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Synthesis and activity of endomorphin-2 and morphiceptin analogues with proline surrogates in position 2

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Abstract

The opioid agonists endomorphins (Tyr–Pro–Trp–Phe–NH_2; EM1 and Tyr–Pro–Phe–Phe–NH_2; EM2) and morphiceptin (Tyr–Pro–Phe–Pro–NH_2) exhibit an extremely high selectivity for μ-opioid receptor. Here a series of novel EM2 and morphiceptin analogues containing in place of the proline at position 2 the *S* and *R* residues of β-homologues of proline (HPro), of 2-pyrrolidinemethanesulphonic acid (HPrs) and of 3-pyrrolidinesulphonic acid (βPrs) have been synthesized and their binding affinity and functional activity have been investigated. The highest μ-receptor affinity is shown by [(S)βPrs^2]EM2 analogue (**6e**) which represents the first example of a β-sulphonamido analogue in the field of opioid peptides.

Keywords

Endomorphins; Opioid peptides; Peptide synthesis; β-Sulphonamido peptides; Unusual amino acids

1. Introduction

Due to their role in the modulation and perception of pain the opioid receptors continue to be an extremely important target in medicinal chemistry. Three major subtypes (μ, κ, δ) of this G-protein coupled family of receptors have been defined and it is well established that the μ group represents the major target of the analgesics. Although several endogenous peptide ligands of opioid receptors are known most of them, including enkephalins, does not show significant μ-selective agonistic activity. A notable exception is represented by

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morphiceptin, a tetrapeptide amide (Tyr–Pro–Phe–Pro–NH_2) isolated from an enzymatic digest of bovine β-casein [1–3] and by the structurally related opioid ligands endomorphin-1 (EM1: Tyr–Pro–Trp–Phe–NH_2) and endomorphin-2 (EM2: Tyr–Pro–Phe–Phe–NH_2) which exhibit high μ-opioid receptor selectivity and agonist potency [4].

As it is well established [5,6] and clearly discussed by Goodman and Schiller [7], in the structure of natural opioid peptides two biological relevant fragments can be identified, namely the N-terminal message sequence, containing the two pharmacophoric aromatic residues of Tyr and Phe, and the remaining C-terminal fragment which represents the address sequence. EM2 and morphiceptin have a different C-terminal address sequence [3] which is –Phe–NH_2 for EM2 and –Pro–NH_2 for morphiceptin, but an identical Tyr–Pro–Phe– N-terminal message sequence. This feature, common to EMs and morphiceptin, is different from that found in enkephalins and other endogenous opioid peptides such as endorphins and dynorphins. These latter ligands possess the tetrapeptidic fragment Tyr–Gly–Gly–Phe– as a characteristic N-terminal message sequence. Thus, a topochemical characteristic which differentiates the N-terminal sequences present in the two classes of peptides is the type of the spacer group separating the Tyr and Phe aromatic residues. It thus can be inferred that different spacers, namely the Pro residue at position 2 in morphiceptin [8,9], and in EMs [10–12], as well as the Gly–Gly dipeptide fragment in enkephalins, fulfill the stereochemical requirements needed for a correct interaction of the N-terminal phaarmacophore aromatic side chains with the involved receptor area.

On the basis of the above considerations and in order to obtain additional information on the biochemical consequences of the structural modifications performed at the level of the Tyr^1/Phe^3 spacer residue we synthesized and biologically evaluated a new group of analogues of EM2 and morphiceptin reported in Fig. 1.

In the first group of analogues (**6a**,**b** and **7a**,**b**) the (*S*) and (*R*) β-homologues of proline, namely (*S*)-homoproline [(*S*)-HPro–OH] and (*R*)-homoproline [(*R*)-HPro–OH], respectively, replace the native Pro residue at position 2. In the remaining four EM2 analogues (**6c**,**d** and **6e**,**f**) two different types of Pro β-homologues have been inserted as spacer groups and in both of them the –SO_2–NH– sulphonamido group replaces the –CO–NH– peptide bond. This bioisosteric replacement, initially introduced in 1989 by Lucente and coworkers [13,14] and then extensively investigated [15–18], generates a metabolically stable junction associated with significant changes in backbone conformational properties, polarity and H-bonding capacity [19]. Although the potentiality of sulfonamidopeptides is now well established [20,21] and applied to different classes of bioactive peptides [22–24] and enzyme inhibitors [25], analogues of EMs containing the sulfonamide junction have not been described. The here reported compounds **6c** and **6d** contain the (*R*) and (*S*) residues of the 2-pyrrolidinemethanesulphonic acid [(*S*)-HPrs–OH and (*R*)-HPrs–OH] and are then β-sulphonamido analogues of **6a** and **6b**, respectively. To a second and new type of β-sulphonamido peptides belong the analogues **6e** and **6f**. These are characterized by the presence of the 3-pyrrolidinesulphonic acid [(*S*) or (*R*) βPrs–OH] residue. This cyclic β-amino sulfonic acid, synthesized for the first time for the present research, is the sulfonyl analogue of β-proline (3-pyrrolidinecarboxylic acid; β-Pro–OH), a well known β-amino

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carboxylic acid, previously incorporated into the EM1 molecule to give the highly active Tyr–(*R*)-β-Pro–Trp–Phe–NH_2 tetrapeptide [26].

2. Chemistry

The synthesis of peptides **6a–f** and **7a**,**b** (Scheme 1) was performed in solution using the carbodiimide method for coupling steps.

Only in the case of the synthesis of the sulphonamido junction couplings were performed by using sulfonyl chlorides. The N-protected pseudoamino acids **1a–f** were coupled with H–Phe–OMe.HCl and the resulting dipeptides **2a–f** were subjected to catalytic hydrogenation. Successive coupling with Boc–Tyr–OH gave the tripeptides **3a–f** which were *O*-deprotected by alkaline hydrolysis and successively coupled with H–Phe–NH_2 or H–Pro–NH_2 leading to the tetrapeptides **4a–f** and **5a,b,** respectively. Treatment with TFA 95% afforded the required final free peptides **6a–f** and **7a,b** as trifluoroacetate salts (final products were characterized by mass spectra analysis, see Table 1).

The N-Cbz (*S*) and (*R*) homoprolines **1a**,**b**, necessary for the synthesis of peptides **6a,b** and **7a,b**, were prepared according to literature [27] through the diazomethyl ketone route, starting from the appropriate *N*-Cbz derivative.

The (*S*) and (*R*) pyrrolidinesulfonyl chlorides **1c–f**, required for the preparation of peptides **6c–f**, were synthesized starting from the corresponding amino alcohol according to Scheme 2 [28–30].

3. Biological evaluation and conclusions

The binding affinities for opioid receptors and functional bioactivities, exhibited by the EM2 and the morphiceptin analogues **6a–f** and **7a**,**b** are summarized in Table 2.

