrapeptide decompositions (Table 3). To prevent incorrect attributions, $R_f$ values were compared for two distinct HPLC systems, as reported in the Experimental Section. Experiments were performed in parallel, taking portions of the same enzyme solution. Data were shown in plots of area vs time. To appreciate the resistances displayed by the different peptides, areas were normalized to 100 at $t = 0$ and the curves were superimposed.

The digestion of **1** with α-chymotrypsin proceeded very rapidly, and gave a mixture of the tripeptide Tyr-Pro-Trp and Phe-$NH_2$ (Figure 3). Indeed, only 14% of the original tetrapeptide quantity was intact after 3 h (Figures 3 and 4). The degradation of **5** and **6** showed a similar trend; although 23% of **5** and 17% of **6** remained after 3 h (Figure 4), this was not considered enhanced resistance to hydrolysis with respect to **1**, since these values are within the estimated error ranges. On the other hand, **4** was extremely resistant under the same conditions, with 93% intact after 3 h.

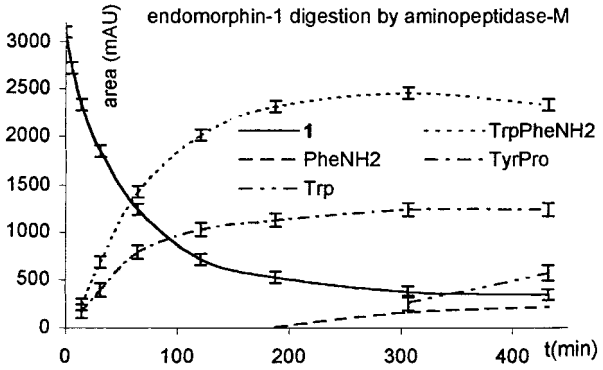



**Figure 5.** Kinetic curve of **1** digestion and digestion products upon incubation with aminopeptidase-M.

The digestion of **1** with aminopeptidase-M gave a mixture of Tyr-Pro and Trp-Phe-$NH_2$.[47] After 3 h, further hydrolysis of the latter dipeptide to Trp and Phe-$NH_2$ was evident (Figure 5). Comparison of the degradation rates for **1**, **4**, **5**, and **6** (Figure 6) showed that **1** and **6** have basically the same resistance, with 17% and 14% remaining after 3 h, respectively, while **4** and **5** both show excellent resistance, 89% and 96% remaining after 3 h, respectively. In fact these two peptides can be considered stable during the time period investigated, since no trace of any product arising from their enzymatic digestion was detected by HPLC.

The enzymatic digestion of endomorphin-1 by carboxypeptidase-Y begins with hydrolysis of the C-terminus amide Phe-$NH_2$, to give the tetrapeptide Tyr-Pro-Trp-Phe, which is in turn hydrolyzed to the tripeptide Tyr-Pro-Trp upon cleavage of the Trp−Phe bond (Figure 7).[47]

Comparison of the rates of digestion of **1** and **4** by carboxypeptidase-Y indicates a comparable hydrolysis resistance, within the error ranges. Indeed, after 24 h

