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ORIGINAL ARTICLE

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

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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

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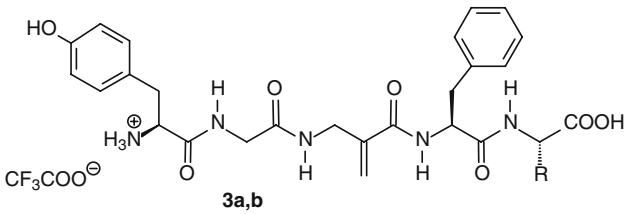
1978) and the molecule in the solid state was shown to display a Gly^2-Gly^3 β-turn stabilized by two intramolecular hydrogen bonds between the CO and NH groups of Tyr^1 and the NH and CO groups of Phe^4. In addition, although enkephalin itself has a random structure in water, a β-turn conformation at 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

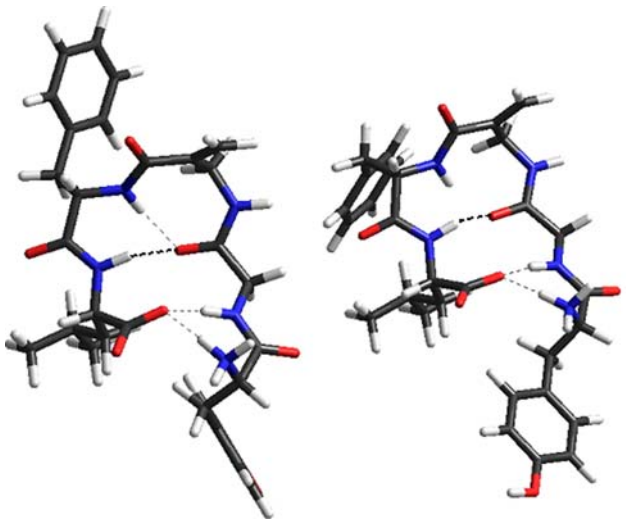
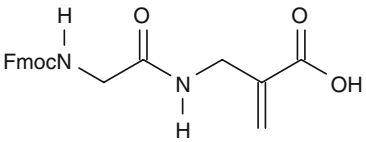


Fig. 1 Geometries of the two lowest energy conformers for pseudopeptide **3a** [*Left* lowest energy minimum. $E = 0.0$ kcal/mol (−262.12 kcal/mol): $\phi_1 = -54.4$, $\psi_1 = -177.3$, $\phi_2 = 132.7$, $\psi_2 = 179.3$, $\phi_3 = -110.2$, $\theta_3 = 27.6$, $\psi_3 = -174.7$, $\phi_4 = 12.7$, $\psi_4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

Scheme 2



4

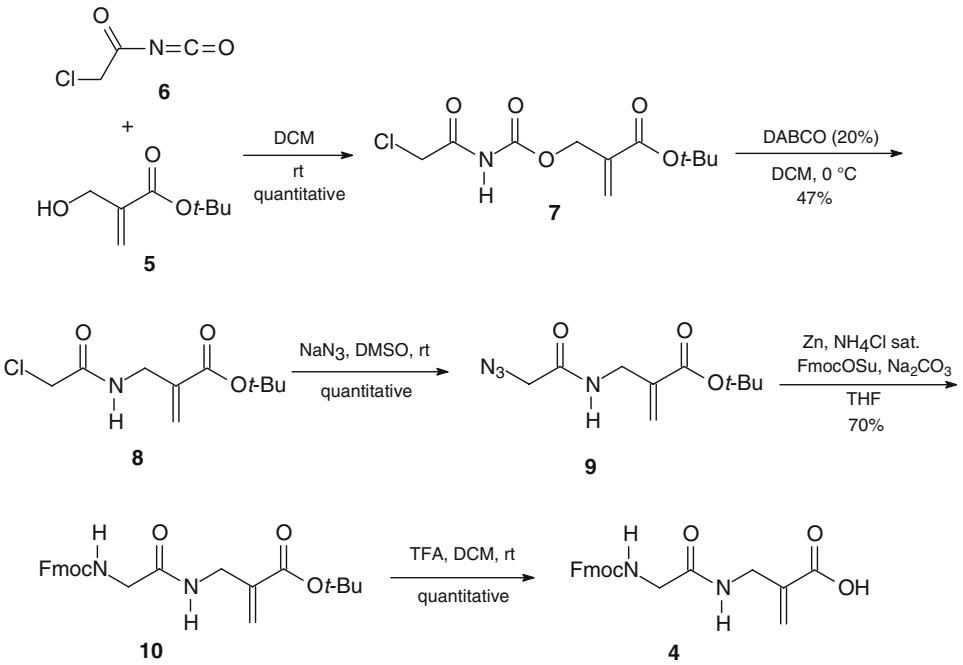
arrangement, and stabilized by the classical H-bond between the C = O of Gly^2 and the NH of the Leu^5 forming a 11-membered ring (not a 10 because of the presence of the β-amino acid Ψ-Gly^3) (Fig. 1). Furthermore this secondary structure is stabilized strongly also by the presence of other H-bonding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

