Amino Acids (2010) 38:1057–1065
DOI 10.1007/s00726-009-0314-z

ORIGINAL ARTICLE

Analogues of both Leu- and Met-enkephalin containing a constrained dipeptide isostere prepared from a Baylis-Hillman adduct

Roberta Galeazzi · Gianluca Martelli · Eleonora Marcucci · Mario Orena · Samuele Rinaldi · Roberta Lattanzi · Lucia Negri

Received: 4 March 2009 / Accepted: 8 June 2009 / Published online: 8 July 2009

Abstract An efficient route was developed for the synthesis of the Fmoc-protected dipeptide **4**, isostere of Gly-Gly containing an α-methylene β-amino acid; the conformationally restricted analogues of Leu-enkephalin, **3a**, and Met-enkephalin, **3b**, respectively, were prepared by changing **4** for Gly^2-Gly^3 in the native compounds **3a** and **3b** whose biological activities were significantly lower than the parent compounds.

Keywords Isostere · Dipeptide · Conformational restriction · Baylis–Hillman · Activity

Introduction

A significant goal of drug discovery is to design small molecules having the key structural and functional elements of biologically active peptides, whereas a major challenge in this area is to define their biologically active conformation, or the bound structure. The strategies aimed at elucidating this structure typically involve introducing conformational restraints at specific sites of the peptide (Olson et al. 1993; Giannis and Kolter 1993; Gante 1994; Hanessian et al. 1997; Goodman and Zhang 1997; Hanessian and Auzzas 2008) and the solution structures of the constrained analogues can then be determined and correlated with binding and biological activity in an iterative process that ultimately provides insights concerning the bound conformation of the peptide. Thus, there is a need for peptide mimics that pre-organize the peptide backbone and the side chains in the biologically active conformation of the native peptide (Gillespie et al. 1997; Hruby et al. 1997; Hruby and Balse 2000; Hruby 2005).

Within an ongoing program directed to identify appropriate peptide replacements that can be useful to elucidate the biologically active conformation of pharmacologically relevant oligopeptides (Galeazzi et al. 2003, 2005a, b, 2007, 2008), we considered Leu-enkephalin **1** and Met-enkephalin **2**, endogeneous peptide ligands towards the δ-opioid receptor isolated from brain tissue (Hughes et al. 1975), that are involved in a lot of physiological processes, their roles in behavior, neuroendocrinology and pain transmission being well documented (Kieffer and Evans 2009; Monnier and Bride 1995; Acosta and Lopez 1999).

H-Tyr^1-Gly^2-Gly^3-Phe^4-Leu^5-OH **1**
H-Tyr^1-Gly^2-Gly^3-Phe^4-Met^5-OH **2**

As for other bioactive endogenous peptides, there are several major considerations that limit the clinical applications of naturally occurring enkephalins including selectivity for receptor subtypes, modest absorption, and relatively low central bioavailability (Chen et al. 2001). Moreover, both enkephalins undergo rapid degradation under physiological conditions (Fernández et al. 2002; Carrera et al. 2005). Since cleavage performed by peptidases can be prevented by the incorporation in the peptide chains of non-natural amino acids, every change at these positions could increase the lifetime of the peptides in vivo, thus increasing the biological activity (Shinada et al. 2007).

A single crystal X-ray diffraction study of Leu-enkephalin and Met-enkephalin was reported (Smith and Griffin

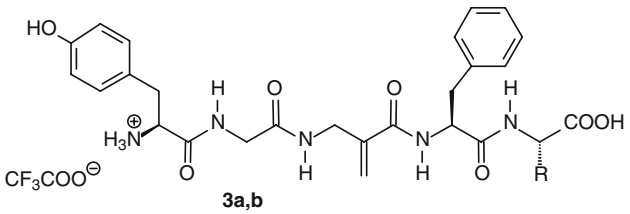
R. Galeazzi · G. Martelli · E. Marcucci · M. Orena (✉) · S. Rinaldi
Dipartimento I.S.A.C., Università Politecnica delle Marche, Via Brecce Bianche, 60131 Ancona, Italy
e-mail: m.orena@univpm.it

R. Lattanzi · L. Negri
Dipartimento di Fisiologia e Farmacologia "Vittorio Erspamer", Università di Roma, La Sapienza, 00815 Rome, Italy

1978) and the molecule in the solid state was shown to display a Gly^2-Gly^3 β-turn stabilized by two intramolecular hydrogen bonds between the CO and NH groups of Tyr^1 and the NH and CO groups of Phe^4. In addition, although enkephalin itself has a random structure in water, a β-turn conformation at the Gly^2-Gly^3 positions seems to be important for its biological activity (Hersh 1982; Currie et al. 1993; Martin et al. 1998; Maricic et al. 2002; Eguchi et al. 1999, 2002; Rew and Goodman 2002; Gu et al. 2004) and conformational calculations of enkephalins were carried out in order to assign their three-dimensional structures (Meirovitch et al. 1994; Koca and Carlsen 1995). Determining the three-dimensional structures of enkephalins, **1** and **2**, bound to the μ- and δ-opioid receptors was the focus of numerous investigations.(Belleney et al. 1989; Wu et al. 2007) A number of conformationally constrained enkephalin derivatives were prepared that display high potency and selectivity (Lee et al. 2007) thus suggesting that a 5 → 2 β-turn might be an important structural motif in the biologically active conformation of these important neuropeptides (Blomberg et al. 2006). On the basis of modeling studies, we reasoned that replacing the Gly^2-Gly^3 subunit with **4** might stabilize such a reverse turn via the geometric constraints due to the methylene group. Consequently, the pseudopeptides **3a** and **3b**, analogues of Leu- and Met-enkephalin, respectively, were selected as the primary targets for synthesis (Scheme 1).

In order to confirm the relevance of the structural constraints introduced by the methylene insertion in inducing a peculiar secondary structure rearrangement for the designed pseudopeptides, i.e. the predicted β-turn arrangement, a molecular modeling investigation was carried out. Thus, the conformational space of compound **3a** in its zwitterionic form was undertaken by means of the standard quenched molecular dynamics (QMD) protocol, which have already shown to predict reliable distribution of conformers (O'Connor et al. 1992) (Carstens et al. 2005) using AMBER force field (Weiner et al. 1986) together with implicit solvation model GA/SA in water (Still et al. 1990), to take account of the biological environment.

All the stable conformations showed common structural features which correspond indeed to a real β-turn



3a,b

a. R = $CH_2CH(CH_3)_2$; **b.** R = $CH_2CH_2SCH_3$

Scheme 1

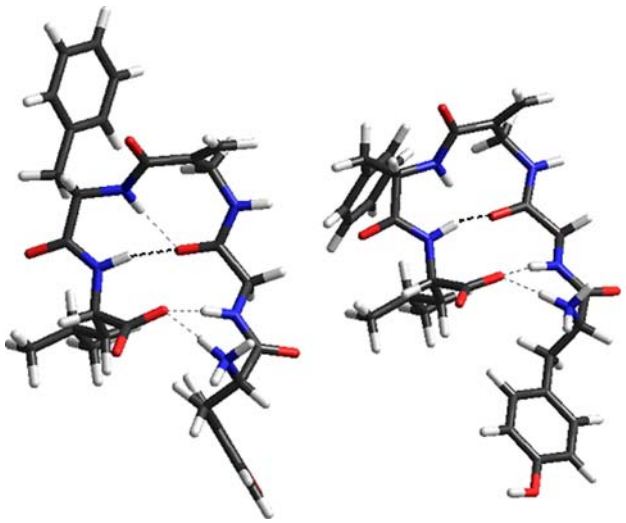
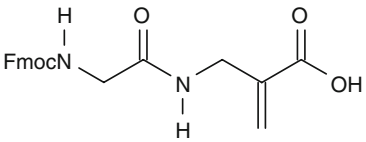