All compounds show very low k- and δ-opioid receptor affinities (micromolar range) and high δ/μ selectivity. Results concerning the four EM2 derivatives containing β-homologues of proline, both of β-carboxylic and β-sulfonic type (namely **6a**,**b** and **6c**,**d** respectively), confirm the strong influence of the stereochemistry at position 2 on the binding affinity. In this group of four analogues only the [(*S*) $HPro^2$]-EM2 (**6a**), containing the (*S*)-β-homoproline residue, maintains good μ-opioid receptor affinity ($K_i^{\mu}=58\,\mathrm{nM}$) whereas the corresponding [(*R*)$HPro^2$]-EM2 (**6b**) is sensibly less potent ($K_i^{\mu}=290\,\mathrm{nM}$). The same trend is found in the case of the two derivatives containing the β-homo-sulfonyl residue HPrs where the [(*R*)$HPrs^2$]-EM2 (**6d**) is 5-fold less active then [(*S*)$HPrs^2$]-EM2 (**6c**). These results are in agreement with those previously reported for β-amino carboxylic acid containing EM1 analogues [33] where the [(*S*) $HPro^2$]-EM1 has been found to be about 33-fold more potent than the [(*R*)$HPro^2$]-EM1. Although with an opposite trend, a high influence on the affinity is also shown by the stereochemistry at position 2 of the two morphiceptin analogues **7a** and **7b** containing enantiomeric β-homologues of proline. Here it has been found that the K_i^{μ} values for [(*S*)$HPro^2$]-morphiceptin (**7a**) and [(*R*)$HPro^2$]-morphiceptin (**7b**) are $K_i^{\mu}=6100\,\mathrm{nM}$ and $K_i^{\mu}=30\,\mathrm{nM}$, respectively. Thus, when these data

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are compared to those shown by the above cited affinities of **6a** and **6b**, it can be seen that the absolute configuration of the HPro residue at position 2 exerts an opposite effect on binding of the morphiceptin and EM2 analogues and leads to the [(*R*)HPro^2]-morphiceptin (**7b**) endowed with the highest potency in the GPI assays (IC50 =17) as well as high δ/μ binding selectivity. This result is not in disagreement with previous studies which highlight the role of the spacer residue structure at position 2 in giving correct spatial orientation to the aromatic residues of the ligand [6,34] and confirms the observation that each class of opioid peptides shows distinct chiral requirements for the spacers between the biologically important Tyr and Phe residues [7].

In addition to the analogues **6a–d** and **7a**,**b** containing the β-homoproline residues HPro and HPrs, the EM2 analogues **6e**,**f** contain at position 2 the enantiomers of the β-proline sulfonyl residue βPrs. These are characterized by relevant structural differences as compared with the β-homoproline (HPro and HPrs) containing residues **6a–d** and **7a**,**b**. In fact, in addition to the presence of the SO_2–NH junction, replacing the usual CO–NH peptide bond, in the βPrs residues the acylating group is directly bound to a carbon atom of the 5-membered ring. Conversely, in **6a–d** and **7a**,**b** a –CH_2– bridge is inserted between the pyrrolidine ring and the acylating group. This feature may greatly change the conformational preferences of the entire molecule, leading to systems with higher flexibility and conformational freedom. As shown in Table 2 the μ-opioid receptor affinity ($K_i^{\mu}=19\,nM$) of the tetrapeptide amide **6e**, containing the (*S*)-βPrs sulfonyl residue, is the highest among the here studied ligands. The epimeric analogue [(*R*)-βPrs^2]-EM2 (**6f**), in which the stereochemistry of the residue at position 2 has been changed, shows a sensible decrease of the binding affinity ($K_i^{\mu}=63\,nM$). Thus, as previously found for the EM1 analogues containing the βPro residue (*i.e.* the carboxylic analogue of βPrs) [26], the absolute configuration at position 2 highly influences the binding. However, an opposite spatial orientation of the acylating group at position 2 characterizes the most active analogues in the two cases. In fact, when the β-carboxylic residue is involved, the [(*R*)-βPro^2]-EM1 [26] was found to be more active than the corresponding [(*S*)-βPrs^2]-EM1 ($K_i^{\mu}=0.33\,nM$ versus $K_i^{\mu}=10.4\,nM$) [26].

Table 2 summarizes, in addition to the affinity data, the functional activity on μ- and δ-opioid receptors of the here studied analogues. The highest potency as μ-agonist is that of the analogue **7b** which, in the GPI functional assay, shows a value (IC_{50} = 17) practical equal to that of the EM2 (IC_{50} = 15) although with a sensibly lower selectivity as indicated by the MVD/GPI IC_{50} ratio which is 34 versus 5.7, respectively. Notable is in this case the strong influence of the absolute configuration of the residue at position 2 as shown by the binding and activity values (see Table 2) found for **7b**, the epimeric analogue of **7a**. Although to a lower degree, a strong effect of the chirality at position 2 is here observed in the case of **6a** and **6b** and was previously reported for the couple of EM1 analogues containing (*R*) and (*S*) HPro residues [33]. The two groups of peptides **6c**,**d** and **6e,f** contain a sulphonamido junction replacing the native Pro^2-Phe^3 peptide bond. In this case, structurally characterized by a CH_2 group inserted between the SO_2–NH bond and the pyrrolidine ring, a clear μ-receptor preference is shown by the analogue **6c**, possessing the (*S*)βPrs residue at position 2 (IC_{50}, GPI = 730 and 2700 nM, for **6c** and **6d**, respectively). This behavior is analogous to that exhibited by **6a** and **6b** containing the (*S*)HPro and

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(*R*)HPro residue, respectively. In the models **6e** and **6f** the SO_2–NH junction is directly bound to the five-membered ring and this strongly limits the backbone flexibility as compared with **6c** and **6d**. In this case both the epimers [(*S*)βPrs^2] EM2 **6e** and [(*R*)βPrs^2]EM2 **6f** show unexpectedly low GPI and MVD potencies while it is still retained significant receptor affinity for both μ- and δ-opioid receptor types. This suggests that this couple of analogues may have a mixed agonist/antagonist property for both receptor types.

To the best of our knowledge the here reported βPrs containing tetrapeptides **6e,f** are the first examples of analogues containing a β-sulphonamido replacement in the field of opioid peptides. The high μ-receptor affinity shown by the tetrapeptide [(*S*)-βPrs^2]EM2 (**6e**) suggests further study of this type of derivatives and underlines, at the same time, the highly different interactions which β-sulphonamido pseudopeptides, as compared with the β-carboxyamido counterparts, may establish with the receptors.

4. Experimental protocols

4.1. General

IR spectra were recorded in 1% $CHCl_3$ solution employing a Perkin-Elmer FT-IR Spectrum 1000 spectrophotometer. $[\alpha]_D$ was measured at 20 °C with a Schmidt–Haensch polarimeter at a 1% concentration in $CHCl_3$ (unless otherwise specified) with a 1 cm cell. ^{1}H NMR spectra were determined in $CDCl_3$ solution with a Bruker AM 400 spectrometer and chemical shifts were indirectly referred to TMS. The mass spectra were performed on a Q-TOFMICRO spectrometer (Micromass, now Waters, Manchester, UK) equipped with an ESI source, in the positive ion mode and data were analyzed using the MassLynx software (Waters). Thin-layer and preparative layer chromatographies were performed on silica gel Merck 60 F254 plates. The drying agent was sodium sulfate. Elemental analyses for C, H and N (where necessary a sample was further purified by preparative TLC) were performed in the laboratories of the Servizio Microanalisi del CNR, Area della Ricerca di Roma, Montelibretti, Italy, and were within 0.4% of the theoretical values.

