Stability against enzymatic hydrolysis of endomorphin-1 analogues containing β-proline

Giuliana Cardillo,*[a] Luca Gentilucci,*[a] Alessandra Tolomelli,[a] Maria Calienni,[b] Ahmed R. Qasem[b] and Santi Spampinato[b]

[a] Dipartimento di Chimica "G. Ciamician", Università di Bologna, via Selmi 2, 40126 Bologna, Italy. E-mail: gentil@ciam.unibo.it. E-mail: cardillo@ciam.unibo.it; Fax: +39 0512099456
[b] Dipartimento di Farmacologia, Università di Bologna, via Irnerio 48, 40126 Bologna, Italy. E-mail: spampi@biocfarm.unibo.it

The enantiomer of endomorphin-1 (Tyr-Pro-Trp-PheNH$_2$) and the analogues containing (S)- or (R)-β-proline have been synthesized, and their affinities towards μ-opioid receptors have been measured. As expected, the incubations of the different peptides with some commercially available enzymes showed that the presence of D-residues gave strong resistance towards digestion. The presence of β-proline alone is sufficient to confer good resistance against the hydrolysis of the biologically strategic Pro–Trp bond.

Introduction

Despite the enormous need for pain control therapies in modern medicine, there is not at present any efficient alternative to the use of morphine and related alkaloids. Unfortunately, a prolonged clinical use of morphine leads to well known undesirable effects.[1] Recently, Zadina isolated the endogenous peptides endomorphin-1, Tyr-Pro-Trp-PheNH$_2$, and endomorphin-2, Tyr-Pro-Phe-PheNH$_2$,[2] which revealed high affinity for the μ-opioid receptor,[3] similar to morphine, and play an important role in analgesia.[4] Recent papers support the concept that the immunomodulatory,[5] cardiovascular, respiratory, and analgesic effects of endomorphin-1-like agonists can be dissociated.[6]

One of the most intriguing outcomes of this discovery, is the possibility of developing endomorphins as novel, safer analgesics.[7] The possibility of using native opioid peptides as analgesics is generally invalidated by their limited resistance towards enzymatic hydrolysis *in vivo*. Endomorphin-1 is easily degraded by peptidases,[8] such as dipeptidyl peptidase IV (DPP IV), which appears to be a major physiological regulator[9] for some neuropeptides, regulatory peptides, circulating hormones and chemokines.[10] Several of the endomorphin-1 degradation products have been isolated from the central nervous system. None of the detected products had an effect on GTP binding, nor was able to produce analgesia, suggesting that degradation prevents the biological activity.[11]

Besides the concomitant use of peptidase inhibitors,[12] a possible approach is the use of more stable peptide analogues.[13] In several cases, however, peptidomimetics displayed long term toxicity and difficulties in penetrating the blood–brain barrier,[14] in particular when massive structural modifications were introduced.

Generally, the introduction of D-amino acids in an opioid peptide[15] gives an increased stability. Indeed, only a few enzymes capable of hydrolysing peptide bonds involving D-amino acids have been characterised in multicellular organisms.[16] A series of diastereoisomeric endomorphin-1[17] and endomorphin-2[18] analogues containing D-amino acids have been synthesized and their potency measured. However, these peptides in general exhibited poor affinities towards μ-receptors.

Another possibility we explored is the substitution of α-amino acids with β-amino acids in the peptide sequence. During the course of a program directed towards the synthesis of modified endomorphins, we have reported the preparation of some endomorphin-1 analogues containing β-amino acids, having the carboxylic acid group shifted to the β position,[19] and

homo-β-amino acids.[20] The affinity for the opioid receptors varied depending on the β-amino acid present.

Recent studies performed on endomorphin-1 by molecular modeling and two-dimensional NMR in different environments, indicated proline to be the key residue that induces the other residues to assume the proper spatial orientation for the best ligand–receptor interaction.[17,21]

Moreover, the introduction of modified proline could play a special role in the peptide stability *in vivo*, since the activity of DPP IV is particularly directed towards the degradation of proline-containing peptides.[14]

For this reason, we decided to focus our attention on peptide sequences containing β-prolines carrying the carboxylic acid group in the 2 position of the pyrrolidinic ring. Previously we have reported the enhanced resistance towards enzymatic hydrolysis of endomorphin-1 analogues containing homoproline.[20] In the present work, we report the enzymatic stability of peptides containing β-proline in comparison to endomorphin-1 (**5**) and its enantiomer **6**.

Results and discussion

The synthesis of β-proline was performed by means of a modified version of a described procedure (Scheme 1).[22] To prepare Boc-(S)-β-proline, we started from Cbz-(R)-3-hydroxypyrrolidine **1**[23] (Scheme 1). Tosylation under standard conditions gave **2**, which was treated with KCN, giving the 3-cyano derivative **3** with complete inversion of the configuration. In the same step the cyano group was hydrolysed and the benzyloxycarbonyl group was removed under acidic conditions, and the resulting amino acid was protected to give Boc-(S)-β-proline **4** (Scheme 1). In a similar way, starting from Boc-(S)-3-hydroxypyrrolidine we obtained Boc-(R)-β-proline.
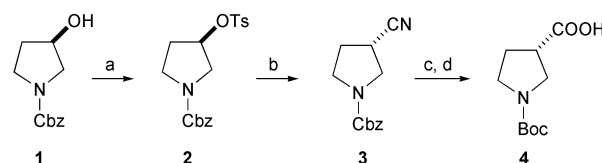
**Scheme 1** Synthesis of (S)-Boc-β-proline. *Reagents*: (a) TsCl, Et$_3$N, DMAP. (b) KCN, DMSO. (c) 6 M HCl. (d) (Boc)$_2$O, Na$_2$CO$_3$.