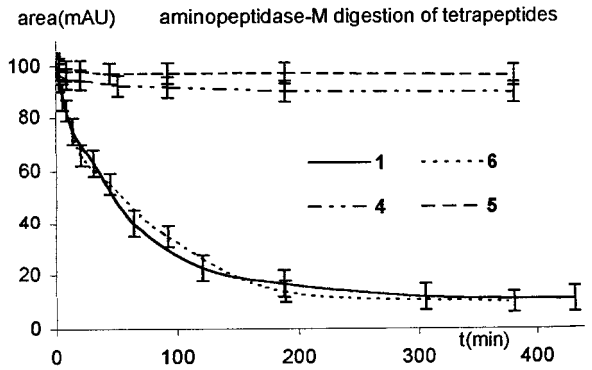



**Figure 6.** Kinetic curve of **1** and **4**−**6** digestion upon incubation with aminopeptidase-M.

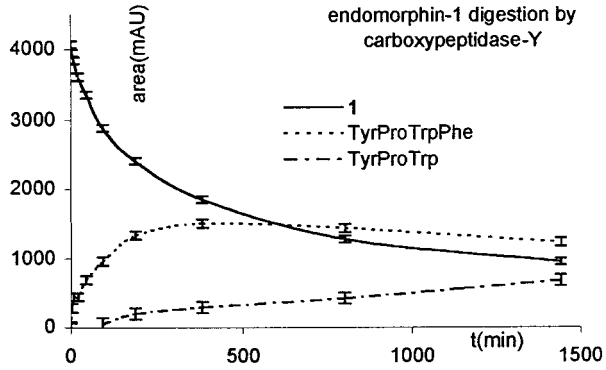



**Figure 7.** Kinetic curve of **1** digestion and digestion products upon incubation with carboxypeptidase-Y.

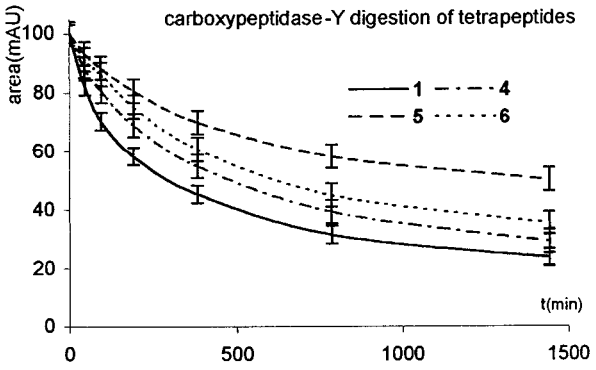



**Figure 8.** Kinetic curve of **1** and **4**−**6** digestion upon incubation with carboxypeptidase-Y.

only 23% of the initial amount of endogenous **1** was detected, while 29% of the homopeptide **4** was found (Figure 7). Compound **6** was slightly more resistant, 35% remaining after 24 h. More appreciably, peptide **5** showed the higher resistance, with 50% of the starting amount intact after 24 h.

Overall, our results indicate that no hydrolysis of the amide bond next to the substituted amino acid occurred. Peptide **6**, which contains homo-Tyr, maintains almost the same resistance as native **1** upon digestion with each enzyme. On the contrary, **4** and **5**, which contain homo-Pro, show enhanced resistance against aminopeptidase-M, which hydrolyzes the Pro−Trp bond. Interestingly, a good resistance enhancement is observed for **4** also against α-chymotrypsin, which hydrolyzes the Trp−Phe bond, while **5** shows resistance against carboxypeptidase-Y, which hydrolyzes the Phe−$NH_2$ bond. Comparison of **4** and **5** with **6** also suggests that the simple substitution of a generic residue is not sufficient to enhance peptide stability. Again, Pro seems to influence the conformation of the entire peptide, and the resistance of **4** and **5** can be attributed to a significant change in peptide−enzyme recognition.

## Conclusions

The tetrapeptides described in this paper can be regarded as promising modified endomorphins and interesting models for the study of ligand−receptor interaction. Indeed, they display different affinities for μ-opioid receptors, depending on the homo-amino acid introduced and its absolute stereochemistry. Good affinities were found for **4**−**6**. In particular, **4** (Tyr-L-homo-Pro-Trp-Phe-$NH_2$) showed affinity comparable to that of DAMGO. The cyclic AMP assay confirmed that **4** and **5** act as agonists, and probably bind in the same way as **1**. Due to the presence of an extra backbone carbon, **4** and **5** should be more flexible than endomorphin-1. Nevertheless, it appears that they can assume an active conformation. We should emphasize that the binding modes of flexible DAMGO and more conformationally constrained endomorphins are suspected to be the same, although additional binding modes can be considered.[48] Moreover, a certain degree of flexibility should also be attributed to the receptor itself, thus enabling molecules with different shapes to interact in the same way.[45]

Finally, we tested the resistance of **1** and **4**−**6** to the proteolytic enzymes α-chymotrypsin, aminopeptidase-M, and carboxypeptidase-Y. Our results indicate that the presence of homo-Pro gives the peptides **4** and **5** a good resistance, while homo-Tyr seems to have little effect. In particular, due to its enzyme resistance, and affinity for the opioid receptor, peptide **4** may validate further study for the development of a peptide analgesic based on the endomorphin sequence.