Fig. 1 Geometries of the two lowest energy conformers for pseudopeptide **3a** [*Left* lowest energy minimum. $E = 0.0$ kcal/mol (−262.12 kcal/mol): $\phi_1 = -54.4$, $\psi_1 = -177.3$, $\phi_2 = 132.7$, $\psi_2 = 179.3$, $\phi_3 = -110.2$, $\theta_3 = 27.6$, $\psi_3 = -174.7$, $\phi_4 = 12.7$, $\psi_4 = -179.7$, $\phi_5 = -146.6$, $\psi_5 = 135.8$. *Right* Conformer c2. $E = 0.53$ kcal/mol (−261.59 kcal/mol)]

Scheme 2



4

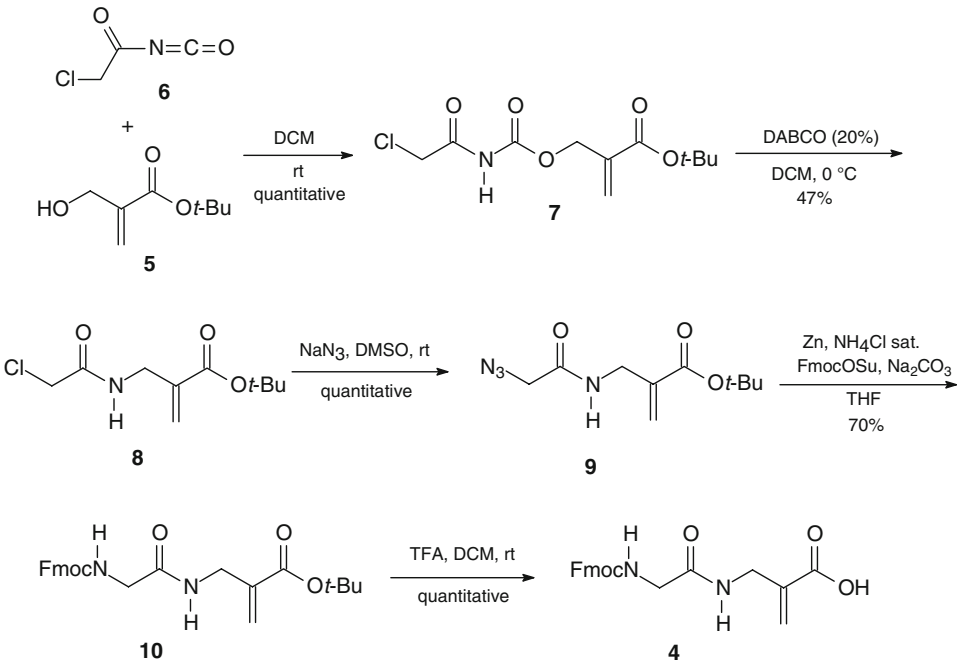
arrangement, and stabilized by the classical H-bond between the C = O of Gly^2 and the NH of the Leu^5 forming a 11-membered ring (not a 10 because of the presence of the β-amino acid Ψ-Gly^3) (Fig. 1). Furthermore this secondary structure is stabilized strongly also by the presence of other H-bonding interactions formed directly between the charged terminal carboxylate and ammonium groups (Sanbonmatsu and García 2002).

Thus, with the aim to identify appropriate changes, we evaluated the Ψ-dipeptide **4** that was chosen for insertion in both Leu- and Met-enkephalin as novel mimic of the Gly-Gly unit, also considering that replacement of Gly^3 for dehydroalanine strongly enhances the biological activity of the enkephalins (Shimoigashi and Stammer 1982; Shimoigashi et al. 1982) (Scheme 2).

Synthesis of the enkephalin analogues 3a and 3b

A significant challenge associated with the synthesis of peptidomimetics **3a** and **3b** was the design of a convenient entry to the dipeptide isostere **4** originating from non-amino acid precursors. To address this problem, we

Scheme 3



developed a straightforward approach to **4** starting from the alcohol **5** (Byun et al. 1994) that was treated with an equimolar amount of chloroacetyl isocyanate, **6** (Speziale and Smith 1963) to give the corresponding acyl carbamate **7** in very good yield (Ciclosi et al. 2002) (Scheme 3). Reaction of **7** with DABCO in DCM gave the amide **8** through two subsequent S_N' reactions whereas substitution of the halogen, performed with sodium azide in DMSO, gave the azide **9** in good yield.

The elaboration of the *N*-terminus of **9** involved reduction of the azido group with Zn in NH_4Cl aqueous solution, which was performed in the presence of Fmoc–OSu, with the aim to directly obtain the corresponding *N*-Fmoc derivative **10**. Eventual cleavage of the *t*-butyl ester, carried out with TFA in DCM, afforded the key acid **4**.

The hydrochlorides of dipeptides Phe-Leu-OMe, **11a**, and Phe-MetOMe, **11b**, underwent *N*-acylation with **4** under standard peptide coupling conditions (EDCl in DCM), to give in good yield **12a** or **12b**, respectively (Scheme 4).

However, at the outset of these investigations removal of the Fmoc protecting group in both **12a** and **12b** under usual conditions (Carpino, 1987; Flinn et al. 1995) resulted difficult and heating with 1 equiv DABCO in dry DCM was required in order to prepare derivatives **13a** and **13b** bearing the free amino group. Again, by using standard peptide coupling (EDCl in DCM) and starting from commercial *t*-Bu,*t*-Boc-tyrosine, the full protected analogues **14a** and **14b** were obtained in good yield. Eventually, by cleavage of the methyl ester moiety and of both the *t*-butyl and *t*-Boc protecting groups, the corresponding trifluoroacetates **3a** and **3b** were obtained.