4.1.1. Preparation of sulfonic acids. General procedure—An HCl saturated solution of the Boc-mesylates (1.00 mmol) in dioxane (30 mL) was allowed to stand at room temperature for 30 min. The precipitate was filtered and crystallized from ethanol-ethyl ether. A solution of the recovered precipitate and Na_2SO_3 (2.6 mmol) in water (5 mL) was stirred for 24 h at room temperature, then passed firstly on Amberlite IR-120 (H^+ form) and then on Dowex 11 (acetate form) columns. Evaporation of the eluate under vacuum gave a crude residue which was crystallized from water–ethanol.

4.1.1.1. (S)-3-pyrrolidinesulfonic acid: From *N*-Boc-(*S*)-3-pyrrolidinemethansulfonate [30] (4.90 g, 18.48 mmol). White solid (78%). ^{1}H NMR (D_2O) δ: 2.12 (m, 2H, Pyr γCH_2), 3.06–3.28 (two m, 3H, Pyr βCH and δCH_2), 4.02 (m, 2H, Pyr αCH_2). Anal. Calcd for $C_4H_9NO_3S$: C 31.78, H 6.00, N 9.26; found C 31.85, H 6.23, N 9.21.

4.1.1.2. (R)-3-pyrrolidinesulfonic acid: From *N*-Boc-(*R*)-3-pyrrolidinemethansulfonate [30] (3.83 g, 14.43 mmol). White solid (86%). ^{1}H NMR (D_2O) δ: 2.12 (m, 2H, Pyr γCH_2),

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3.06–3.28 (two m, 3H, Pyr βCH and δCH_2), 4.02 (m, 2H, Pyr αCH_2). Anal. Calcd for $C_4H_9NO_3S$: C 31.78, H 6.00, N 9.26; found C 31.91, H 6.20, N 9.33.

4.1.2. Preparation of sulfonyl chlorides. General procedure—To a solution of the appropriate sulfonic acid (1.00 mmol) in H_2O (10 mL), adjusted to pH 8.5 with NaOH, Cbz-chloride (1.1 mmol) was added in five portions at room temperature and under vigorous stirring, maintaining the pH at 8–8.5 by small amounts of 1 N NaOH. After additional 2 h from the last addition, water was added (10 mL) and the aqueous phase was washed with Et_2O (2 × 10 mL), evaporated under reduced pressure and coevaporated with toluene. The crude sodium sulfonate salt, white solid, was dried overnight under high vacuum on P_2O_5. To a suspension of the dried residue in DCM (30 mL) a solution of phosgene in toluene (20% p/p, 4.0 mL) and DMF (0.6 mL) in DCM (30 mL) was added under N_2. If the reaction was not complete (TLC) after 1 h, an additional amount (1–2 mL) of the latter solution was added. After stirring for 2 h at room temperature, concentration of the mixture and purification on silica gel column (CH_2Cl_2) gave the products which were stored under argon and used as such.

4.1.2.1. Cbz–(R)-2-pyrrolidinemethanesulfonyl chloride [Cbz–(R)-HPrs-Cl] (1d): From (*R*)-2-pyrrolidinemethanesulfonic acid [29] (2.05 g, 12.42 mmol). Colourless oil (56%). IR ν: 3020, 2957, 2873, 1701, 1414 cm^{-1}; ^1H NMR δ: 1.81–2.34 (m, 4H, Pyr β and γCH_2), 3.16 (m,2H, CH_2SO_2), 3.25 (m,1H, Pyr αCH), 3.96–4.25 (m, 2H, Pyr δCH_2), 5.21 (s, 2H, Cbz CH_2), 7.21–7.53 (m, 5H, Ar). Anal. Calcd for $C_{13}H_{16}ClNO_4S$: C 49.13, H 5.07, N 4.41; found C 49.01, H 5.18, N 4.56.

4.1.2.2. Cbz-(S)-3-pyrrolidinesulfonyl chloride [Cbz–(S)-βPrs-Cl] (1e): From (*S*)-3-pyrrolidinesulfonic acid (1.93 g, 12.77 mmol). Colourless oil (62%). IR ν: 3018, 2955, 2871, 1769, 1414 cm^{-1}; ^1H NMR δ: 1.96–2.43 (m, 4H, Pyr γ and δCH_2), 3.02 (m, 1H, Pyr βCH), 3.4 (m, 2H, Pyr αCH_2), 5.19 (s, 2H, Cbz CH_2), 7.20–7.54 (m, 5H, Ar). Anal. Calcd for $C_{12}H_{14}ClNO_4S$: C 47.45, H 4.65, N 4.61; found C 47.38, H 4.72, N 4.57.

4.1.2.3. Cbz-(R)-3-pyrrolidinesulfonyl chloride [Cbz–(R)-βPrs-Cl] (1f): From (*R*)-3-pyrrolidinesulfonic acid (1.45 g, 9.60 mmol). Colourless oil (48%). IR ν: 3018, 2955, 2871,1769, 1414 cm^{-1}; ^1H NMR δ: 1.96–2.43 (m, 4H, Pyr γ and δCH_2), 3.02 (m, 1H, Pyr βCH), 3.4 (m, 2H, Pyr αCH_2), 5.19 (s, 2H, Cbz CH_2), 7.20–7.54 (m, 5H, Ar). Anal. Calcd for $C_{12}H_{14}ClNO_4S$: C 47.45, H 4.65, N 4.61; found C 47.29, H 4.86, N 4.45.

4.1.3. Preparation of peptidosulfonamides. General procedure—To an ice-cooled mixture containing the appropriate Cbz–sulfonyl chloride (1.0 mmol) and Phe–OMe.HCl (2.0 mmol) in anhydrous DCM (10 mL) a solution of TEA (2.0 mmol) in DCM (4.0 mL) was added dropwise under stirring. The reaction was stirred overnight at room temperature, then was diluted with DCM (25 mL) and consecutively washed with 1 N HCl (2 × 20 mL), sat. $NaHCO_3$ (2 × 20 mL) and brine (20 mL). The organic phase was dried and evaporated. The crude products were purified on silica gel flash chromatography ($CHCl_3$) and were obtained as pale yellow oil which solidified on standing.