The synthesis of the peptides **5**–**9** was performed by way of a convergent coupling of the amino acids in solution, under the conventional Boc conditions, and using EDCI–HOBt as condensing agents (Scheme 2).[24] After each coupling, peptides

DOI: 10.1039/b301507f

were purified by flash-chromatography over silica gel. Boc deprotection with HCl in dioxane gave HCl peptide salts, which were used without purification. Final purification of the tetrapeptides by semi-preparative reversed-phase HPLC gave peptides **5**–**9** as TFA salts (Scheme 2). Purities were determined to be 94–97% by analytical reversed-phase HPLC.

Under the conditions reported above, we synthesized endomorphin-1 (**5**), its enantiomer **6**, the modified endomorphin containing (*R*)-β-Pro **7**, its enantiomer **8**, and the tetrapeptide
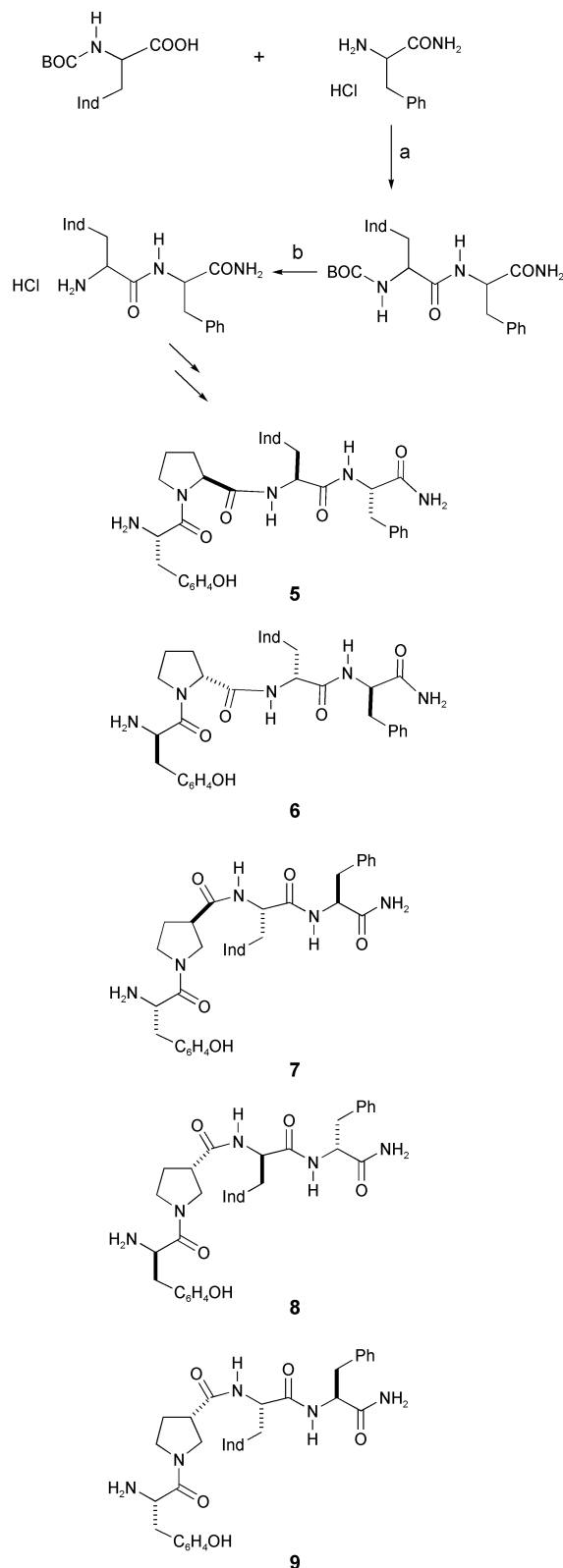
Scheme 2 Synthesis of endomorphin-1 (**5**), of the enantiomer **6**, and of the analogues **7-9**. *Reagents*: (a) EDCI, HOBt, NEt₃. (b) HCl–dioxane. Ind = indolyl.

containing (*S*)-β-Pro **9**. The synthesis of **7** and **9** has been reported in a short communication.[19]

To test the affinities towards μ-opioid receptors, the new peptides were incubated in rat brain membrane homogenates containing the receptors, using [³H]-DAMGO {[³H]-(Tyr-D-Ala-Gly-MePhe-glyol); MePhe = *N*-methylphenylalanine} as the μ-specific radioligand.

The affinities of **7** (K_i: 0.33 nM, IC$_{50}$: 1.80 nM) and **9** (K_i: 10.4 nM, IC$_{50}$: 72.0 nM) have been reported.[19] The K_i and IC$_{50}$ values measured for endomorphin-1 (**5**) (K_i: 0.16 ± 0.02 nM; IC$_{50}$: 4.6 ± 0.3 nM; n_H: 0.74 ± 0.03) and DAMGO (K_i: 1.60 ± 0.30 nM; IC$_{50}$: 9.9 ± 0.6 nM; n_H: 0.90 ± 0.05) agree with the literature.[12,25] The affinities measured for the modified peptides[26] were in general lower in comparison to the parent peptide **5**. In a similar way to what was observed for endomorphin-2,[18] from the comparison of **5** and its enantiomer **6** (K_i: 67.0 ± 2.0 nM; IC$_{50}$: 780 ± 30 nM; n_H: 0.90 ± 0.05), it appears that the substitution of each amino acid with the corresponding D-stereoisomer caused a decrease of affinity.