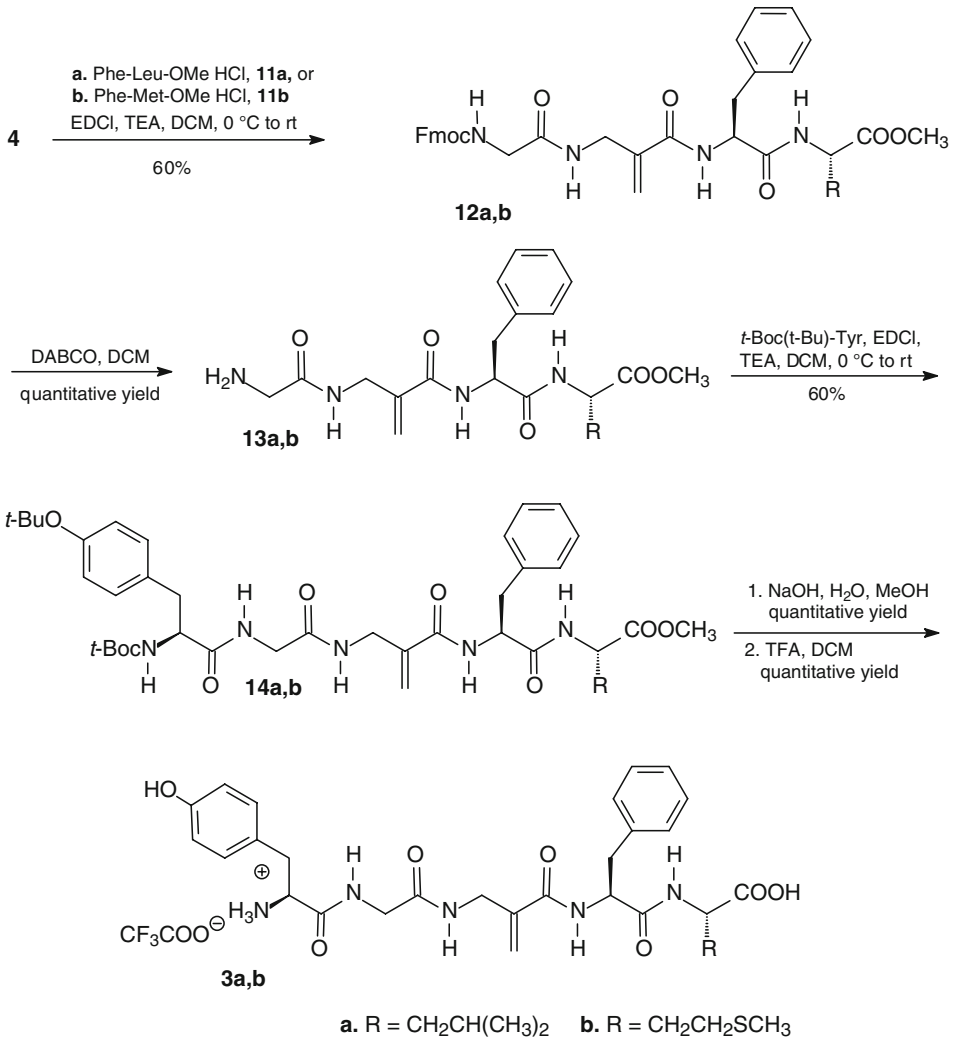
Biological assays

The opioid activities of the compound **3a** and **3b** were evaluated in vitro using mouse vas deferens (MVD, rich in δ-opioid receptors) and guinea pig ileum (GPI, rich in μ-opioid receptors) in comparison with the activity of deltorphin I, a full agonist highly selective for δ-opioid receptors, and dermorphin, a full agonist highly selective for μ-opioid receptors. Comparison was also carried out against Leu-enkephalin, **1**, and Met-enkephalin, **2** (Table 1). Compounds **3a** and **3b** behaved as partial agonists both on MVD and on GPI. Whereas **3b** displayed higher potency on MVD than on GPI, indicating a preferential affinity for δ-receptors than for μ-receptors, **3a** displayed comparable low potency on both MVD and GPI.

Conclusions

Two conformationally constrained analogues of Leu-enkephalin and Met-enkephalin that contain the novel dipeptide mimic **4** were prepared. A concise and efficient protocol was developed for the synthesis of this dipeptide isostere, which may be applied to the preparation of analogues of other peptides. Our investigation indicated that the incorporation of compound **4** had little effect concerning the activity of derivatives **3a**, **b**, and highlighted that insertion of a methylene group, with respect to dehydroalanine (Shimoigashi et al. 1982) leads to a dramatic loss of activity.

Scheme 4



Experimental

Melting points were measured on an Electrothermal IA 9,000 apparatus and are uncorrected. Ir spectra were recorded in $CHCl_3$ on a Nicolet Fourier Transform Infrared 20-SX spectrophotometer. ^1H and ^{13}C NMR spectra were recorded at 200 and 50 MHz, respectively, on a Varian Gemini 200 spectrometer, using $CDCl_3$ as a solvent unless otherwise stated. Chemical shifts (δ) are reported in ppm relative to TMS and coupling constants (J) in Hz. Assignments were aided by decoupling and homonuclear two-dimensional experiments. Optical rotations were measured on a Perkin Elmer 341 polarimeter. The samples were analyzed with a liquid chromatography Agilent Technologies HP1100 equipped with a Zorbax Eclipse XDB-C8 Agilent and Technologies column (flow rate 0.5 mL/min) and equipped with a diode-array UV detector (220 and 254 nm). Acetonitrile and methanol for HPLC were purchased from a commercial supplier. All the samples were prepared by diluting 1 mg in 5 mL of a 1:1 mixture of H_2O and acetonitrile in pure acetonitrile or in pure methanol. The MSD1100 mass detector was utilized under the following conditions: mass range 100–2,500 uma, positive scanning, energy of fragmentor 50 V, drying gas flow (nitrogen) 10.0 mL/min, nebulizer pressure 45 psig, drying gas temperature 350°C, capillary voltage 4,500 V. Column chromatography was performed with silica gel 60 (230–400 mesh). Compound **5** was synthesized according (Byun et al. 1994); L-Phe-L-Leu-OMe

Table 1 Functional biological activity of compounds **3a** and **3b**

	MVD (δ) IC_{50} (nM)	GPI (μ) IC_{50} (nM)
Dermorphine	15 ± 2	1.24 ± 0.2
Deltorphine	0.4 ± 0.03	1,300 ± 150
Met-enkephalin, **1**	17.3 ± 1.9	90.1 ± 8.7
Leu-enkephalin, **2**	12.1 ± 1.5	504.2 ± 43
3a	3,000.0 ± 445	2,200.0 ± 355
3b	202.0 ± 37.5	990.0 ± 105

hydrochloride **11a** was purchased from Chem-Impex International, Wood Dale, IL, USA, and L-Phe-L-Met-OMe hydrochloride **11b** from Astatech, Princeton, MA, USA.

t-Butyl 3-(chloroacetylaminocarbonyloxy)-2-methylenepropanoate (**7**)

A solution containing compound **5** (Byun et al. 1994) (2.2 g; 13 mmol) and chloroacetyl isocyanate **6** (Speziale and Smith 1963) (15 mmol) in DCM (20 mL) was stirred for 3 h at rt. After removal of the solvent, the residue was purified by silica gel chromatography (cyclohexane:ethyl acetate 50:50) to give the *N*-acyl carbamate **7** (3.2 g; 89% yield) as a colorless oil. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.50 (s, 9H), 4.49 (s, 2H), 4.88 (s, 2H), 5.83 (s, 1H), 6.33 (s, 1H), 7.97 (s, 1H, NH). ^{13}C NMR (50 MHz, $CDCl_3$): δ 28.0, 43.6, 64.8, 81.8, 127.9, 135.8, 150.7, 164.0, 166.6. ESI–MS: *m/z* 278.1 $[MH]^+$, 281.1 $[MH+2]^+$, 300.1 $[M+Na]^+$, 302.1 $[M+2+Na]^+$. Anal. calcd for $C_{11}H_{16}ClNO_5$: C, 47.58; H, 5.81; N, 5.04. Found: C, 47.61; H, 5.75; N, 5.01.

t-Butyl 3-(chloroacetylamino)-2-methylenepropanoate (**8**)

To a solution containing the *N*-acyl carbamate **7** (1.4 g; 5 mmol) in DCM (20 mL), DABCO (0.11 g; 1.0 mmol) was added and the misture was stirred for 10 min at rt. After dilution with ethyl acetate (100 mL), the organic layer was washed with 1 M HCl (30 ml) and then with brine (50 mL). After drying (Na_2SO_4), the solvent was removed under reduced pressure and the residue was purified by silica gel chromatography (cyclohexane:ethyl acetate 80:20), to give compound **8** (0.91 g; 78% yield) as white solid. Mp: 58–60°C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.51 (s, 9H), 4.06 (s, 2H), 4.11 (d, $J = 6.6$ Hz, 2H), 5.73 (s, 1H), 6.20 (s, 1H), 7.08 (br s, 1H, NH); ^{13}C NMR (50 MHz, $CDCl_3$): δ 28.0, 40.8, 42.6, 81.6, 126.5, 137.3, 165.0, 165.6. ESI–MS: *m/z* 234.1 $[MH]^+$, 236.1 $[MH+2]^+$, 256.1 $[M+Na]^+$, 258.1 $[M+2+Na]^+$. Anal. calcd for $C_{10}H_{16}ClNO_3$: C, 51.40; H, 6.90; N, 5.99. Found: C, 51.37; H, 6.86; N, 6.01.

t-Butyl 3-(azidoacetylamino)-2-methylenepropanoate (**9**)