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4.1.3.1. Cbz–(R)-HPrs–Phe–OMe (2d): From **1d** (1.750 g, 5.50 mmol). Pale yellow solid (48%); $[\alpha]_D$ + 7° (1, H_2O); IR ν: 3430, 3008, 1747, 1668, 1430 $cm^{-1;}$ ^1H NMR δ: 1.87–2.40 (m, 4H, HPrs β and γCH_2), 2.94 and 3.12 (dd, 2H, A and B of an ABX, J = 7.9, 5.5 and 13.5 Hz, Phe βCH_2), 3.26 (m, 2H, HPrs δCH_2), 3.54 (m, 2H, CH_2SO_2), 3.76 (s, 3H, OCH_3), 4.18 (m,1H, HPrs αCH), 4.22 (m,1H, Phe αCH), 5.21 (s, 2H, Cbz CH_2), 5.84 (br d, 1H, Phe NH), 7.04–7.12 (m, 10H, Ar). Anal. Calcd for $C_{23}H_{28}N_2O_6S$: C 59.98, H 6.13, N 6.08; found C 60.12, H 6.27, N 5.96.

4.1.3.2. Cbz–(S)-βPrs–Phe–OMe (2e): From **1e** (0.827 g, 2.72 mmol). Pale yellow solid (46%); $[\alpha]_D$ − 3° (1, H_2O); IR ν:3689, 3012, 1699, 1426 $cm^{-1;}$ ^1H NMR δ: 2.02 (m, 2H, βPrs γCH_2), 2.90–3.05 (m, 5H, Phe βCH_2, βPrs βCH and δCH_2), 3.80 (s, 3H, OCH_3), 4.31 (br d, 2H, βPrs αCH_2), 4.90–5.12 (m, 3H, Phe αCH and Cbz CH_2), 5.10 (s, 1H, Phe NH), 7.01–7.23 (m, 10H, Ar). Anal. Calcd for $C_{22}H_{26}N_2O_6S$: C 59.18, H 5.87, N 6.27; found C 59.33, H 6.07, N 6.12.

4.1.3.3. Cbz–(R)-βPrs–Phe–OMe (2f): From **1f** (0.975 g, 3.21 mmol). Colourless oil (52%); $[\alpha]_D$ + 2° (1, H_2O); IR ν:3691, 3020, 1687, 1432 $cm^{-1;}$ ^1H NMR δ: 1.98 (m, 2H, βPrs γCH_2), 2.93–3.12 (m, 5H, Phe βCH_2, βPrs βCH and δCH_2), 3.87 (s, 3H, OCH_3), 4.26 (br d, 2H, βPrs αCH_2), 4.92–5.15 (m, 3H, Phe αCH and Cbz CH_2), 5.11 (s,1H, Phe NH), 7.08–7.21 (m, 10H, Ar). Anal. Calcd for $C_{22}H_{26}N_2O_6S$: C 59.18, H 5.87, N 6.27; found C 59.14, H 6.02, N 6.19.

4.1.4. Carbodiimide coupling. General procedure—To an ice-cooled mixture containing the C-protected amino acid or peptide salt (1.0 mmol), the required N-protected amino acid (1.0 mmol), HOBt (1.2 mmol) and TEA (2.2 mmol) in anhydrous DCM (6.0 mL), EDC (1.2 mmol) was added and the reaction mixture was allowed to warm slowly to room temperature overnight. The mixture was then diluted with DCM (20 mL) and washed with 1 M $KHSO_4$ (2 × 15 mL), saturated aqueous $NaHCO_3$ (2 × 15 mL) and brine (15 mL). The organic phase was dried and evaporated under reduced pressure.

4.1.4.1. Cbz–(R)-HPro–Phe–OMe (2b): From **1b** [27] (0.650 g, 2.47 mmol) and H–Phe–OMe. HCl (0.532 g, 2.47 mmol). Pale yellow oil (81%); $[\alpha]_D$ + 10°; IR ν:3689, 3425, 3029, 3009,1740,1676 $cm^{-1;}$ ^1H NMR δ: 2.06–2.10 (m, 4H, HPro β and γCH_2), 2.90–2.97 (dd, 2H, A and B of an ABX, J = 8.0, 5.5 and 13.5 Hz, Phe βCH_2), 3.18 (m, 2H, CH_2CO), 3.55 (m, 2H, HPro δCH_2), 3.74 (s, 3H, OCH_3), 4.13 (br, 1H, HPro αCH), 4.92 (m, 1H, Phe αCH), 5.10 (m, 2H, Cbz CH_2), 5.89 (br, 1H, Phe NH), 6.98–7.57 (m, 10H, Ar). Anal. Calcd for $C_{24}H_{28}N_2O_5$: C 67.91, H 6.65, N, 6.60; found: C 67.82, H 6.58, N 6.49.

4.1.4.2. Boc–Tyr–(S)-HPro–Phe–OMe (3a): Hydrogenolysis of **2a** [28] (1.37 g, 3.23 mmol) in MeOH on 10% Pd/C in the presence of TFA afforded the corresponding trifluoroacetate salt (white foam, 92%) which was coupled with Boc–Tyr–OH (0.838 g, 2.98 mmol). Purified on SiO_2 (DCM/EtOAc 1:1). White solid (63%); $[\alpha]_D$ + 46°; IR ν:3689, 3432, 3032, 1743, 1670, 1629 $cm^{-1;}$ ^1H NMR δ: 1.30 [s, 9H, C $(CH_3)_3$], 1.20–1.54 (m, 4H, HPro β and γCH_2), 2.39–3.45 (four m, 8H, Tyr βCH_2, Phe βCH_2, HPro δCH_2 and CH_2CO), 3.73 (s, 3H, OCH_3), 4.20 (br s, 1H, HPro αCH), 4.51 (m, 1H, Phe αCH), 4.93 (m, 1H, Tyr αCH), 5.23 (m, 1H, Tyr NH), 6.80–7.20 (m, 10H, Ar), 7.54 (s, 1H, Phe NH), 9.83 (m, 1H,

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OH). Anal. Calcd for $C_{30}H_{39}N_3O_7$: C 65.08, H 7.10, N 7.59; found: C 65.22, H 6.97, N 7.71.

4.1.4.3. Boc–Tyr–(R)-HPro–Phe–OMe (3b): Hydrogenolysis of **2b** (0.806 g, 1.90 mmol) in MeOH on 10% Pd/C in the presence of TFA afforded the corresponding trifluoroacetate salt (white foam, 88%) which was coupled with Boc–Tyr–OH (0.470 g, 1.67 mmol). Purified on SiO_2 (DCM/EtOAc 1:1). Colourless oil (76%); $[\alpha]_D + 2°$; IR ν: 3639, 3429, 3012, 2835, 1742, 1697, 1631 $cm^{-1;}$ ^{1}H NMR δ: 1.32 [s, 9H, $C(CH_3)_3$], 1.51–1.74 (m, 4H, HPro β and γCH_2), 2.40–3.42 (four m, 8H, Tyr βCH_2, Phe βCH_2, HPro δCH_2 and CH_2CO), 3.70 (s, 3H, OCH_3), 4.22 (br s, 1H, HPro αCH), 4.50 (br s, 1H, Phe αCH), 4.91 (m, 1H, Tyr αCH), 5.24 (m, 1H, Tyr NH), 6.81–7.23 (m, 10H, Ar), 7.52 (m, 1H, Phe NH), 9.80 (s, 1H, OH). Anal. Calcd for $C_{30}H_{39}N_3O_7$: C 65.08, H 7.10, N 7.59; found: C 65.14, H 7.02, N 7.67.