## Experimental Section

**General Methods.** Unless stated otherwise, chemicals were obtained from commercial sources and used without further purification. $CH_2Cl_2$ was distilled from $P_2O_5$. THF was distilled from sodium benzophenone ketyl. Flash chromatography was performed on Merck silica gel 60 (230−400 mesh), and solvents were simply distilled. Preparative reversed-phase HPLC was performed on a Waters Delta Prep 4000 Millipore, with a C18 column RP-18 (40−63 μm). The FAB-MS instrument employed was a Micromass ZMD spectrometer equipped with a single quadrupole analyzer and a Z-spray ionspray source outfitted with a 50 mm deactivated fused Si capillary connected to a Harvard Apparatus pump 11 for sample injection. Data acquisition and spectral analysis were conducted with Masslynx 3.3 software running on a Digital Equipment Corp. personal computer. Nitrogen was used as both desolvation and nebulizer gas. The desolvation temperature was set at 200 °C and the capillary voltage at 3.0 kV. MALDI-TOFMS analysis was performed on a Bruker Biflex III apparatus, equipped with a reflectron and pulse ion extraction (PIE). The instrument was run in positive ion reflectron mode (voltage 20 kV) with PIE set at "short". The cutoff was set at *m*/*z* 300 using the deflection mode, and the investigated range was 288−3590. Laser power ($N_2$, 337 nm) was adjusted for optimal resolution, and each spectrum was obtained by addition of 100−400 shots. The peptides were diluted in 1:1 matrix solution, which was α-cyano-4-hydroxycinnamic acid in water/acetonitrile (2:1) + 0.1% TFA. Analytical HPLC was performed on an HP Series 1100, with an HP Hypersil ODS column (4.6 μm particle size, 100 Å pore diameter, 250 mm), DAD 215.8 nm. Enzymatic hydrolysis was performed in an MGM Lauda RC20 thermostatic bath. Carboxypeptidase-Y lyophilized powder, 20%

protein content, pH 5, 19 units/mg of solid, 100 units/mg of protein, aminopeptidase-M suspension in 3.5 M $(NH_4)_2SO_4$ solution, pH 7.4, 5.1 mg of protein/mL, 29 units/mg, and α-chymotripsin, 60 U/mg, were purchased from Sigma. Homogenates were centrifuged in Beckman J6B and Beckman J2-21 centrifuges. Radioactivity was measured by liquid scintillation spectrometry using a Beckman apparatus.

**Synthesis of Boc-β-Tyr, Boc-β-Trp, and Boc-β-Phe by Arndt−Eistert Homologation.** To a stirred solution of Boc-amino acid (10 mmol) in anhydrous THF (35 mL) were added EtOCOCl (11 mmol) and TEA (21 mmol) under an inert atmosphere at −15 °C. After 15 min, a solution of $CH_2N_2$ in ether was added at −5 °C until the pale yellow color persisted. After 1 h the $CH_2N_2$ excess was destroyed with glacial acetic acid (0.5 mL), and the mixture was diluted with ether (30 mL) and washed with saturated $NaHCO_3$, saturated $NH_4Cl$, and brine. The organic layer was dried over $Na_2SO_4$, and solvent was removed at reduced pressure. The residue was purified by flash chromatography over silica gel (eluant 3:7 EtOAc/cyclohexane), giving the diazoketone as an oil (85−92%). To a stirred solution of the diazoketone (5 mmol) in 9:1 THF/$H_2O$ (15 mL) was added a solution of $CF_3COOAg$ (0.55 mmol) in TEA (14 mmol) at −25 °C under an inert atmosphere. The mixture was stirred at room temperature for 4 h, and then solvent was removed at reduced pressure. The residue was diluted with saturated $NaHCO_3$, and the mixture was extracted twice with ether. HCl (1 M) was added to the aqueous layer at 0 °C until the pH was around 2−3, and the mixture was extracted three times with EtOAc. The organic layers were collected and dried over $Na_2SO_4$, and solvent was evaporated at reduced pressure. Boc-homo-amino acid was obtained pure after flash chromatography over silica gel (eluant 1:1 EtOAc/cyclohexane) with a yield of 70−82%.