To a solution of compound **8** (1.2 g; 5 mmol) in DMSO (5 mL), NaN_3 (0.65 g; 10 mmol) was added and the suspension was stirred for 20 h at rt. Then H_2O (20 mL) was added and the mixture was extracted with ethyl acetate (2 × 100 mL). After drying (Na_2SO_4) the solvent was removed under reduced pressure and the residue was purified by silica gel chromatography (cyclohexane:ethyl acetate 70:30), to give the azide **9** (1.2 g; quantitative yield) as colorless oil. IR ($CHCl_3$): 3302, 2106, 1704, 1667 cm^{-1}. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.51 (s, 9H), 3.99 (s, 2H), 4.10 (d, $J = 6.2$ Hz, 2H), 5.73 (s, 1H), 6.19 (s, 1H), 6.79 (br s, 1H, NH); ^{13}C NMR (50 MHz, $CDCl_3$): δ 27.9, 40.5, 52.7, 81.5, 126.4, 137.4, 165.2, 166.3. ESI–MS: *m/z* 241.2 $[MH]^+$, 263.1 $[M+Na]^+$. Anal. calcd for $C_{10}H_{16}N_4O_3$: C, 49.99; H, 6.71; N, 23.32. Found: C, 49.95; H, 6.68; N, 23.36.

t-Butyl 3-(Fmoc-glicylamino)-2-methylenepropanoate (**10**)

To a solution containing the azide **9** (1.32 g; 5.5 mmol) and FmocOSu (5.56 g; 16.5 mmol) in THF (25 mL) a saturated NH_4Cl aqueous solution (5.5 mL) was added at rt, followed by Zn (1.07 g; 16.5 mmol) under vigorous stirring. After 10 min a saturated Na_2CO_3 aqueous solution was added until pH = 9 and then the mixture was stirred for 12 h. After addition of H_2O (30 mL) and ethyl acetate (50 mL), the mixture was extracted with ethyl acetate (2 × 50 mL), the organic layer wad dried (Na_2SO_4) and solvents removed under reduced pressure. The residue was purified by silica gel chromatography (cyclohexane:ethyl acetate 90:10 as eluent) to give the product **10** (1.68 g; 70% yield) as a white amorphous solid. Mp: 49–51°C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.49 (s, 9H), 3.86 (d, $J = 5.6$ Hz, 2H), 4.08 (d, $J = 6.2$ Hz, 2H), 4.22 (t, $J = 6.9$ Hz, 1H), 4.43 (d, $J = 6.9$ Hz, 2H), 5.46 (t, $J = 6.7$ Hz, 1H, NH), 5.70 (s, 1H), 6.16 (s, 1H), 6.42 (t, $J = 6.7$ Hz, 1H, NH), 7.22–7.81 (m, 8 ArH); ^{13}C NMR (50 MHz, $CDCl_3$): δ 28.0, 40.5, 44.6, 47.1, 67.2, 81.4, 120.0, 125.0, 126.0, 127.0, 127.7, 128.4, 128.6, 131.9, 132.1, 137.6, 141.2, 143.7, 156.5, 165.3, 168.6. ESI–MS: *m/z* 437.4 $[MH]^+$, 459.3 $[M+Na]^+$. Anal. Calcd for $C_{25}H_{28}N_2O_5$: C, 68.79; H, 6.47; N, 6.42. Found: C, 68.75; H, 6.44; N, 6.45.

3-(Fmoc-glicylamino)-2-methylenepropanoic acid (**4**)

To a solution of the ester **10** (0.5 g; 1.14 mmol) in dry DCM (2.5 mL), trifluoroacetic acid (1.31 mL; 17.1 mmol) was added at rt. After 10 min volatiles were removed under reduced pressure and the residue was washed with dry ethyl ether to give the product **4** as a white solid (0.44 g; quantitative yield). Mp: 67–69°C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.87 (d, $J = 5.9$ Hz, 3H), 0.88 (d, $J = 5.9$ Hz, 3H), 1.41–1.55 (m, 3H), 3.11 (d, $J = 7.0$ Hz, 2H), 3.68 (s, 3H), 3.77–3.89 (m, 2H), 4.00 (dd, $J = 5.8$ Hz, $J = 15.4$ Hz, 1H), 4.14–4.27 (m, 2H), 4.43 (d, $J = 7.0$ Hz, 2H), 4.46–4.71 (m, 2H), 5.57 (s, 1H), 5.64 (bt, $J = 6.9$ Hz, 1H, NH), 5.77 (s, 1H), 6.38 (d, $J = 8.3$, 1H, NH), 6.63 (t, $J = 6.5$ Hz, 1H, NH), 6.90 (d, $J = 7.2$ Hz, 1H, NH), 7.12–7.42 (m, 9 ArH), 7.58 (d, $J = 7.0$ Hz, 2 ArH), 7.77 (d, $J = 6.6$ Hz, 2 ArH) ^{13}C NMR (50 MHz, $CDCl_3$): δ 41.3, 44.9, 47.4, 68.3, 120.6, 125.4, 127.6, 128.4, 130.9, 135.3, 141.8, 143.8, 157.9, 170.3, 171.6. ESI–MS: *m/z* 381.1

$[MH]^+$, 403.2 $[M+Na]^+$. Anal. Calcd for $C_{21}H_{20}N_2O_5$: C, 66.31; H, 5.30; N, 7.36. Found: C, 66.26; H, 5.25; N, 7.41.

[3-(Fmoc-glicylamino)-2-methylenepropanoyl]-L-phenylalaninyl-L-leucine methyl ester (**12a**)

To a solution containing the acid **4** (1.0 g; 2.6 mmol), the L-Phe-L-Leu-OMe hydrochloride 11a (1.06 g; 2.6 mmol) and EDCl (0.55 g; 2.86 mmol) in dry DCM (15 mL), Et_3N (361 μL; 2.6 mmol) was added and the mixture was stirred for 3 h at rt. Then water (20 mL) was added and the mixture was extracted with ethyl acetate (3 × 50 mL). After drying (Na_2SO_4) and removal of the solvent under (reduced pressure, the residue was purified by silica gel chromatography (ethyl acetate as eluent) to give the compound **12a** (1.14 g; 66% yield) as a white solid. Mp: 65–67°C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.87 (d, $J = 5.9$ Hz, 3H), 0.88 (d, $J = 5.9$ Hz, 3H), 1.48–1.74 (m, 3H), 3.11 (d, $J = 7.0$ Hz, 2H), 3.68 (s, 3H), 3.84 (dd, $J = 6.2$ Hz, $J = 8.0$ Hz, 2H), 4.00 (dd, $J = 5.8$ Hz, $J = 15.4$ Hz, 1H), 4.13–4.28 (m, 2H), 4.43 (d, $J = 7.0$ Hz, 1H), 4.45–4.55 (m, 1H), 4.64–4.71 (m, 1H), 5.57 (s, 1H), 5.63 (br s, 1H, NH), 5.77 (s, 1H), 6.38 (d, $J = 8.3$ Hz, 1H, NH), 6.27 (t, $J = 6.2$ Hz, 1H, NH), 6.90 (d, $J = 7.2$ Hz, 1H, NH), 7.13–7.45 (m, 9 ArH), 7.58 (d, $J = 7.0$ Hz, 2 ArH) 7.67 (d, $J = 6.6$ Hz, 2 ArH); ^{13}C NMR (50 MHz, $CDCl_3$): δ 21.8, 22.6, 24.7, 37.9, 40.8, 41.1, 47.0, 50.8, 52.2, 54.6, 60.3, 67.1, 119.9, 122.4, 125.0, 126.9, 127.0, 127.7, 128.4, 128.6, 129.0, 129.2, 129.8,13 6.5, 140.0, 141.2, 143.7, 156.6, 166.8, 169.7, 170.8, 173.2; $[\alpha]_D$ −31.6 (c 0.5, $CHCl_3$). ESI–MS: *m/z* 655.2 $[MH]^+$, 677.2 $[M+Na]^+$. Anal. Calcd for $C_{37}H_{42}N_4O_7$: C, 67.87; H, 6.47; N, 8.56. Found: C, 67.81; H, 6.42; N, 8.61.