4.1.4.4. Boc–Tyr–(S)-HPrs–Phe–OMe (3c): Hydrogenolysis of **2c** [28] (0.161 g, 0.35 mmol) in MeOH on 10% Pd/C in the presence of TFA afforded the corresponding trifluoroacetate salt (white foam, 78%) which was coupled with Boc–Tyr–OH (0.075 g, 0.27 mmol). Purified on SiO_2 (DCM/MeOH 99:1). Pale yellow solid (90%); $[\alpha]_D - 5°$; IR ν: 3433, 3027, 1704, 1671 $cm^{-1;}$ ^{1}H NMR δ: 1.34 [s, 9H, C $(CH_3)_3$], 1.81 (m, 2H, HPrs γCH_2), 2.10–2.81 (two m, 4H, Tyr βCH_2 and HPrs βCH_2), 2.84–3.33 (m, 6H, Phe βCH_2, HPrs δCH_2 and CH_2SO_2), 3.72 (s, 3H, OCH_3), 4.27 (br d, 1H, HPrs αCH), 4.47 (m, 1H, Phe αCH), 4.78 (m, 1H, Tyr αCH), 5.45 (m, 1H, Tyr NH), 6.32 (m, 1H, Phe NH), 6.89–7.21 (m, 10H, Ar), 9.01 (s, 1H, OH). Anal. Calcd for $C_{29}H_{39}N_3O_8S$: C 59.07, H 6.67, N 7.13; found: C 59.25, H 6.58, N 7.01.

4.1.4.5. Boc–Tyr–(R)-HPrs–Phe–OMe (3d): Hydrogenolysis of **2d** (0.850 g, 1.84 mmol) in MeOH on 10% Pd/C in the presence of TFA afforded the corresponding trifluoroacetate salt (white foam, 90%) which was coupled with Boc–Tyr–OH (0.467 g, 1.66 mmol). Purified on SiO_2 (DCM/MeOH 99:1). Pale yellow solid (70%); $[\alpha]_D + 6°$; IR ν: 3432, 3032, 3028, 1702, 1671 $cm^{-1;}$ ^{1}H NMR δ: 1.34 [s, 9H, C $(CH_3)_3$], 1.83 (m, 2H, HPrs γCH_2), 2.12–2.80 (two m, 4H, Tyr βCH_2, HPrs βCH_2), 2.85–3.37 (m, 6H, Phe βCH_2, HPrs δCH_2 and CH_2SO_2), 3.75 (s, 3H, OCH_3), 4.29 (br s, 1H, HPrs αCH), 4.54 (m, 1H, Phe αCH), 4.80 (m, 1H, Tyr αCH), 5.48 (m, 1H, Tyr NH), 6.38 (m, 1H, Phe NH), 6.90–7.24 (m, 10H, Ar), 9.07 (s, 1H, OH). Anal. Calcd for $C_{29}H_{39}N_3O_8S$: C 59.07, H 6.67, N 7.13; found: C 59.13, H 6.42, N 6.98.

4.1.4.6. Boc–Tyr–(S)-βPrs–Phe–OMe (3e): Hydrogenolysis of **2e** (0.161 g, 0.36 mmol) in MeOH on 10% Pd/C in the presence of TFA afforded the corresponding trifluoroacetate salt (white foam, 85%) which was coupled with Boc–Tyr–OH (0.086 g, 0.31 mmol). Purified on SiO_2 (DCM/MeOH 95:5). Colourless oil (80%); $[\alpha]_D - 5°$; IR ν:3689, 3432, 3032, 3011, 1744 $cm^{-1;}$ ^{1}H NMR δ: 1.49 [s, 9H, C $(CH_3)_3$], 2.47 (m, 2H, βPrs γCH_2), 2.70–3.51 (m, 6H, Phe βCH_2, βPrs α and δCH_2), 3.81 (s, 3H, OCH_3), 4.31 (br d, 1H, βPrs βCH), 4.41–4.72 (m, 2H, Phe and Tyr αCH), 5.22 (s, 1H, Phe NH), 5.52 (m, 1H, Tyr NH), 7.02–7.24 (m, 10H, Ar), 9.01 (s, 1H, OH). Anal. Calcd for $C_{28}H_{37}N_3O_8S$: C 58.42, H 6.48, N 7.30; found: C 58.63, H 6.39, N 7.57.

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4.1.4.7. Boc–Tyr–(R)-βPrs–Phe–OMe (3f): Hydrogenolysis of **2f** (0.270 g, 0.60 mmol) in MeOH on 10% Pd/C in the presence of TFA afforded the corresponding trifluoroacetate salt (white foam, 92%) which was coupled with Boc–Tyr–OH (0.156 g, 0.55 mmol). Purified on SiO_2 (DCM/MeOH 95:5). Colourless oil (78%); $[\alpha]_D$ – 5°; IR ν:3689, 3432, 3033, 3011, 1744 cm^{-1}; ^{1}H NMR δ: 1.53 [s, 9H, C $(CH_3)_3$], 2.49 (m, 2H, βPrs γCH_2), 2.68–3.52 (m, 6H, Phe βCH_2, βPrs α and δCH_2), 3.79 (s, 3H, OCH_3), 4.33 (br d, 1H, βPrs βCH), 4.40–4.68 (m, 2H, Phe and Tyr αCH), 5.20 (s, 1H, Phe NH), 5.53 (m, 1H, Tyr NH), 7.00–7.23 (m, 10H, Ar), 9.02 (s, 1H, OH). Anal. Calcd for $C_{28}H_{37}N_3O_8S$: C 58.42, H 6.48, N 7.30; found: C 58.59, H 6.72, N 7.47.

4.1.4.8. Boc–Tyr–(S)-HPro–Phe–Phe–NH_2 (4a): Alkaline hydrolysis of **3a** (0.372 g, 0.69 mmol) in MeOH with aq. NaOH 2.0 N afforded the corresponding acid (white solid, 72%) which was coupled with H–Phe–NH_2. HCl (0.100 g, 0.50 mmol). Triturated with hexane. White solid (64%); $[\alpha]_D$ – 12°; IR ν:3684, 3295, 3029, 2975, 1643 cm^{-1}; ^{1}H NMR (d_6-DMSO) δ: 1.37 [s, 9H, $C(CH_3)_3$], 1.75–1.80 (m, 4H, HPro β and γCH_2), 2.61–2.95 (m, 8H, Tyr βCH_2, two Phe βCH_2 and CH_2CO), 3.12 (br s, 2H, HPro δCH_2), 3.73 (br s, 1H, HPro αCH), 3.91 (m, 2H, two Phe αCH), 4.28 (m, 1H, Tyr αCH), 6.71 (m, 1H, Tyr NH), 6.90–7.22 (m, 14H, Ar), 8.01–8.22 (m, 4H, NH_2 and two Phe NH), 9.21 (m, 1H, OH). Anal. Calcd for $C_{38}H_{47}N_5O_7$: C 66.55, H 6.91, N 10.21; found: C 66.27, H 7.03, N 10.35.