**Synthesis of Boc-β-Phe-$NH_2$.** A solution of Boc-β-Phe (0.28 g, 1.0 mmol) in $CH_2Cl_2$ (10 mL), TEA (0.31 mL, 2.2 mmol), and trimethylacethyl chloride (0.12 mL, 1.0 mmol) was stirred under an inert atmosphere at 0 °C. After 30 min, the solution was saturated with $NH_3$ at 0 °C, and stirring was maintained for 30 min. The reaction was quenched with water, and the mixture was extracted three times with EtOAc. The organic layers were collected and dried over $Na_2SO_4$. Boc-β-Phe-$NH_2$ was obtained after solvent evaporation at reduced pressure (0.25 g, 90%), and used without further purification.

**Synthesis of Boc-D-β-Pro. (*R*)-*N*-Boc-prolinol.** A mixture of Boc-D-proline (0.75 g, 3.5 mmol), *N*-methylmorpholine (0.46 mL, 4.2 mmol), isobutylchloroformate (0.55 mL, 4.2 mmol), and THF (4 mL) was stirred at −5 °C. After 10 min the mixture was filtered over Celite, and the precipitate was washed with THF. In a round-bottom flask a solution of $NaBH_4$ (0.16 g, 4.2 mmol) in dry THF (10 mL) was stirred at 0 °C under an inert atmosphere. Iodine (0.44 g, 1.7 mmol) was slowly added over 30 min, and after an additional 10 min the collected organic layers containing the Boc-D-Pro mixed anhydride were added with stirring. After 30 min at 0 °C the temperature was allowed to reach room temperature, and the mixture was stirred for 5 h. Then solvent was evaporated at reduced pressure, the residue was diluted with EtOAc, and the mixture was washed with water (5 mL). The water layer was extracted twice with EtOAc, and the organic layers were collected and dried over $Na_2SO_4$. Solvent was evaporated at reduced pressure. The residue was separated by flash chromatography over silica gel (eluant 75:25 cyclohexane/EtOAc), giving pure (*R*)-*N*-Boc-prolinol (0.53 g, 75%): ($[\alpha]_D^{20} = +47.0°$ ($c = 1$, $CHCl_3$) (lit.[49] $[\alpha]_D^{20} = +47.5°$ ($c = 1$, $CHCl_3$)).

**(*R*)-N-Boc-pyrrolidylacetonitrile.** A mixture of (*R*)-*N*-Boc-prolinol (0.35 g, 1.75 mmol), TEA (0.29 mL, 2.1 mmol), catalytic DMAP (0.071 g, 0.58 mmol), and tosyl chloride (0.40 g, 2.1 mmol) in $CH_2Cl_2$ (20 mL) was stirred at 0 °C. After 3 h, the mixture was washed with saturated $Na_2CO_3$, and the water layer was extracted twice with $CH_2Cl_2$. Organic layers were collected and dried over $Na_2SO_4$, and solvent was evaporated at reduced pressure. The resulting crude oil was purified by flash chromatography over silica gel (eluant 80:20 cyclohexane/EtOAc), giving pure (*R*)-*N*-Boc-2-tosyloxymethylpyrrolidine (0.48 g, 78%). To a solution of (*R*)-*N*-Boc-2-tosyloxymethylpyrrolidine (1.1 g, 3.0 mmol) in DMSO (15 mL) was added KCN (0.58 g, 9.0 mmol), and the mixture was stirred at 90 °C for 4 h, then EtOAc was added, and the organic layer was washed three times with small portions of water. The collected water layers were treated with $KMnO_4$ before elimination. The organic layer was dried over $Na_2SO_4$, and solvent was evaporated at reduced pressure, giving crude (*R*)-*N*-Boc-pyrrolidylacetonitrile as an oil, purified (0.38 g, 60%) by flash chromatography over silica gel (eluant 50:50 cyclohexane/EtOAc): $[\alpha]_D^{20} = -96.4°$ ($c = 1$, $CHCl_3$).