(3-Glicylamino-2-methylenepropanoyl)-L-phenylalaninyl-L-leucine methyl ester (**13a**).

To a solution containing the compound **12a** (505 mg; 0.77 mmol) in dry DCM (10 mL), DABCO (87 mg; 0.77 mmol) was added and the clear solution was refluxed for 6 h and stirred for further 12 h at rt. After removal of the solvent under reduced pressure, the residue was purified by silica gel chromatography (ethyl acetate:methanol 90:10 as eluent) to give **13a** (0.32 g; 94% yield) as a white solid. Mp: 47–48°C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.89 (d, $J = 6.1$ Hz, 6H), 1.34–1.63 (m, 3H), 2.81 (br s, 2H, NH_2), 3.05–3.21 (m, 2H), 3.20–3.41 (m, 2H), 3.69 (s, 3H), 4.04 (dd, $J = 4.4$ Hz, $J = 15.2$ Hz, 1H), 4.15 (dd, $J = 6.0$ Hz, $J = 15.2$ Hz, 1 H), 4.46–4.60 (m, 1H), 4.72 (dt, $J = 6.6$ Hz, $J = 7.6$ Hz, 1H), 5.55 (s, 1H), 5.94 (s, 1H), 6.59 (d, $J = 7.6$ Hz, 1H, NH), 7.15–7.35 (m, 5 ArH), 7.48 (d, $J = 8.0$ Hz, 1H, NH), 7.51 (dd, $J = 4.4$ Hz, $J = 6.0$ Hz, 1 H, NH); ^{13}C NMR (50 MHz, $CDCl_3$): δ 21.9, 22.7, 24.7, 37.5, 40.4, 41.3, 44.1, 50.9, 52.2, 54.8, 123.6, 126.8, 128.5, 129.1, 129.2, 136.8, 139.9, 166.5, 170.8, 173.0, 173.1. $[\alpha]_D$ −22.8 (c 0.5, $CHCl_3$). ESI–MS: *m/z* 433.2 $[MH]^+$, 455.2 $[M+Na]^+$. Anal. Calcd for $C_{22}H_{32}N_4O_5$: C, 61.09; H, 7.46; N, 12.95. Found: C, 61.02; H, 7.51; N, 12.88.

[3-(*t*-BuO,*t*-Boc-L-tyrosylglicylamino)-2-methylenepropanoyl]-L-phenylala-ninyl-L-leucine methyl ester (**14a**)

To a solution containing the amino derivative **13a** (310 mg; 0.71 mmol) and *t*-Boc,*t*-Bu-tyrosine (241 mg; 0.71 mmol) in DCM (8.0 mL), EDCl (164 mg; 0.85 mmol) was added and the mixture was stirred for 3 h at rt. Then water (10 mL) was added and the mixture was extracted with ethyl acetate (3 × 50 mL). After drying (Na_2SO_4) and removal of the solvents under reduced pressure, the residue was purified by silica gel chromatography (ethyl acetate as eluent), to give the title product **14a** (0.34 g; 63% yield) as a white foam. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.89 (d, $J = 6.0$ Hz, 6H), 1.32 (s, 9H), 1.36 (s, 9H), 1.42–1.64 (m, 3H), 2.92–3.20 (m, 4 H), 3.68 (s, 3H), 3.74 (dd, $J = 5.4$ Hz, $J = 15.1$ Hz, 1H), 3.92 (dd, $J = 6.2$ Hz, $J = 15.1$ Hz, 1H), 4.02–4.10 (m, 2H), 4.16–4.27 (m, 1H), 4.45–4.69 (m, 2H), 4.98 (d, $J = 6.5$ Hz, 1H, NH), 5.52 (s, 1H), 5.82 (s, 1H), 6.54 (d, $J = 6.9$ Hz, 1H, NH), 6.93 (d, $J = 8.5$ Hz, 2 ArH), 7.11 (d, $J = 8.5$ Hz, 2 ArH), 7.15–7.42 (m, 8H, 5 ArH + 2 NH); ^{13}C NMR (50 MHz, $CDCl_3$): δ 21.9, 22.7, 24.7, 28.2, 28.8, 37.7, 40.9, 41.2, 42.9, 50.9, 52.2, 54.9, 56.5, 78.2, 80.5, 122.7, 124.3, 126.8, 128.5, 129.2, 129.6, 131.1, 136.7, 1400, 154.4, 166.9, 169.4, 171.0, 172.2, 173.2; $[\alpha]_D$ −12.6 (c 0.5, $CHCl_3$). ESI–MS: *m/z* 752.4 $[MH]^+$, 774.3 $[M+Na]^+$. Anal. Calcd for $C_{40}H_{57}N_5O_9$: C 63.90; H 7.64; N 9.31. Found: C, 63.81; H, 7.69; N, 9.41.

(3-L-Tyrosylglicylamino-2-methylenepropanoyl)-L-phenylalaninyl-L-leucine trifluoroacetate (**3a**)

The ester **14a** (0.24 g; 0.31 mmol) was dissolved in methanol (3 mL) and then 1 M NaOH aqueous solution was added (1.55 equiv; 210 μL). After stirring for 4 h at rt, the mixture was extracted with ethyl acetate (2 × 10 mL), the aqueous phase cooled to 0°C and 1 M HCl was added until pH = 2. After extraction with ethyl acetate (2 × 10 mL), the solvent was dried and eventually removed under reduced pressure, to give a residue which crystallized on standing To a solution of the this solid in DCM (3 mL), TFA (0.513 g; 4.5 mmol) was added. After stirring for 7 h at rt, the solvent was removed under reduced pressure and the residue was washed with dry ethyl ether (2 × 5 mL), to give the trifluoroacetate **3a** (0.22 g; quantitative yield) as a white solid. Mp: 44–45°C.

^{1}H NMR (200 MHz, DMSO-d_6): δ 0.87 (d, J = 6.0 Hz, 3H), 0.93 (d, J = 6.0 Hz, 3H), 1.47–1.77 (m, 3H), 2.78 (dd, J = 8.2 Hz, J = 15.0 Hz, 1H), 2.91–3.15 (m, 3H), 3.22–3.33 (m, 1H), 3.34 (bs, 3H, NH_3^+), 3.68–4.07 (m, 4H), 4.19–4.32 (m, 1H), 4.51–4.68 (m, 1H), 5.34 (s, 1H), 5.76 (s, 1H), 6.72 (d, J = 8.3 Hz, 2 ArH), 7.07 (d, J = 8.3 Hz, 2 ArH), 7.16–7.35 (m 5 ArH), 8.07–8.18 (m, 2H, NH), 8.31 (d, J = 7.5 Hz, 1H, NH), 9.38 (s, 1H, OH); ^{13}C NMR (50 MHz, DMSO-d_6): δ 21.4, 22.8, 24.3, 36.2, 38.1, 40.2, 40.6, 41.8, 50.3, 53.7, 54.9, 115.3, 124.7, 126.2, 128.0, 129.1, 10.4, 138.2, 140.4, 156.5, 166.2, 168.1, 168.5, 171.3, 173.9; $[\alpha]_D$ −11.9 (c 0.5, $CHCl_3$). ESI–MS: *m/z* 582.3 $[MH]^+$, 604.3 $[M+Na]^+$. Anal. Calcd for $C_{32}H_{40}F_3N_5O_9$: C, 55.34; H, 5.79; N, 10.07. Found: C, 55.25; H, 5.82; N, 10.01.