Interestingly, peptide **8**, which differs from **6** in the presence of D-β-proline, has affinity in the nanomolar range (K_i: 3.8 ± 0.2 nM; IC$_{50}$: 180 ± 5 nM; n_H: 1.04 ± 0.05), despite of the presence of D-amino acids.

The stability of endomorphin-1 and of the modified peptides towards enzymatic degradation has been investigated by measuring their hydrolysis rates in the presence of some commercially available enzymes: α-chymotrypsin (Fig. 1), carboxypeptidase-Y[27] (Fig. 2), and aminopeptidase-M[27] (Fig. 3). Peptides were dissolved in a buffer pH = 7.4 and incubated at 37 °C with each enzyme in parallel. At designated times, mixture aliquots were analysed by HPLC. Results were collected in graphs reporting the amount of starting peptide remaining (area%) *vs.* time. To appreciate the resistances displayed by the different peptides, peak areas were normalized to 100 at *t* = 0 and the curves were superimposed.

The digestions of **5**–**9** with α-chymotrypsin were followed over 5–6 h (Fig. 1). After 1 h the remaining amounts of endomorphin-1 (**5**) and **7** were around 20%, while **9** was still around
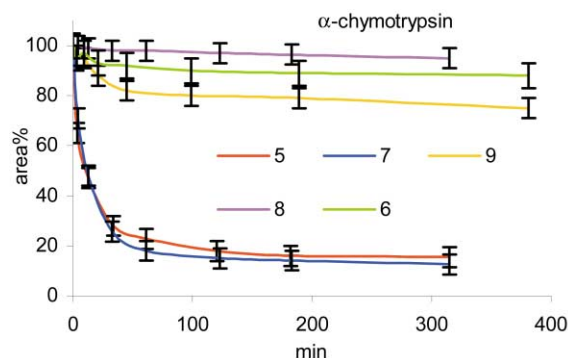

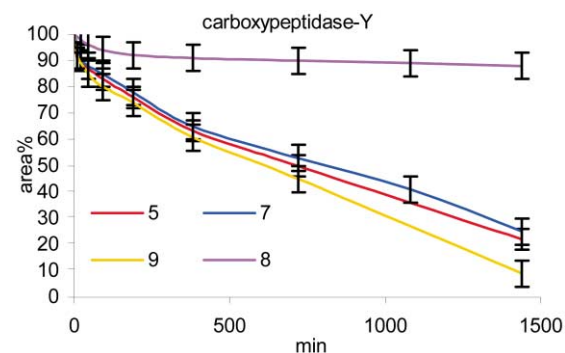**Fig. 1** Peptide degradation rates upon incubation with α-chymotrypsin.

Fig. 2 Peptide degradation rates upon incubation with carboxypeptidase-Y.
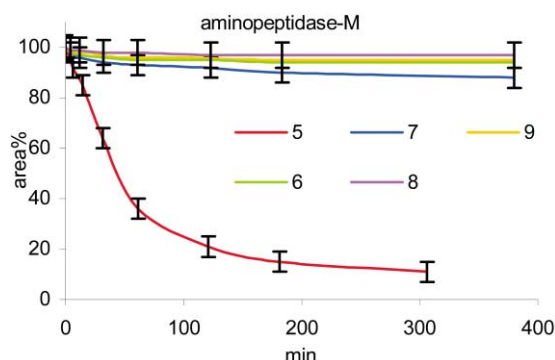
Fig. 3 Peptide degradation rates upon incubation with aminopeptidase-M.

80%, and **6** and **8** were almost completely intact (>90%). While the results obtained for **6** and **8** confirm the resistance expected for peptides containing D-residues,[15,16] the resistance displayed by **9** was less predictable, since the only unusual residue was (*S*)-β-Pro.

The degradation of the peptides with carboxypeptidase-Y under the selected experimental conditions were slower (Fig. 2). However, after 24 h, endomorphin-1 (**5**), **7**, and **9** were strongly reduced (around 18–25%), while **8** was still around 90%. Taking into consideration area errors, with the exception of **8**, which contained all D-residues, the other peptides were degraded to substantially the same extent as endomorphin-1.

The digestions with aminopeptidase-M were monitored over 5–6 h (Fig. 3). During this period, endomorphin-1 (**5**) was degraded to approximately 15% of the original amount, for the cleavage of the Pro–Trp bond.[20,27] In contrast, the peptides **6**–**9** were scarcely degraded, being still present in 85–90%. In particular, the stabilities displayed by **7** and **9** were of interest, since the presence of only an (*S*)-β-proline or an (*R*)-β-proline respectively in a sequence of natural residues was sufficient to ensure a good stability.

The study of peptide digestions by aminopeptidase-M could also be of value to predict the resistance to degradation *in vivo*. Indeed, peptides demonstrating a good resistance towards aminopeptidase-M could also possess stability against the natural regulator DPP IV, which hydrolyses the same Pro–Trp bond.[28]

The comparison of the affinities displayed by the different peptides for μ-opioid receptors, and the comparison of the degradation rates in the presence of proteolyic enzymes, indicated β-proline to be the key residue both for biological activity and resistance to enzymatic hydrolysis.