**(*R*)-β-Pro-HCl.** A mixture of (*R*)-*N*-Boc-pyrrolidylacetonitrile (0.21 g, 1.0 mmol), 12 N HCl (10 mL), and glacial $CH_3COOH$ (2 mL) was refluxed for 6 h. Then the reaction was allowed to reach room temperature, and it was washed twice with $Et_2O$. Water was evaporated at reduced pressure, giving (*R*)-β-Pro as hydrochloric salt (0.13 g, 80%), used without further purification: $[\alpha]_D^{20} = -35.5°$ ($c = 0.7$, 2 N HCl); for (*S*)-homo-Pro, lit.[50] $[\alpha]_D^{20} = +35°$ ($c = 1$, 2 N HCl).

**(*R*)-Boc-β-Pro.** (*R*)-β-Pro-HCl (0.17 g, 1.0 mmol) was dissolved in water (5 mL), and the pH was adjusted to around 10 at 0 °C with NaOH. Then the solution was diluted with acetone (10 mL), and di-*tert*-butyl dicarbonate (0.33 g, 1.5 mmol) was added at 0 °C with stirring. After 15 min the temperature was allowed to reach room temperature. After 2 h acetone was evaporated at reduced pressure, and 3 M HCl was added at 0 °C until pH 3. The mixture was extracted three times with EtOAc, and the collected organic layers were dried over $Na_2SO_4$. Solvent was evaporated at reduced pressure, giving (*R*)-Boc-β-Pro (0.34 g, 98%), which was used without further purification: $[\alpha]_D^{20} = +42.3°$ ($c = 2.0$, DMF) (lit.[40] $[\alpha]_D^{20} = +40.5°$ ($c = 1.9$, DMF)).

**Synthesis and Characterization of Tetrapeptides.** As a general procedure, the peptide coupling was performed by stirring overnight the HCl salt of the amino amide, the *N*-*tert*-butyloxycarbonyl-amino acid (1.2 equiv), triethylamine (3 equiv), 1-hydroxy-1*H*-benzotriazole (1.5 equiv), and the HCl salt of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (1.5 equiv), in a 9:1 mixture of $CHCl_3$ and DMF at 0 °C and under a nitrogen atmosphere. After 8 h, the solvent was evaporated at reduced pressure, and the residue was dissolved in EtOAc. The solution was washed with 0.5 M HCl, saturated $NaHCO_3$, and brine. The organic layer was dried over $Na_2SO_4$, and solvent was removed at reduced pressure. Peptides were obtained pure by flash chromatography over silica gel (eluant 96:4 EtOAc/MeOH) with yields from 60% to 90%.

*N*-*tert*-Butyloxycarbonyl group deprotection was performed by treatment with HCl in dioxane at 0 °C. After 45 min the solvent was evaporated at reduced pressure, and the resulting HCl peptide salt, obtained in quantitative yield, was used without purification for the next coupling. HCl-tetrapeptide salts were prepurified by recrystallization from MeOH/$Et_2O$. Final purification was performed by semipreparative reversed-phase HPLC on a Waters Delta Prep 4000 Millipore, with a C18 column RP-18 (40−63 μm, 250 mm) with solvent systems A (0.1% TFA in water) and B (0.1% TFA in acetonitrile), gradient 100% A to 50% B in 50 min at a 5.0 mL/min flow. Purities were determined by analytical reversed-phase HPLC under two distinct systems (Table 2).