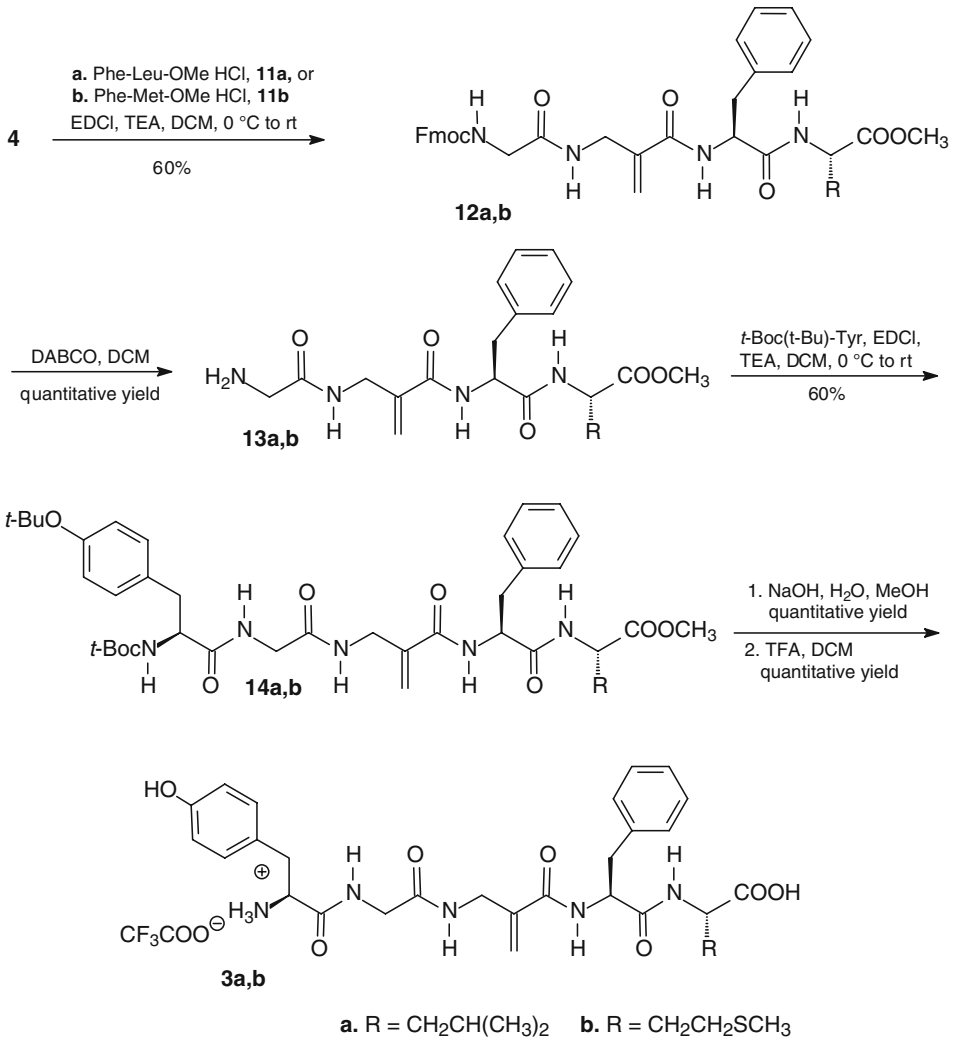
Biological assays

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.

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