Conclusions

We have synthesized and tested a series of endomorphin analogues containing β-proline with L or D configuration and/or D-α-amino acids. The modified peptides were more stable than endomorphin-1 in the presence of proteolytic enzymes. In particular, **8** remained in all cases almost intact for several hours under the experimental conditions, while **7** and **9** showed a good resistance against the hydrolysis of the Pro–Trp bond. The modifications introduced gave **8** an excellent general resistance, still maintaining an affinity in the nanomolar range. Therefore, the new modifications introduced could strongly enhance peptide bioavailability *in vivo*.

Experimental

General methods

Unless stated otherwise, chemicals were obtained from commercial sources and used without further purification. CH_2Cl_2 was distilled from P_2O_5. Flash chromatography was performed on Merck silica gel 60 (230–400 mesh), and solvents were simply distilled. NMR spectra were recorded with a Gemini Varian spectrometer at 300 MHz (^{1}H-NMR) and at 75 MHz (^{13}C-NMR). Chemical shifts are reported as δ values relative to the solvent peaks ($CHCl_3$ in $CDCl_3$: 7.26 ppm; CH_3OH in CD_3OD: 3.34 ppm). Preparative reversed-phase HPLC was performed on a Waters Delta Prep 4000 Millipore instrument, with a C18 column RP-18 (40–63 μm). The FAB-mass instrument employed was a Micromass ZMD spectrometer equipped with single quadrupole analyzer and a Z-spray ionspray source outfitted with a 50 mm deactivated fused Si capillary connected to a Harvard Apparatus pump 11 for sample injection. Data acquisition and spectra analysis were conducted with Masslynx 3.3 software running on a Digital Equipment Corp. personal computer. Nitrogen was used both as desolvation and nebulizer gas. The desolvation temperature was set at 200 °C and capillary voltage at 3.0 kV. Analytical HPLC was performed on a HP Series 1100 spectrometer, with a HP Hypersil ODS column (4.6 μm particle size, 100 Å pore diameter, 250 mm), DAD 215.8 nm. Optical rotations were measured by a Perkin-Elmer 343 polarimeter, and are given in 10^{-1} deg cm^2 g^{-1}. GC-MS Analyses were performed using a Hewlett-Packard series II 5890 GC with a capillary column HP 5–5% Ph-Me-Si, 30 m, 0.25 micron, ID 0.25 mm, coupled with a MS detector HP 5971. IR was performed using a Nicolet 210 FT-IR spectrometer. Enzymatic hydrolyses were performed in a MGM Lauda RC20 thermostatic bath. Carboxypeptidase-Y lyophilized powder, 20% protein content, pH 5, 19 units mg^{-1} solid, 100 units mg^{-1} prot.; aminopeptidase-M suspension in 3.5 M $(NH_4)_2SO_4$ solution, pH 7.4, 5.1 mg prot. mL^{-1}, 29 units mg^{-1}; α-chymotripsin, 60 U mg^{-1}, were purchased from Sigma. Homogenates were centrifuged in Beckman J6B and Beckman J2-21 centrifuges. Radioactivity was measured by liquid scintillation spectrometry using a Beckman apparatus.

(*R*)-1-Benzyloxycarbonyl-3-tosyloxypyrrolidine ((*R*)-2)

A mixture of (*R*)-3-hydroxypyrrolidine hydrochloride (0.50 g, 4.0 mmol), TEA (0.56 mL, 4.0 mmol) and benzyloxycarbonyl chloride (0.86 mL, 6.0 mmol) was stirred in CH_2Cl_2 (50 mL) at 0 °C. After 2 h solvent was evaporated at reduced pressure, the residue was diluted with EtOAc and washed with 0.5 M HCl, with sat. Na_2CO_3, and dried over Na_2SO_4. Evaporation of solvent at reduced pressure gave (*R*)-**1** (0.85 g, 95%), used without further purification. MS *m/z* 203 (M$^+$ − 18, 17%), 148 (12), 112 (19), 91 (100).

A mixture of (*R*)-**1** (0.85 g, 3.8 mmol), TEA (0.53 mL, 3.8 mmol,), cat. DMAP, and tosyl chloride (0.93 g, 4.9 mmol) in CH_2Cl_2 (30 mL), was stirred at 0 °C. After 3 h, the mixture was washed with sat. Na_2CO_3, and the water layer was extracted twice with CH_2Cl_2. Organic layers were collected and dried over Na_2SO_4, and solvent was evaporated at reduced pressure. The resulting crude residue was purified by flash chromatography over silica gel (eluant: cyclohexane : EtOAc, 80 : 20), giving pure (*R*)-**2** (1.00 g, 70%). IR: *v* 3070, 3050, 1710, 1445, 1370, 1210, 1110 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ_H 1.98–2.05 (m, 2H), 2.41 (s, 3H), 3.38–3.62 (m, 4H), 5.00–5.09 (m, 1H), 5.10 (s, 2H), 7.30–7.80 (m, 9H); ^{13}C NMR ($CDCl_3$) δ_C 21.6, 31.4, 32.4, 43.9, 51.8, 67.0, 127.6, 127.8, 127.9, 128.4, 129.9, 136.5, 145.0, 154.0; MS *m/z* 272 (2%), 232 (5), 216 (19), 189 (8), 169 (9), 145 (20), 113 (33), 57 (100). [*a*]$_D^{20}$ = −11.8 (*c* 1, $CHCl_3$).