**Spectroscopic Characterization of Tyr-L-β-Pro-Trp-Phe-$NH_2$ (4).** $^1$H NMR ($CD_3OD$, 400 MHz, major conformer) δ 1.70−1.80 (m, 1H), 1.80−1.88 (m, 2H), 1.92−2.08 (m, 1H), 2.77 (dd, $J = 8.1$, 14.4 Hz, 1H), 2.89 (d, $J = 6.6$ Hz, 2H), 2.93 (dd, $J = 5.4$, 8.7 Hz, 1H), 3.02−3.15 (m, 3H), 3.22 (d, $J = 6.6$ Hz, 2H), 3.48−3.59 (m, 1H), 4.24 (dd, $J = 6.6$, 7.2 Hz, 1H), 4.44 (dd, $J = 4.8$, 8.4 Hz, 1H), 4.53 (t, $J = 6.9$ Hz, 1H), 4.58 (t, $J = 6.6$ Hz, 1H), 6.71 (d, $J = 7.0$ Hz, 1H), 6.76 (d, $J = 6.8$ Hz, 2H), 6.93 (d, $J = 7.0$ Hz, 1H), 7.01−7.31 (m, 7H), 7.60 (d, $J = 7.1$ Hz, 2H), 7.82 (d, $J = 6.5$ Hz, 1H); $^{13}$C NMR ($CD_3OD$) δ 24.7, 25.4, 28.5, 29.6, 38.4, 39.4, 41.2, 46.3, 55.4, 56.0, 56.5, 110.5, 112.1, 116.5, 119.0, 122.2, 124.5, 125.4, 127.5, 128.1, 129.1, 130.1, 131.8, 137.5, 137.8, 157.8, 162.0, 168.2, 173.5, 176.8.

**Receptor Binding Assays.** Rat brain, without cerebellum, was weighed and homogenized in 10 volumes of ice-cold 0.32 M sucrose/10 mM Tris−HCl (pH 7.4 at 4 °C). The homogenate was centrifuged at 2000 rpm, for 10 min, at 4 °C, and the supernatant was in turn centrifuged at 19000 rpm, for 20 min, at 4 °C. The resulting pellet was suspended in 10 volumes of 50 mM Tris−HCl/100 mM NaCl (pH 7.4 at 4 °C) as incubation buffer and incubated for 1 h at room temperature (in a water bath at 37 °C) to remove any endogenous opioid ligands. After a final centrifugation at 19000 rpm, for 20 min, at 4 °C, the pellet was stored at −80 °C for up to two weeks.

The protein concentration was determined according to Lowry et al.[51] [$^3$H]DAMGO was used as $\mu$-selective radioligand (1 nM); the specific activity was 64 Ci/mmol, $K_d$ = 4.85 nM and $B_{max}$ = 48 fmol/mg of protein, $n$ = 3. Nonspecific binding was determined in the presence of 100 $\mu$M DAMGO. The incubation buffer consisted of 50 mM Tris−HCl, 0.1% BSA (pH 7.4 at 4 °C), and 2 mM EDTA. To prevent any peptidase degradation, the following protease inhibitors were added to the binding buffer: captopril (25 $\mu$g/mL), bacitracin (0.2 mg/mL), leupeptin (10 $\mu$g/mL), phenylmethylsulfonyl floride (0.19 mg/mL), and aprotinin (5 KIU/mL). $\delta$- and $\kappa$-opioid receptors were blocked with 0.01 M DADLE and 0.01 M U50,488, respectively.

The mixture (1 mL) was incubated for 1 h at room temperature, and then it was filtered under vacuum through glass fibers (GFB, Whatman, soaked for 1 h in 0.1% polyethyleneimine) and washed with ice cold washing buffer (50 mM Tris−HCl, pH 7.4, at 4 °C). The ligand−receptor complex radioactivity retained in the filter was measured by liquid scintillation spectrometry using a scintillator after 12 h of incubation in the scintillation cocktail. All assays were performed in triplicate, and repeated at least three times. Stock solutions ($10^{-2}$ M) were made in dimethyl sulfoxide or methanol/HCl (0.1 N) (1:1 v/v).