[3-(Fmoc-glicylamino)-2-methylenepropanoyl]-L-phenylalaninyl-L-methionine methyl ester (**12b**)

To a solution containing the acid **4** (0.88 g; 2.3 mmol), the hydrochloride **11b** (0.95 g; 2.3 mmol) and EDCl (0.49 g; 2.5 mmol) in dry DCM (15 mL), Et_3N (320 μL; 2.3 mmol) was added and the mixture was stirred for 3 h at rt. Then water (20 mL) was added and the mixture was extracted with ethyl acetate (3 × 50 mL). After drying (Na_2SO_4) and removal of the solvent under reduced pressure, the residue was purified by silica gel chromatography (ethyl acetate as eluent) to give the compound **12b** (0.97 g; 64% yield) as a white solid. Mp: 68–70°C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.88–2.17 (m, 2H), 2.01 (s, 3H), 2.32–2.43 (m, 2H), 3.07 (dd, J = 7.6 Hz, J = 14.7 Hz, 1H), 3.15 (dd, J = 6.5 Hz, J = 14.7 Hz, 1H), 3.68 (s, 3H), 3.73–4.00 (m, 3H), 4.17–4.28 (m, 2H), 4.43 (d, J = 7.3 Hz, 2H), 4.57–4.71 (m, 2H), 5.56 (s, 1H), 5.72 (t, J = 6.7 Hz, 1H, NH), 5.82 (s, 1H), 6.68 (bt, J = 7.2 Hz, 1H, NH), 6.74 (d, J = 7.7 Hz, 1H, NH), 7.03 (d, J = 7.6 Hz, 1H, NH), 7.16–7.44 (m, 9 ArH), 7.58 (d, J = 7.0 Hz, 2 ArH), 7.76 (d, J = 7.4 Hz, 2 ArH); ^{13}C NMR (50 MHz, $CDCl_3$): δ 15.3, 29.7, 31.3, 37.6, 41.0, 44.6, 47.1, 51.5, 52.5, 54.9, 67.2, 120.0, 123.3, 125.0, 127.1, 127.8, 128.7, 129.2, 136.4, 139.8, 141.3, 143.7, 166.6, 169.9, 170.7, 172.2; $[\alpha]_D$ −16.4 (c 0.5, $CHCl_3$). ESI–MS: *m/z* 673.2 $[MH]^+$, 695.1 $[M+Na]^+$. Anal. Calcd for $C_{36}H_{40}N_4O_7S$: C, 64.27; H, 5.99; N, 8.33. Found: C, 64.18; H, 6.09; N, 8.24.

(3-Glicylamino-2-methylenepropanoyl)-L-phenylalaninyl-L-methionine methyl ester (**13b**)

To a solution containing the compound **12b** (513 mg; 0.77 mmol) in dry DCM (10 mL), DABCO (87 mg; 0.77 mmol) was added and the clear solution was refluxed for 7 h and stirred for further 12 h at rt. After removal of the solvent under reduced pressure, the residue was purified by silica gel chromatography (ethyl acetate:methanol 80:20 as eluent) to give the compound **13b** (0.32 g; 94% yield) as a colorless oil. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.83–2.19 (m, 2H), 2.04 (s, 3H), 2.38–2.46 (m, 2H), 2.88 (br s, 2H, NH_2), 2.95–3.21 (m, 2H), 3.25–3.39 (m, 2H), 3.70 (s, 3H), 3.98 (dd, J = 4.2 Hz, J = 15.1 Hz, 1H), 4.20 (dd, J = 6.1 Hz, J = 15.1 Hz, 1 H), 4.55–4.66 (m, 1H), 4.66–4.78 (m, 1H), 5.55 (s, 1H), 5.97 (s, 1H), 6.97 (d, J = 7.7 Hz, 1H, NH), 7.18–7.35 (m, 5 ArH), 7.65 (d, J = 7.8 Hz, 1H, NH), 7.81 (dd, J = 4.2 Hz, J = 6.1 Hz, 1H, NH); ^{13}C NMR (50 MHz, $CDCl_3$): δ 15.3, 29.7, 31.4, 37.3, 40.3, 44.2, 51.5, 52.4, 55.1, 124.3, 126.8, 128.5, 129.2, 136.8, 139.8, 166.3, 170.7, 172.0, 173.3; $[\alpha]_D$ −13.4 (c 0.5, $CHCl_3$). ESI–MS: *m/z* 451.2 $[MH]^+$, 473.2 $[M+Na]^+$. Anal. Calcd for $C_{21}H_{30}N_4O_5S$: C, 55.98; H, 6.71; N, 12.44. Found: C, 56.06; H, 6.63; N, 12.37.

[3-(*t*-BuO,*t*-Boc-L-tyrosylglicylamino)-2-methylenepropanoyl]-L-phenylalaninyl-L-methionine methyl ester (**14b**)

To a solution containing the amino derivative **13b** (0.55 g; 1.25 mmol) and *t*-Boc,*t*-Bu-tyrosine (0.42 g; 1.25 mmol) in DCM (15 mL), EDCl (288 mg; 1.5 mmol) was added and the mixture was stirred for 2 h at rt. Then water (10 mL) was added and the mixture was extracted with ethyl acetate (3 × 50 mL). After drying (Na_2SO_4) and removal of the solvents under reduced pressure, the residue was purified by silica gel chromatography (ethyl acetate as eluent), to give the title product **14b** (0.58 g; 51% yield) as a white foam. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.32 (s, 9H), 1.35 (s, 9H), 1.88–2.18 (m 2H), 2.04 (s, 3H), 2.31–2.49 (m, 2H), 2.92 (dd, J = 7.8 Hz, J = 14.7 Hz, 1H), 3.01 (dd, J = 6.2 Hz, J = 15.2 Hz, 1H), 3.09 (dd, J = 8.4 Hz, J = 15.2 Hz, 1H), 3.17 (dd, J = 5.9 Hz, J = 14.7 Hz, 1H), 3.68 (s, 3H), 3.86 (d, J = 5.9 Hz, 2H), 4.01–4.10 (m, 2H), 4.19–4.31 (m, 1H), 4.54–4.72 (m, 2H), 5.04 (d, J = 5.2 Hz, 1H, NH), 5.54 (s, 1H), 5.88 (s, 1H), 6.84–6.94 (m, 3H, 2 ArH + 1 NH), 7.08 (d, J = 8.6 Hz, 2 ArH), 7.15–7.33 (m, 6H, 5 ArH + 1 NH), 7.52 (d, J = 5.7 Hz, 1H, NH); ^{13}C NMR (50 MHz, $CDCl_3$): δ 15.3, 28.3, 28.8, 29.7, 31.2, 37.2, 37.3 (50%), 37.4 (50%), 41.0, 42.9, 51.5, 52.5, 55.3, 56.7, 78.5, 80.8, 124.1, 124.4, 126.9, 128.6, 129.2, 129.6, 129.7, 130.9, 136.7, 139.6, 154.5, 166.5, 169.8, 171.1, 172.0, 172.3; $[\alpha]_D$ −22.4 (c 0.5, $CHCl_3$). ESI–MS: *m/z* 770.4 $[MH]^+$, 792.3 $[M+Na]^+$. Anal. Calcd for $C_{39}H_{55}N_5O_9S$: C, 60.84; H, 7.20; N, 9.10. Found: C, 60.75; H, 7.27; N, 9.01.