4.1.4.9. Boc–Tyr–(R)-HPro–Phe–Phe–NH_2 (4b): Alkaline hydrolysis of **3b** (0.448 g, 0.81 mmol) in MeOH with aq. NaOH 2.0 N afforded the corresponding acid (white solid, 87%) which was coupled with H–Phe–NH_2. HCl (0.141 g, 0.70 mmol). Triturated with hexane. White solid (66%); $[\alpha]_D$ – 9°; IR ν:3690, 3336, 3034, 2989, 1682 cm^{-1}; ^{1}H NMR (d_6-DMSO) δ: 1.39 [s, 9H, $C(CH_3)_3$], 1.72–1.81 (m, 4H, HPro β and γCH_2), 2.59–2.91 (m, 8H, Tyr βCH_2, two Phe βCH_2 and CH_2CO), 3.14 (br s, 2H, HPro δCH_2), 3.70 (br s, 1H, HPro αCH), 3.89 (m, 2H, two Phe αCH), 4.33 (m, 1H, Tyr αCH), 6.68 (m, 1H, Tyr NH), 6.92–7.29 (m, 14H, Ar), 8.09–8.25 (m, 4H, NH_2 and two Phe NH), 9.26 (m, 1H, OH). Anal. Calcd for $C_{38}H_{47}N_5O_7$: C 66.55, H 6.91, N 10.21; found: C 66.33, H 6.76, N 10.29.

4.1.4.10. Boc–Tyr–(S)-HPrs–Phe–Phe–NH_2 (4c): Alkaline hydrolysis of **3c** (0.271 g, 0.46 mmol) in MeOH with aq. NaOH 2.0 N afforded the corresponding acid (white solid, 84%) which was coupled with H–Phe–NH_2. HCl (0.077 g, 0.38 mmol). Triturated with hexane. White solid (58%); $[\alpha]_D$ – 36°; IR ν:3691, 3372, 3012, 2931, 1685 cm^{-1}; ^{1}H NMR (d_6-DMSO) δ: 1.42 [s, 9H, $C(CH_3)_3$], 1.80–2.00 (m, 4H, HPrs β and γCH_2), 2.70–3.49 (four m, 8H, Tyr βCH_2, two Phe βCH_2 and HPrs δCH_2), 3.96 (m, 2H, CH_2SO_2), 3.91–4.32 (four m, 4H, two Phe, HPrs and Tyr αCH), 5.22 (br s, 1H, Tyr NH), 5.90–6.22 (m, 2H, two Phe NH), 6.72–7.64 (m, 14H, Ar), 9.79 (m, 1H, OH). Anal. Calcd for $C_{37}H_{47}N_5O_8S$: C 61.56, H 6.56, N 9.70; found: C 61.37, H 6.76, N 9.98.

4.1.4.11. Boc–Tyr–(R)-HPrs–Phe–Phe–NH_2 (4d): Alkaline hydrolysis of **3d** (0.200 g, 0.34 mmol) in MeOH with aq. NaOH 2.0 N afforded the corresponding acid (white solid, 87%) which was coupled with H–Phe–$NH._2$ HCl (0.059 g, 0.29 mmol). Triturated with hexane. White solid (64%); $[\alpha]_D$ – 38°; IR ν:3689, 3367, 3021, 2930, 1676 cm^{-1}; ^{1}H NMR (d_6-DMSO) δ: 1.42 [s, 9H, $C(CH_3)_3$], 1.84–2.03 (m, 4H, HPrs β and γCH_2), 2.67–3.45 (four m,

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8H, Tyr βCH_2, two Phe βCH_2 and HPrs δCH_2), 3.89 (m, 2H, CH_2SO_2), 3.89–4.28 (four m, 4H, two Phe, HPrs and Tyr αCH), 5.20 (br s, 1H, Tyr NH), 5.87–6.19 (m, 2H, two Phe NH), 6.78–7.59 (m, 14H, Ar), 9.84 (m, 1H, OH). Anal. Calcd for $C_{37}H_{47}N_5O_8S$: C 61.56, H 6.56, N 9.70; found: C 61.28, H 6.34, N 9.56.

4.1.4.12. Boc–Tyr–(S)-βPrs–Phe–Phe–NH_2 (4e): Alkaline hydrolysis of **3e** (0.109 g, 0.19 mmol) in MeOH with aq. NaOH 2.0 N afforded the corresponding acid (pale yellow solid, 89%) which was coupled with H–Phe–NH_2. HCl (0.034 g, 0.17 mmol). Triturated with hexane. White solid (66%); $[\alpha]_D$ – 10°; IR ν:3689, 3032, 2337, 1683 $cm^{-1;}$ ^{1}H NMR (d_6-DMSO) δ: 1.36 [s, 9H, $C(CH_3)_3$], 2.20–2.79 (m, 8H, Tyr βCH_2, two Phe βCH_2 and βPrs γCH_2), 3.10–3.29 (m, 4H, βPrs α and δCH_2), 3.51–4.72 (four m, 4H, βPrs βCH, two Phe and Tyr αCH), 6.32–6.54 (m, 2H, Tyr NH and $NHSO_2$), 6.73–7.68 (m, 14H, Ar), 7.51–8.00 (m, 3H, Phe NH and NH_2), 8.90 (br m, 1H, OH). Anal. Calcd for $C_{36}H_{45}N_5O_8S$: C 61.09, H 6.41, N 9.89; found: C 61.17, H 6.29, N 9.48.

4.1.4.13. Boc–Tyr–(R)-βPrs–Phe–Phe–NH_2 (4f): Alkaline hydrolysis of **3f** (0.196 g, 0.34 mmol) in MeOH with aq. NaOH 2.0 N afforded the corresponding acid (pale yellow solid, 89%) which was coupled with H–Phe–NH_2. HCl (0.061 g, 0.30 mmol). Purified on SiO_2 (DCM/MeOH 95:5). White foam (48%); $[\alpha]_D$ – 10°; IR ν:3691, 3029, 2330, 1687 $cm^{-1;}$ ^{1}H NMR δ: 1.34 [s, 9H, $C(CH_3)_3$], 2.23–2.84 (m, 8H, Tyr βCH_2, two Phe βCH_2 and βPrs γCH_2), 3.12–3.33 (m, 4H, βPrs α and δCH_2), 3.49–4.76 (four m, 4H, two Phe and Tyr αCH, βPrs βCH), 6.30–6.48 (m, 2H, Tyr NH and $NHSO_2$), 6.70–7.66 (m, 14H, Ar), 7.48–7.95 (m, 3H, Phe NH and NH_2), 8.92 (br m, 1H, OH). Anal. Calcd for $C_{36}H_{45}N_5O_8S$: C 61.09, H 6.41, N 9.89; found: C 61.25, H 6.18, N 9.67.