**Determination of Inhibition of Cyclic AMP Accumulation.** The activity of endomorphin-1 analogues was determined by measuring the potency for the inhibition of forskolin-stimulated cyclic AMP accumulation in SH-SY5Y cells (obtained from the European Collection of Cell Cultures, passage no. 12). Cells were routinely grown as indicated by the purchaser. A 75 cm flask at 95−100% confluence was split into 24 wells and incubated overnight. When the confluence arrived at 85−100%, the medium was removed and the cells were washed three times with PBS. Then 240 $\mu$L of medium without serum (F12/DMEM (1:1) + 2 mM glutamine + 1% nonessential amino acids (NEAA)) with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma), the following protease inhibitors (captopril 25 ($\mu$g/mL), bacitracin (0.2 mg/mL), leupeptin (10 $\mu$g/mL), phenylmethylsulfonyl fluoride (0.19 mg/mL), and aprotinin (5 IU/mL)), and 0.01 M DADLE ($\delta$-opioid receptors blocker) were added, and the cells were incubated at 37 °C for 10 min. Forskolin (10 $\mu$M) and the ligand of interest at the concentration range studied were added (30 $\mu$L of each, to a final volume of 300 $\mu$L), and the cells were incubated at 37 °C for 15 min. The cells were washed three times with PBS, then 500 $\mu$L of 95% ethanol was added to each well, the cells were incubated at 37 °C for 5 min, then transferred to 1.5 mL tubes, and centrifuged at 6000 rpm, for 5 min, at 4 °C, and then the resulting supernatant was transferred to a new tube. The pellets were washed with 500 $\mu$L of ethanol/water (2:1 v/v) and centrifuged again at 6000 rpm, for 5 min, at 4 °C, and the supernatant was added to the first one. Then the samples were dried under vacuum by speed vac for 4−6 h until completely dried, and then cyclic AMP levels were determined using a commercial kit (Amersham). Each well was determined individually, and the triplicates were averaged, $IC_{50}$ values were determined (Graph PAD Software, San Diego, CA.).

**Enzymatic Digestion of the Tetrapeptides.** Peptides, 4.0 mg, were dissolved in 4.0 mL of Tris (50 nM), pH 7.4, buffer. Solutions were incubated for 30 min before enzyme addition in a thermostatic bath at 37 °C and magnetically stirred. The enzyme solutions were prepared in Tris (50 nM), pH 7.4, as follows: α-chymotripsin (2 mg/10 mL), aminopeptidase-M (0.1 mL (0.5 mg)/5 mL), carboxypeptidase-Y (0.5 mg/5 mL). Experiments were performed in parallel, by adding 1.0 mL portions of the same enzyme solution to each peptide solution. At designated intervals, a 0.50 mL aliquot of incubated mixture was quenched with 15 $\mu$L of 1 N HCl and diluted with 0.2 mL of $CH_3CN$. Sampling times were chosen so that a representative kinetic curve could be constructed. The resulting solution was filtered over PTFE syringe filters, pore diameter 0.20 $\mu$m. Samples were analyzed by HPLC. Blanks were obtained by incubation of peptides in Tris (50 nM), pH 7.4/$CH_3CN$ (5:2) with 1.5 $\mu$L of 1 N HCl for 6 h.

Hydrolysis results were collected in graphics: the amount of starting peptide remaining vs time, given in area % of the initial value calculated from HPLC. To appreciate the different resistances displayed by the peptides, the areas were normalized to 100 at $t$ = 0 and the curves were superimposed. Each hydrolysis experiment was repeated at least twice, and the reported data are mean values. Error ranges were estimated on the basis of the standard deviation, and are substantially similar for the different peptides. Peak attributions were determined by comparison with authentic fragments prepared by coupling the proper α- or $\beta$-amino acids by taking into account the more plausible tetrapeptide decompositions. To confirm the coincidence between authentic fragments and fragments resulting from enzymatic digestion, we used analytical reversed-phase HPLC under two distinct column and solvent systems (Table 3).

**HPLC Purity and Peak Attributions.** Method 1: HP Hypersil ODS column (4.6 $\mu$m particle size, 100 Å pore diameter, 250 mm) with solvent systems A (0.1% TFA in water) and B (0.1% TFA in acetonitrile), gradient 100% A to 50% B in 20 min at a 1.0 mL/min flow, followed by 20 min at 50% B. Method 2: Phenomenex Luna C18 column (5 $\mu$m particle size, 100 Å pore diameter, 250 mm) with solvent systems A (0.1% TFA in water) and B (0.1% TFA in acetonitrile), gradient 95% A to 80% B in 50 min at 1.0 mL/min flow.

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