(*S*)-1-Benzyloxycarbonyl-3-tosyloxypyrrolidine ((*S*)-2)

Under the same reaction conditions as reported for the synthesis of (*R*)-**1**, (*S*)-3-hydroxypyrrolidine (0.60 g, 4.8 mmol) gave (*S*)-**1** (1.04 g, 97%). Under the same reaction conditions as reported for the synthesis of (*R*)-**2**, (*S*)-**1** (1.04 g, 4.7 mmol) was tosylated to give (*S*)-**1** (1.41 g, 80%). [*a*]$_D^{20}$ = +12 (*c* 1, $CHCl_3$).

(*S*)-1-Benzyloxycarbonyl-3-cyanopyrrolidine ((*S*)-3)

To a solution of (*R*)-**2** (1.00 g, 2.7 mmol) in DMSO (15 mL), KCN (0.35 g, 5.3 mmol) was added and the mixture was stirred at 90 °C for 4 h, then EtOAc was added and the organic layer was washed three times with small portions of water. The combined water layers were treated with $KMnO_4$ before disposal. The organic layer was dried over Na_2SO_4 and solvent was evaporated at reduced pressure, giving crude (*S*)-**3**, obtained pure (0.43 g, 70%) after flash chromatography over silica gel (eluant: cyclohexane : EtOAc, 50 : 50). IR ν 2243, 1704, 1417, 1360, 1195, 696 cm^{-1}; ^{1}H-NMR (CDCl$_3$) δ 2.18–2.38 (m, 2H), 3.02–3.18 (m, 1H), 3.43–3.81 (m, 4H), 5.20 (s, 2H), 7.30–7.50 (m, 5H); ^{13}C-NMR (CDCl$_3$, major conformer) δ 28.3, 29.3, 44.9, 48.7, 49.1, 67.2, 119.6, 127.9, 128.0, 128.4, 136.2, 154.1; MS *m/z* 230 (M$^+$, 20%), 185 (3), 123 (7), 91 (100). $[a]_D^{20}$ = +25.4 (*c* 1.3, CHCl$_3$).

(*R*)-1-Benzyloxycarbonyl-3-cyanopyrrolidine ((*R*)-3)

As reported for the synthesis of (*S*)-**3**, the reaction of (*S*)-**2** (1.00 g, 2.7 mmol) with KCN gave (*R*)-**3** (0.44 g, 72%). $[a]_D^{20}$ = −22.0 (*c* 0.7, CHCl$_3$).

(*S*)-1-(*tert*-Butoxycarbonyl)pyrrolidine-3-carboxylic acid ((*S*)-4)

A stirred mixture of (*S*)-**3** (0.43 g, 1.9 mmol), conc. HCl (10 mL), and conc. CH$_3$COOH (2 mL), was refluxed for 3 h, then it was cooled at rt. The water layer was washed with Et$_2$O, and then it was treated with 6 M NaOH at 0 °C until pH = 9–10 was reached. The mixture was diluted with acetone (10 mL), and di-*tert*-butyl dicarbonate (0.61 g, 2.9 mmol) was added at 0 °C. After 3 h, acetone was evaporated at reduced pressure, and the mixture was treated with 3 M HCl at 0 °C until pH = 3 was reached. The mixture was extracted three times with EtOAc, and the collected organic layers were dried over Na$_2$SO$_4$. Evaporation of the solvent gave (*S*)-**4** (0.35 g, 85%), not needing further purification. IR ν 3400–3000 br, 1741, 1670, 1429, 1375, 1167, 1132; ^{1}H-NMR (CDCl$_3$) δ 1.50 (s, 9H), 2.10–2.20 (m, 2H), 3.00–3.10 (m, 1H), 3.38–3.70 (m, 4H), 10.9 (s, 1H); ^{13}C-NMR (CDCl$_3$) δ 28.2, 28.5, 43.1, 45.1, 47.9, 79.8, 154.4, 177.8. $[a]_D$ = +7.7 (*c* 0.8 CHCl$_3$).

(*R*)-1-(*tert*-Butoxycarbonyl)pyrrolidine-3-carboxylic acid ((*R*)-4)

Hydrolysis of (*R*)-**3** (0.44 g, 1.9 mmol) under acidic conditions as reported for (*S*)-**3** and protection with di-*tert*-butyl dicarbonate gave (*R*)-**4** (0.34 g, 83%). $[a]_D$= −8.0 (*c* 0.5 CHCl$_3$).

Synthesis and purification of 5–9

As a general procedure, the peptide coupling was performed by stirring overnight the HCl salt of the amino amide, the *N-tert*-butyloxycarbonyl amino acid (1.0 equiv), triethylamine (3 equiv), 1-hydroxy-1*H*-benzotriazole (HOBt; 1.0 equiv), the HCl salt of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (EDCI; 1.2 equiv), in a 9 : 1 mixture of CH$_2$Cl$_2$ and DMF at 0 °C. After 12 h, the solvent was evaporated at reduced pressure, and the mixture was diluted with EtOAc. The organic solution was in turn washed with 0.5 M HCl, with saturated Na$_2$CO$_3$, and with brine. The organic layer was dried over Na$_2$SO$_4$, and solvent was evaporated at reduced pressure. Peptides were obtained pure by flash-chromatography over silica gel (eluant: EtOAc : MeOH, 95 : 5) with yields from 60 to 90%.