4.1.4.14. Boc–Tyr–(S)-HPro–Phe–Pro–NH_2 (5a): Alkaline hydrolysis of **3a** (0.112 g, 0.19 mmol) in MeOH with aq. NaOH 2.0 N afforded the corresponding acid (pale yellow solid, 89%) which was coupled with H–Pro–NH.$_2$ HCl (0.026 g, 0.17 mmol). Purified on SiO_2 (DCM/MeOH 95:5). White solid (56%); $[\alpha]_D$ – 12°; IR ν:3687,3295, 2975, 2345, 1643 $cm^{-1;}$ ^{1}H NMR (d_6-DMSO) δ: 1.32 [s, 9H, $C(CH_3)_3$], 1.52–2.00 (m, 4H, HPro and Pro γCH_2), 2.70–3.02 (m, 6H, Tyr and Phe βCH_2, CH_2CO), 3.11 (m, 4H, HPro and Pro δCH_2), 4.12–4.81 (four m, 4H, Phe, HPro, Pro and Tyr αCH), 6.63 (m, 1H, Tyr NH), 6.65–7.28 (m, 11H, Ar, Phe NH and Pro–NH_2), 9.25 (br m, 1H, OH). Anal. Calcd for $C_{34}H_{45}N_5O_7$: C 64.23, H 7.13, N 11.02; found: C 64.17, H 6.98, N 11.23.

4.1.4.15. Boc–Tyr–(R)-HPro–Phe–Pro–NH_2 (5b): Alkaline hydrolysis of **3a** (0.134 g, 0.26 mmol) in MeOH with aq. NaOH 2.0 N afforded the corresponding acid (pale yellow solid, 83%) which was coupled with H–Pro–NH.$_2$ HCl (0.032 g, 0.21 mmol). Purified on SiO_2 (DCM/MeOH 95:5). White solid (51%); $[\alpha]_D$ – 9°; IR ν:3690, 3336, 2989, 2345, 1682 $cm^{-1;}$ ^{1}H NMR (d_6-DMSO) δ: 1.30 [s, 9H, C $(CH_3)_3$], 1.49–1.98 (m, 4H, HPro and Pro γCH_2), 2.75–3.10 (m, 6H, Tyr and Phe βCH_2, CH_2CO), 3.09 (m, 4H, HPro and Pro δCH_2), 4.10–4.75 (four m, 4H, Phe, HPro, Pro and Tyr αCH), 6.59 (m, 1H, Tyr NH), 6.73–7.32 (m, 11H, Ar, Phe NH and Pro–NH_2), 9.28 (br m, 1H, OH). Anal. Calcd for $C_{34}H_{45}N_5O_7$: C 64.23, H 7.13, N 11.02; found: C 64.39, H 6.93, N 11.18.

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4.1.5. Deprotection of Boc-peptides. General procedure

The Boc-protected peptides were dissolved in 95% trifluoroacetic acid (2.0 mL). After 2 h at room temperature, the solution was collected, the crude peptides were precipitated from the solution with peroxide-free dry diethyl ether at 0 °C and centrifuged. After several washing with ether, the precipitated peptides were dissolved in a solution of water with 1% TFA and then lyophilized to give compounds **6a–f** and **7a,b**.

4.2. In vitro assays

All radioligands were purchased from NEN (Boston, MA). Radioligands binding analysis was carried out using crude membrane preparations from HN9.10 cells that have been transfected with the human κ-, δ- or the μ-opioid receptor cDNA, and in each transfected cells line expressed constitutively a stable level of these receptors after clonal selection [35]. The membranes were resuspended in ice-cold Tris-buffer (50 mM, pH 7.4) containing 0.5% bovine serum albumin (BSA), and the following protease inhibitors: 30 μM bestatin, 10 μM captopril, 50 μg/mL bacitracin, 100 μM phenyl-methylsulfonyl-fluoride (PMSF). Radioligand competition analysis was carried out using membranes prepared from each of the cell lines that expressed either κ-, δ- or μ-opioid receptors. [^{3}H]U69,593 (1.6 nM) was used to label the κ-opioid receptors, 1 nM [^{3}H]DAMGO was used to label the μ-opioid receptors and 1 nM ([^{3}H]DPDPE was used to label the δ-opioid receptors in the respective cell membranes preparations. For each competition assay, 10 concentrations (10^{-13} M to 10^{-4} M, in duplicate) of the examined substrate were each incubated with membranes (ranging between 15 and 25 μg) and radioligand in a total volume of 0.5 mL for 3 h at 25 °C in a shaking water bath and terminated by rapid filtration through Whatman GF/B filters (presoaked in polyethyleneimine) and washed with 2 × 4 mL of ice-cold 50 mM Tris. Non-specific binding of the radioligand was defined as the amount of radioactivity bound to the cell membranes in the presence of 10 μM naloxone. Radioactivity was determined by liquid scintillation counting. Data were fitted by non-linear least-squares analysis using GraphPad Prism. All analyses were based on 3 independent experiments [36]. The *in vitro* tissue bioassays (MVD and GPI/LMMP) were performed as described previously [37]. IC_{50} values represent means of no less than four experiments. IC_{50} values, relative potency estimates, and their associated standard errors were determined by fitting the data to the Hill equation by a computerized non-linear least-squares method.

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Abbreviations

HPr–OH	homoproline (2-pyrrolidineacetic acid)
HPrs-OH	2-pyrrolidinemethanesulphonic acid
βPro-OH	β-proline (3-pyrrolidinecarboxylic acid)
βPrs-OH	3-pyrrolidinesulphonic acid

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DMSO	dimethylsulfoxide
Cbz	benzyloxycarbonyl
Boc	tert-butyloxycarbonyl
DCM	dichloromethane
HOBt	hydroxybenzotriazole
TEA	triethylamine
EDC	N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride

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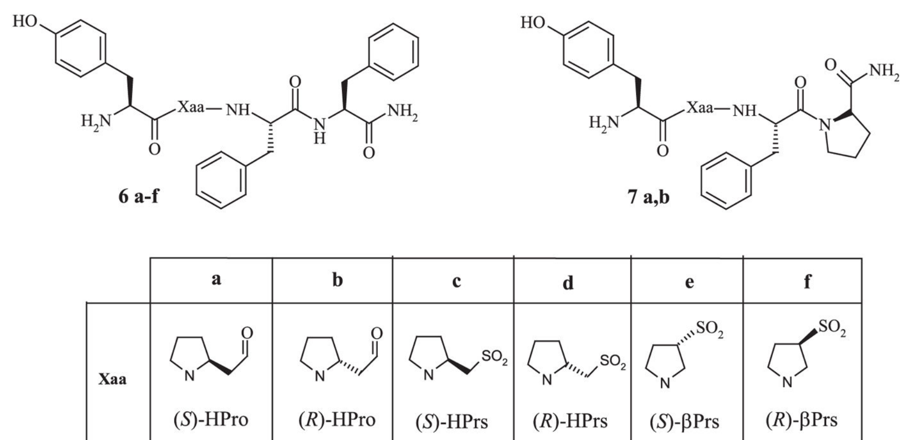

Fig. 1.