(3-L-Tyrosylglicylamino-2-methylenepropanoyl)-L-phenylalaninyl-L-methionine trifluoroacetate (**3b**)

The ester **14b** (0.39 g; 0.51 mmol) was dissolved in methanol (5 mL) and then 1 M NaOH aqueous solution (0.8 mL; 0.8 mmol) was added. After stirring for 5 h at rt, the mixture was extracted with ethyl acetate (2 × 10 mL),

the aqueous phase cooled to 0°C and 1 M HCl was added until pH = 2. After extraction with ethyl acetate (2 × 10 mL), the solvent was dried and eventually removed under reduced pressure, to give a residue which crystallized on standing. The solid was dissolved in DCM (5 mL) and TFA (0.85 g; 7.4 mmol) was added. After stirring for 7 h at rt, the solvent was removed under reduced pressure and the residue was washed with dry ethyl ether (2 × 5 mL), to give the trifluoroacetate **3b** (0.36 g; quantitative yield) as a white solid. ^{1}H NMR (200 MHz, D_2O): δ 1.83–2.27 (m, 2H), 2.08 (s, 3H), 2.41–2.61 (m, 2H), 2.82–3.21 (m, 4H), 3.22–3.33 (m, 1H), 3.88 − 4.09 (m, 5H), 4.58 (dd, J = 4.8 Hz, J = 9.2 Hz, 1H), 4.71 (dd, J = 4.8 Hz, J = 9.6 Hz, 1H), 4.91 (br s, 9H, NH+OH), 5.51 (s, 1H), 5.76 (s, 1H), 6.78 (d, J = 8.1 Hz, 2 ArH), 7.11 (d, J = 8.1 Hz, 2 ArH), 7.18–7.32 (m, 5 ArH); ^{13}C NMR (50 MHz, CD_3OD): δ 15.2, 31.1, 32.2, 37.7, 38.5, 41.4, 43.3, 50.3, 52.7, 56.1, 109.8, 116.9, 121.7, 126.0, 127.8, 129.5, 129.6, 130.2, 130.3, 131.5, 138.5, 141.5, 158.3, 169.2, 170.3, 171.2, 173.8, 174.8; $[\alpha]_D$ −16.8 (c 0.5, $CHCl_3$). ESI–MS: *m/z* 600.2 $[MH]^+$, 622.2 $[M+Na]^+$. Anal. Calcd for $C_{31}H_{38}F_3N_5O_9S$: C, 52.17; H, 5.37; N, 9.81. Found: C, 52.06; H, 5.29; N, 9.88.

Molecular modeling

All calculations were carried out on SGI Octane2 IRIX 6.5 workstations. Molecular mechanics calculations were performed using the implementation of AMBER force field (AMBER*) (Weiner et al. 1986) within the framework of Macromodel version 5.5 (Mohamadi et al. 1990).

To explore the conformational space, a Quenched Molecular Dynamics (QMD) protocol was applied. Thus, the molecules were first heated (equilibration stage) to 900 K in 100 ps and then a trajectory of 2 ns was carried out at T constant (collection stage) with an integration step of 1.5 fs. The SHAKE algorithm (Ryckaert et al. 1977) was used to constrain the stretching of bonds involving hydrogen atoms. The coordinates of the pseudopeptides were saved on a trajectory file every 1 ps, giving a total of 2,000 conformations which must be "quenched" and then analyzed. The "quenching" consists in the minimization of such conformations to the nearest local minimum. Thus, each saved structure was energy minimized till the root mean square of the Cartesian elements of the gradient was less then 0.005 Kcal/mol using a full conjugate gradient algorithm (Polack–Ribiere) and the implicit solvation model GB/SA in water (Still et al. 1990). In order to be sure that the conformational PES have been fully explored, i.e. the ensemble of conformations represents all the possible energy minima, the QMD protocol was repeated for ten times and the data collected into the same trajectory analysis.

In vitro bioactivity assays

Preparations of the myenteric plexus-longitudinal muscle obtained from male guinea pig ileum (GPI, rich in μ-opioid receptors) and preparations of mouse vas deferens (MVD, rich in δ-opioid receptors) were used for field stimulation with bipolar rectangular pulses of supramaximal voltage. Agonists were evaluated for their ability to inhibit the electrically evoked twitch. The results are expressed as the IC_{50} values obtained from concentration–response curves (Prism). IC_{50} values represent the mean of not less than five tissue samples ± SEM.

Acknowledgments We wish to acknowledge Nanodream s.r.l. (Iesi, Italy) for funding this research, and Mr. Andrea Garelli, University of Bologna, for mass spectral analyses.

References

Acosta CG, Lopez HS (1999) δ-Opioid receptor modulation of several voltage-dependent Ca^{2+} currents in rat sensory neurons. J Neurosci 19:8337–8348

Belleney J, Gacel G, Fournie-Zaluski MC, Maigret B, Roques BP (1989) δ Opioid receptor selectivity induced by conformational constraints in linear enkephalin-related peptides: proton 400-MHz NMR study and theoretical calculations. Biochemistry 28:7392–7400

Blomberg D, Kreye P, Fowler C, Brickmann K, Kihlberg J (2006) Synthesis and biological evaluation of leucine enkephalin turn mimetics. Org Biomol Chem 4:416–423

Byun HS, Reddy KC, Bittman R (1994) Improved syntheses of ethyl α-(bromomethyl)acrylate and 2-methylene-1, 3-propanediol via ethyl α-(hydroxymethyl)acrylate. Tetrahedron Lett 35:1371–1374

Carpino LA (1987) The 9-fluorenylmethyloxycarbonyl family of base-sensitive amino-protecting groups. Acc Chem Res 20:401–407

Carrera M, Ramirez-Exposito MJ, Valenzuela MT, Garcia MJ, Mayas MD, Arias de Saavedra JM, Sanchez R, Perez M, Martinez-Martos JM (2005) Specific enkephalin-degrading aminopeptidase activity in the HPT and HPO axes of rats with breast cancer induced by *N*-methyl nitrosourea. Regul Pept 124:157–161

Carstens H, Renner C, Milbradt AG, Moroder L, Tavar P (2005) Multiple loop conformations of peptides predicted by molecular dynamics simulations are compatible with nuclear magnetic resonance. Biochemistry 44:4829–4840

Chen H, Noble F, Roques BP, Fournie-Zaluski MC (2001) Long lasting antinociceptive properties of enkephalin degrading enzyme (NEP and APN) inhibitor prodrugs. J Med Chem 44:3523–3530

Ciclosi M, Fava C, Galeazzi R, Orena M, Sepulveda-Arques J (2002) Synthesis of unsaturated β-amino acid derivatives from carbamates of the Baylis–Hillman products. Tetrahedron Lett 43:2199–2202

Currie BL, Krstenansky JL, Lin ZL, Ungwiatayatorn J, Lee YH, del Rosario-Chow M, Sheu WS, Johnson ME (1993) Design and synthesis of a bicyclic non-peptide β-bend mimetic of enkephalin. Tetrahedron 49:3489–3500

Eguchi M, Lee MS, Nakanishi H, Stasiak M, Lovell S, Kahn M (1999) Solid-phase synthesis and structural analysis of bicyclic β-turn mimetics incorporating functionality at the i to i+3 positions. J Am Chem Soc 121:12204–12205

Eguchi M, Shen RYW, Shea JP, Lee MS, Kahn M (2002) Design, synthesis, and evaluation of opioid analogues with non-peptidic β-turn scaffold: enkephalin and endomorphin mimetics. J Med Chem 45:1395–1398

Fernández D, Valdivia A, Irazusta J, Ochoa C, Casis L (2002) Peptidase activities in human semen. Peptides 23:461–468

Flinn JP, Murphy R, Boublik JH, Lew MJ, Wright CE, Angus JA (1995) Synthesis and biological characterization of a series of analogues of omega-conotoxin GVIA. J Pept Sci 1:379–384