Boc deprotection was performed by treatment with saturated HCl in dioxane at 0 °C. After 1 h the solvent was evaporated at reduced pressure and the resulting HCl peptide salts were used without purification for the next coupling. HCl tetrapeptide salts were pre-purified by precipitation from MeOH–Et$_2$O.

Final purification was performed by semi-preparative reversed-phase HPLC on a Waters Delta Prep 4000 Millipore,

with a C18 column RP-18 (40–63 μm, 250 mm) with a solvent system A = 0.1% TFA in water and B = 0.1% TFA in acetonitrile, gradient 100% A to 50% B in 50 min at a 5.0 mL min^{-1} flow.

Purities were determined by analytical reversed-phase HPLC, under two different systems: HP Hypersil ODS column, 4.6 μm particle size, 100 Å pore diameter, 250 mm, solvent A = 0.1% TFA in water and B = 0.1% TFA in acetonitrile, gradient 100% A to 50% B in 20 min at a 1.0 mL min^{-1} flow, followed by 20 min at 50%; Phenomenex Luna C18 column, 5 μm particle size, 100 Å pore diameter, 250 mm, solvent A = 0.1% TFA in water and B = 0.1% TFA in acetonitrile, gradient 90% A to 90% B in 60 min at 1.0 mL min^{-1} flow. The average purity levels are: for **5**, 97%; for **6**, 94%; for **7**, 96%; for **8**, 96%; for **9**, 95%.

H-Tyr-Pro-Trp-PheNH$_2$ (5)

^{1}H-NMR (DMSO-d$_6$, major conformer) δ_H 1.40–1.80 (m, 3H), 1.90–2.02 (m, 1H), 2.70–2.90 (m, 2H), 2.90–3.20 (m, 5H), 3.50–3.68 (m, 1H), 4.10–4.21 (m, 1H), 4.35–4.58 (m, 3H), 6.80 (d, *J* = 7.0 Hz, 2H), 6.90–7.60 (m, 14H), 7.80–7.90 (d, *J* = 6.7 Hz, 1H), 8.10 (d, *J* = 6.6 Hz, 1H), 9.40 (s, 1H), 10.80 (s, 1H); ^{13}C-NMR (CD$_3$OD, major conformer) δ_C 26.0, 28.5, 29.8, 36.9, 38.3, 38.8, 54.5, 55.4, 56.1, 61.6, 110.2, 110.8, 112.5, 116.7, 119.3, 120.0, 122.6, 125.0, 125.3, 125.6, 127.7, 128.7, 129.4, 130.3, 131.4, 131.8, 137.9, 138.0, 158.0, 169.0, 173.4, 175.5. FAB MS [M + H]: 611.2; calculated for C$_{34}$H$_{38}$N$_6$O$_5$: 610.3. $[a]_D^{20}$ = −18.5 (*c* 1, MeOH).

H-D-Tyr-D-Pro-D-Trp-D-PheNH$_2$ (6)

FAB MS [M + H]: 611.4; calculated for **6**: 610.3. $[a]_D^{20}$ = +17 (*c* 0.8, MeOH).

H-Tyr-β-L-Pro-Trp-PheNH$_2$ (7)

^{1}H-NMR (DMSO-d$_6$, major conformer) δ_H 1.63–1.80 (m, 1H), 1.80–1.99 (m, 1H), 2.70–2.95 (m, 3H), 2.97–3.15 (m, 3H), 3.16–3.42 (m, 4H), 3.60–3.71 (m, 1H), 4.15–4.20 (m, 1H), 4.40–4.61 (m, 2H), 6.70 (d, *J* = 7.3 Hz, 2H), 6.90–7.40 (m, 13H), 7.55 (d, *J* = 6.9 Hz, 1H), 7.87 (d, *J* = 7.8 Hz, 1H), 8.00 (d, *J* = 8.0 Hz, 1H), 9.41 (s, 1H), 10.80 (s, 1H); ^{13}C-NMR (CD$_3$OD, major conformer) δ_C 27.8, 29.9, 34.0, 36.7, 37.9, 45.2, 49.0, 53.2, 54.5, 67.0, 111.2, 115.6, 118.3, 119.0, 122.6, 124.7, 126.7, 128.3, 129.3, 130.2, 130.5, 137.2, 157.3, 169.0, 171.0, 173.7, 175.0. FAB MS [M + H]: 611.3; calculated for C$_{34}$H$_{38}$N$_6$O$_5$: 610.3. $[a]_D^{20}$ = −9 (*c* 0.2, MeOH).

H-D-Tyr-β-D-Pro-D-Trp-D-PheNH$_2$ (8)

FAB MS [M + H]: 611.6; calculated for **8**: 610.3. $[a]_D^{20}$ = +10 (*c* 0.2, MeOH).

H-Tyr-D-β-Pro-Trp-PheNH$_2$ (9)

^{1}H-NMR (DMSO-d$_6$, major conformer) δ_H 1.65–1.80 (m, 2H), 2.60–3.00 (m, 4H), 3.10–3.15 (m, 3H), 3.40–3.70 (m, 4H), 4.11–4.30 (m, 1H), 4.46–4.85 (m, 2H), 6.75 (d, *J* = 7.1 Hz, 2H), 6.90–7.60 (m, 14H), 8.00–8.25 (m, 2H), 9.40 (s, 1H), 10.70 (s, 1H); ^{13}C-NMR (CD$_3$OD, major conformer) δ_C 27.6, 29.8, 36.0, 36.6, 37.5, 37.9, 46.1, 53.2, 54.5, 67.0, 111.3, 115.6, 118.3, 119.0, 123.6, 124.7, 126.7, 128.3, 129.3, 130.2, 130.5, 136.0, 137.2, 157.3, 166.7, 173.1, 174.0, 174.9. FAB MS [M + H]: 611.2; calculated for C$_{34}$H$_{38}$N$_6$O$_5$: 610.3. $[a]_D^{20}$ = +37.9 (*c* 0.4, MeOH).