Schematic representation of the reported EM2 (**6a–f**) and morphiceptin (**7a,b**) analogues.

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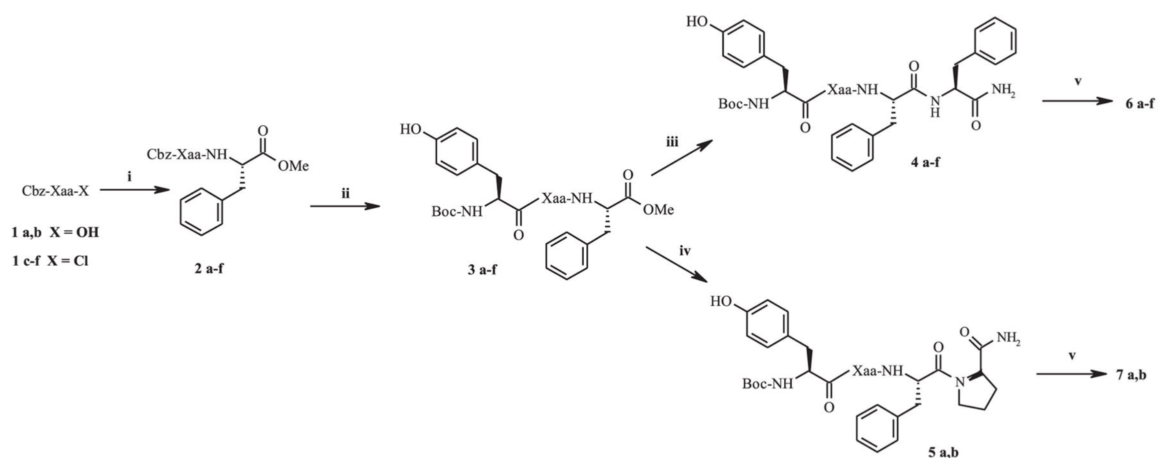

Scheme 1.

Synthesis of pseudopeptides **6a–f** and **7a,b**. Reagents: (i) for **2a,b**: Phe–OMe.HCl, EDC, HOBt, TEA, DCM; for **2c–f**: Phe–OMe.HCl, TEA, DCM; (ii) a: H_2, Pd/C, MeOH, TFA; b: Boc–Tyr–OH, EDC, HOBt, TEA, DCM; (iii) a: 1 M NaOH, MeOH; b: Phe-NH_2HCl, EDC, HOBt, TEA, DCM; (iv) a: 1 M NaOH, MeOH; b: Pro-NH_2HCl, EDC, HOBt, TEA, DCM; (v) TFA/H_2O 95:5. Structures of Xaa residues are reported in Fig. 1.

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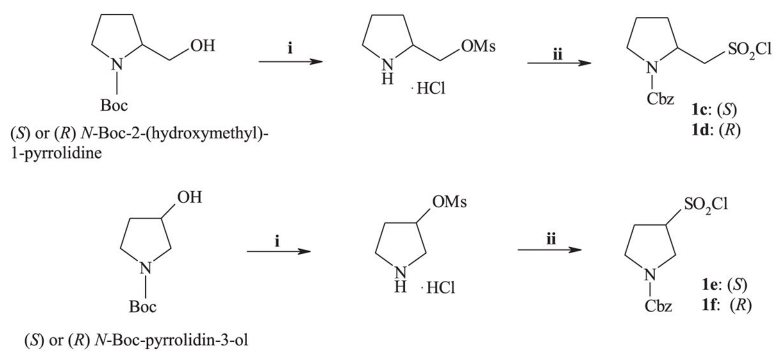

Scheme 2.

Synthesis of sulfonyl chlorides **1c–f**. Reagents: i) a: MsCl, TEA, DCM; b: HCl, dioxane; ii) a: Na_2SO_3, H_2O; b: Cbz–Cl, aq. NaOH; c: $COCl_2$/toluene, DMF, DCM.

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Table 1

Sequence and mass spectra analysis of the reported analogues **6a–f** and **7a,b**.

Peptide	Sequence[a]	M(H+) obsd (MW calcd)
6a	H–Tyr–**(*S*)-HPro**–Phe–Phe–NH_2	586.3792 (585.2951)
6b	H–Tyr–**(*R*)-HPro**–Phe–Phe–NH_2	586.2837 (585.2951)
6c	H–Tyr–**(*S*)-HPrs**–Phe–Phe–NH_2	622.3729 (621.2621)
6d	H–Tyr–**(*R*)-HPrs**–Phe–Phe–NH_2	622.4567 (621.2621)
6e	H–Tyr–**(*S*)-βPrs**–Phe–Phe–NH_2	608.5681 (607.7216)
6f	H–Tyr–**(*R*)-βPrs**–Phe–Phe–NH_2	608.6743 (607.7216)
7a	H–Tyr–**(*S*)-HPro**–Phe–Pro–NH_2	536.4761 (535.2795)
7b	H–Tyr–**(*R*)-HPro**–Phe–Pro–NH_2	536.3954 (535.2795)

[a] Modification are shown in bold letters.

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Table 2

Binding affinity and in vitro activity for compounds **6 a–f** and **7 a,b**.

Compound	Receptor affinity[a,b] (nM)			Selectivity	Bioassay[b] (nM)	
	K_i^{δ}	K_i^{μ}	K_i^{κ}	K_i^{δ}/K_i^{μ}	**MVD (IC_{50})**	**GPI (IC_{50})**
EM2[c]	8360 ± 1314	8.23 ± 0.48	–	1016	510 ± 35d	15 ± 2[d]
Morphyceptin[c]	> 10000	135 ± 18.4	–	> 74.1	–	–
6a	4900 ± 540	58 ± 5	990 ± 12	85	1300 ± 300	260 ± 32
6b	> 10000	293 ± 27	10000 ± 1200	> 34	1900 ± 230	420 ± 91
6c	8600 ± 940	100 ± 8	4800 ± 367	86	1100 ± 260	730 ± 64
6d	7800 ± 850	510 ± 48	6300 ± 540	15.3	36% at 1 μM	2700 ± 220
6e	> 10000	19 ± 3	3400 ± 330	> 532.9	530 ± 91	260 ± 56
6f	7100 ± 782	63 ± 5	7100 ± 628	113.1	390 ± 56	220 ± 41
7a	> 10000	6100 ± 553	4400 ± 356	> 6	33.7% at 1 μM	1100 ± 115
7b	> 10000	30 ± 4	10000 ± 982	> 333	96 ± 12	17 ± 3

[a] Displacement of [^{3}H]DAMGO (μ-selective) and [^{3}H]DPDPE (κ-selective) from rat brain membrane binding sites; displacement of [^{3}H]U69593 (κ-selective) from guinea pig brain membrane binding sites.

[b] ± S.E.M.

[c] Data from reference [31].

[d] Data from reference [32].