Galeazzi R, Martelli G, Mobbili G, Orena M, Rinaldi S (2003) Synthesis of a conformationally restricted analog of pregabalin by stereoselective alkylation of a chiral pyrrolidin-2-one. Tetrahedron Asymmetry 14:3353–3358

Galeazzi R, Martelli G, Orena M, Rinaldi S, Sabatino P (2005a) Conformationally restricted analogues of both (*S*)-β-homoserine and (*S*)-aspartic acid from chiral 3-acylamino pyrrolidin-2-ones. Tetrahedron 61:5465–5473

Galeazzi R, Martelli G, Natali D, Orena M, Rinaldi S (2005b) Chiral 3-hydroxypyrrolidin-2-ones. Part 2: Stereodivergent synthesis of conformationally restricted analogues of β-homoserine. Tetrahedron Asymmetry 16:1779–1787

Galeazzi R, Marcucci E, Martelli G, Mobbili G, Natali D, Orena M, Rinaldi S (2007) A new conformationally restricted mimetic of dipeptide EG: synthesis of an analogue of FEG. Eur J Org Chem 440:2–4407

Galeazzi R, Marcucci E, Martelli G, Natali D, Orena M, Rinaldi S (2008) Efficient synthesis of an Asp-Gly dipeptide analogue leading to a new restricted RGDG mimetic. Amino Acids 34:333–336

Gante J (1994) Peptidomimetics-tailored enzyme inhibitors. Angew Chem Int Ed Eng 33:1699–1720

Giannis A, Kolter T (1993) Peptidomimetics for receptor ligands—discovery, development, and medical perspectives. Angew Chem Int Ed Eng 32:1244–1267

Gillespie P, Cicariello J, Olson GL (1997) Conformational analysis of dipeptide mimetics. Biopolymers 43:191–217

Goodman M, Zhang J (1997) Peptidomimetic building blocks for drug design. Chemtracts-Org Chem 10:629–645

Gu X, Ying J, Agnes RS, Navratilova E, Davis P, Stahl G, Porreca F, Yamamura HI, Hruby VJ (2004) Novel design of bicyclic β-turn dipeptides on solid-phase supports and synthesis of [3.3.0] bicyclo[2, 3]-Leu-enkephalin analogues. Org Lett 6:3285–3288

Hanessian S, Auzzas L (2008) The practice of ring constraint in peptidomimetics using bicyclic and polycyclic amino acids. Acc Chem Res 41:1241–1251

Hanessian S, McNaughton-Smith G, Lombart HG, Lubell WD (1997) Design and synthesis of conformationally constrained amino acids as versatile scaffolds and peptide mimetics. Tetrahedron 53:12789–12854

Hersh LB (1982) Degradation of enkephalins: the search for an enkephalinase. Mol Cell Biochem 47:35–43

Hruby VJ (2005) On the topic of peptide research and drug discovery. J Peptide Res 66:309–310

Hruby VJ, Balse PM (2000) Conformational and topographical considerations in designing agonist peptidomimetics from peptide leads. Curr Med Chem 7:945–970

Hruby VJ, Li G, Haskell-Luevano C, Shenderovich M (1997) Design of peptides, proteins, and peptidomimetics in χ space. Biopolymers 43:219–266

Hughes J, Smith TW, Kosterlitz HW, Fothergill LA, Morgan BA, Morris HR (1975) Nature (Lond) 258:577–579

Kieffer BL, Evans CJ (2009) Opioid receptors: from binding sites to visible molecules in vivo. Neuropharmacology 56(Suppl 1):205–212

Koca J, Carlsen PHJ (1995) Conformational behavior and flexibility of met-enkephalin. J Mol Struct (THEOCHEM) 337:17–24

Lee YS, Petrov R, Park CK, Ma S, Davis P, Lai J, Porreca F, Vardanyan R, Hruby VJ (2007) Development of novel enkephalin analogues that have enhanced opioid activities at both δ and μ opioid receptors. J Med Chem 50:5528–5532

Maricic S, Berg U, Frejd T (2002) Synthesis and conformational studies of a Leu-enkephalin amide analogue containing a ferrocene substructure. Tetrahedron 58:3085–3093

Martin SF, Dorsey GO, Gane TH, Hillier M, Kessler H, Baur M, Mathä B, Erickson JW, Bhat N, Munshi S, Gulnik S, Topol IA (1998) Cyclopropane-derived peptidomimetics. Design, synthesis, evaluation, and structure of novel HIV-1 protease inhibitors. J Med Chem 41:1581–1597

Meirovitch H, Meirovitch E, Michel AG, Vasquez M (1994) A simple and effective procedure for conformational search of macromolecules: application to Met- and Leu-enkephalin. J Phys Chem 98:6241–6243

Mohamadi F, Richards NGJ, Guida WC, Liskamp R, Lipton M, Caufield C, Chang G, Hendrickson T, Still WC (1990) Macromodel—an integrated software system for modeling organic and bioorganic molecules using molecular mechanics. J Comp Chem 11:440–467

Monnier Z, Bride M (1995) In vitro effects of methionine-enkephalin, somatostatin and insulin on cultured gonadal cells of the snail Helix aspersa. Experientia 51:824–830

O'Connor SD, Smith PE, Al-Obeidi F, Pettitt BM (1992) Quenched molecular dynamics simulations of tuftsin and proposed cyclic analogs. J Med Chem 35:2870–2881

Olson GL, Bolin DR, Bonner MP, Bos M, Cook CM, Fry DC, Graves BJ, Hatada M, Hill DE, Kahn M (1993) Concepts and progress in the development of peptide mimetics. J Med Chem 36:3039–3049

Rew Y, Goodman M (2002) Solid-phase synthesis of amine-bridged cyclic enkephalin analogues via on-resin cyclization utilizing the Fukuyama-Mitsunobu reaction. J Org Chem 67:8820–8826

Ryckaert JP, Ciccotti G, Berendsen HJC (1977) Numerical integration of the Cartesian equations of motion of a system with constraints: molecular dynamics of *n*-alkanes. J Comp Phys 23:327–341

Sanbonmatsu KY, García AE (2002) Structure of Met-enkephalin in explicit aqueous solution using replica exchange molecular dynamics. Proteins 46:225–234

Shimoigashi Y, Stammer CH (1982) Dehydro-enkephalins. VI. Dehydroalanine^3-enkephalin: a potent enkephalin analog for the δ opiate receptor. Int J Pept Prot Res 20:199–206

Shimoigashi Y, English ML, Stammer CH, Costa T (1982) Dehydroenkephalins. IV. Discriminative recognition of δ and μ opiate receptors by enkephalin analogs. Biochem Biophys Res Commun 104:583–590

Shinada T, Ishida T, Hayashi K, Yoshida Y, Shigeri Y, Ohfune Y (2007) Synthesis of leucine-enkephalin analogs containing α-amino squaric acid. Tetrahedron Lett 48:7614–7617

Smith D, Griffin JF (1978) Conformation of [Leu5]enkephalin from X-ray diffraction: features important for recognition at opiate receptor. Science 199:1214–1216

Speziale AJ, Smith LR (1963) The reaction of oxalyl chloride with amides. II. Oxazolidinediones and acyl isocyanates. J Org Chem 28:1805–1811

Still WC, Tempczyk A, Hawley RC, Hendrickson T (1990) Semianalytical treatment of solvation for molecular mechanics and dynamics. J Am Chem Soc 112:6127–6129

Weiner SJ, Kollman PA, Nguyen DT, Case DA (1986) An all atom force field for simulations of proteins and nucleic acids. J Comp Chem 7:230–252

Wu Y, Hsieh J, Lin HC, Hwang CC (2007) Conformational stability and three-dimensional model of the δ-opioid pharmacophore for the extended antiparallel dimer structure of Met-enkephalin in water. J Mol Model 13:171–177