Membrane preparations, determination of protein content, and binding assays

Rat brain, without cerebellum, was weighed and homogenized in 10 volumes of ice-cold 0.32 M sucrose–10 mM TRIS-HCl (tris(hydroxymethyl)aminomethane hydrochloride), pH 7.4 at 4 °C. The homogenate was centrifuged at 2000 rpm, for 10 min, at 4 °C, and the supernatant was in turn centrifuged at 19000 rpm,

for 20 min, at 4 °C. The resulting pellet was suspended in 10 volumes of 50 mM TRIS-HCl–100 mM NaCl (pH 7.4 at 4 °C), as incubation buffer and incubated for 1 h at room temperature (in a water bath at 37 °C) to remove any endogenous opioid ligands. After a final centrifugation at 19000 rpm, for 20 min, at 4 °C, the pellet was stored at −80 °C for up to two weeks.

Protein concentration was determined according to Lowry *et al.*[29] [³H]-DAMGO was used as μ-selective radioligand (1 nM); specific activity was 64 Ci mmol^{-1}, K_d = 4.85 nM and B_{max} = 48 fmol mg^{-1} protein; n = 3. Non-specific binding was determined in the presence of 100 μM DAMGO. The incubation buffer consisted of 50 mM TRIS-HCl, 0.1% BSA (bovine serum albumin), pH 7.4 at 4 °C, 2 mM EDTA (ethylene-diaminetetraacetic acid). To prevent any peptidase degradation, the following protease inhibitors were added to the binding buffer: captopril (*N*-[(*S*)-3-mercapto-2-methylpropionyl]-L-proline) 25 μg mL^{-1}, bacitracin 0.2 mg mL^{-1}, and leupeptin (*N*-acetyl-L-leucyl-L-leucyl-L-argininal) 10 μg mL^{-1}, phenyl-methylsulfonyl fluoride 0.19 mg ml^{-1} and aprotinin 5 TIU ml^{-1} (TIU = trypsin inhibitor unit). δ- and κ-opioid receptors were blocked with 0.01 M DADLE ([D-Ala^2, D-Leu^5]-enkephalin) and 0.01 M U50, 488, respectively.

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

Enzymatic digestion of the tetrapeptides

Peptides, 4.0 mg, were dissolved in 4.0 mL TRIS (50 nM, pH 7.4); the solutions were incubated for 30 min before enzyme addition in a thermostated bath at 37 °C and magnetically stirred. The enzyme solutions were prepared in TRIS (50 nM, pH 7.4) as follows: α-chymotrypsin, 2 mg per 10 mL; amino-peptidase-M, 0.1 mL, 0.5 mg per 5 mL; carboxypeptidase-Y, 0.5 mg per 5 mL. Experiments were performed in parallel, by adding 1.0 mL portions of the same enzyme solution to each peptide solution. At designated intervals, a 0.50 mL aliquot of incubated mixture was quenched with 15 μL of 1 M HCl and diluted with 0.2 mL CH$_3$CN. Sampling intervals were chosen so that a kinetic curve could be constructed. The resulting solution was filtered over PTFE syringe filters, pore diameter 0.20 μm. Samples were analyzed by HPLC. Blanks were obtained by incubation of peptides in TRIS 50, pH 7.4–CH$_3$CN 5 : 2 with 1.5 μL 1 M HCl for 6 h.

Each hydrolysis experiment was repeated at least twice, and the reported data are mean values. Error ranges were estimated on the basis of the standard deviation, and are substantially similar for the different peptides.

Acknowledgements

References and notes

1 (*a*) N. Cherny, C. Ripamonti, J. Pereira, C. Davis, M. Fallon, H. McQuay, S. Mercadante, G. Pasternak and V. Ventafridda, *J. Clin. Oncol.*, 2001, **19**, 2542–2554; (*b*) G. A. Olson, R. D. Olson, A. L. Vaccarino and A. J. Kastin, *Peptides*, 1998, **19**, 1791–1843; (*c*) M. Marita, M. Funada and T. Suzuki, *Pharmacol. Ther.*, 2001, **89**, 1–15.

2 J. E. Zadina, L. Hackler, L. J. Ge and A. J. Kastin, *Nature*, 1997, **386**, 499–502.

3 G. W. Pasternak, *Life Sci.*, 2001, **68**, 2213–2219.

4 For a review see: G. Horvath, *Pharmacol. Ther.*, 2000, **88**, 437–463.

5 K. A. Carrigan, C. J. Nelson and D. T. Lysle, *Psychopharmacology*, 2000, **151**, 299–305.

6 (*a*) M. A. Czapla, D. Gozal, O. A. Alea, R. C. Beckerman and J. E. Zadina, *Am. J. Respir. Crit. Care Med.*, 2000, **162**, 994–999; (*b*) A. M. Wilson, R. D. Soignier, J. E. Zadina, A. J. Kastin, W. L. Nores, R. D. Olson and G. A. Olson, *Peptides*, 2000, **2**, 1871–1874.

7 (*a*) For a review see: B. N. Dhawan, F. Cesselin, R. Raghubir, T. Reisine, P. B. Bradley, P. S. Portoghese and M. Hamon, *Pharmacol. Rev.*, 1996, **48**, 567–592; (*b*) R. Przewlocki, D. Labuz, J. Mika, B. Przewlocka, C. Tomboly and G. Toth, *Ann. N. Y. Acad. Sci.*, 1999, **897**, 154–164.

8 A. Sugimoto-Watanabe, K. Kubota, K. Fujibayashi and K. Saito, *Jpn. J. Pharmacol.*, 1999, **81**, 264–270.

9 R. Shane, S. Wilk and R. Bodnar, *J. Brain Res.*, 1999, **815**, 278–286.

10 R. Mentlein, *Regul. Pept.*, 1999, **85**, 9–24.

11 I. Szatmari, D. Biyashev, C. Tomboly, G. Toth, M. Macsai, G. Szabo, A. Borsodi and I. Lengyel, *Biochem. Biophys. Res. Commun.*, 2001, **284**, 771–776.

12 P. W. Shiller, in *Opioids 1*, ed. A. Hertz, Springer-Verlag, Berlin, 1993.

13 (*a*) S. E. Foran, D. B. Carr, A. W. Lipkowski, I. Maszczynska, J. E. Marchand, A. Misicka, M. Beinborn, A. S. Kopin and R. M. Kream, *J. Pharmacol. Exp. Ther.*, 2000, **295**, 1142–1148; (*b*) V. J. Hruby and P. M. Balse, *Curr. Med. Chem.*, 2000, **7**, 945–970.

14 (*a*) Minireview: G. Meisenberg and W. H. Simmons, *Life Sci.*, 1983, **32**, 2611–2623; (*b*) D. J. Begley, *J. Pharm. Pharmacol.*, 1996, **48**, 136–146.

15 C. T. Dooley and R. A. Houghten, *Biopolymers*, 1999, **51**, 379–390.

16 R. Yamada and Y. Kera, *EXS*, 1998, **85**, 145–155.

17 M. G. Paterlini, F. Avitabile, B. G. Ostrowski, D. M. Ferguson and P. S. Portoghese, *Biophys. J.*, 2000, **78**, 590–599.

18 Y. Okada, A. Fukumizu, M. Takahashi, Y. Shimizu, Y. Tsuda, T. Yokoi, S. D. Bryant and L. H. Lazarus, *Biochem. Biophys. Res. Commun.*, 2000, **276**, 7–11.

19 G. Cardillo, L. Gentilucci, P. Melchiorre and S. Spampinato, *Bioorg. Med. Chem. Lett.*, 2000, **10**, 2755–2758.

20 G. Cardillo, L. Gentilucci, A. R. Qasem, F. Sgarzi and S. Spampinato, *J. Med. Chem.*, 2002, **45**, 2571–2578.

21 (*a*) S. Fiori, C. Renner, J. Cramer, S. Pegoraro and L. Moroder, *J. Mol. Biol.*, 1999, **291**, 163–175; (*b*) Y. R. Yang, T. H. Chiu and C.-L. Chen, *Eur. J. Pharmacol.*, 1999, **372**, 229–236; (*c*) B. L. Podlogar, M. G. Paterlini, D. M. Ferguson, G. C. Leo, D. A. Demeter, F. K. Brown and A. B. Reitz, *FEBS Lett.*, 1998, **439**, 13–20; (*d*) M. Keller, C. Boissard, L. Patiny, N. N. Chung, C. Lemieux, M. Mutter and P. W. Schiller, *J. Med. Chem.*, 2001, **44**, 3896–3903.

22 (*a*) S. I. Klein, M. Czekaj, B. F. Molino, F. Bruce and V. Chu, *Bioorg. Med. Chem. Lett.*, 1997, **7**, 1773–1778; (*b*) R. J. Bridges, M. S. Stanley, M. W. Anderson, C. W. Cotman and A. R. Chamberlin, *J. Med. Chem.*, 1991, **34**, 717–725.

23 J. P. Sanchez, J. M. Domagala, C. L. Heifetz, S. R. Priebe, J. A. Sesnie and A. K. Trehan, *J. Med. Chem.*, 1992, **35**, 1764–1773.

24 D. Seebach, P. E. Ciceri, M. Overhand, B. Jaun and D. Rigo, *Helv. Chim. Acta*, 1996, **79**, 2043–2065.

25 C. Harrison, S. McNulty, D. Smart, D. J. Rowbotham, D. K. Grandy, L. A. Devi and D. G. Lambert, *Br. J. Pharmacol.*, 1999, **128**, 472–478.

26 Means ± standard error (SE) of three experiments performed in triplicate. DAMGO and the tetrapeptides bound to a single class of receptors (Hill slopes were not significantly different from unity). P. Taylor and P. A. Insel, in *Principles of drug action. The basis of pharmacology*, eds. W. B. Pratt and P. Taylor, Churchill Livingstone, New York, 1990.

27 A. Peter, G. Toth, C. Tomboly, G. Laus and D. Tourwe, *J. Chromatogr., A*, 1999, **846**, 39–48.

28 The unusual hydrolysis of endomorphin-1 at the level of the Pro–Trp bond by aminopeptidase-M has been also reported by P. A. Toth *et al.*, ref. 27. We are inclined to agree with H.-U. Demuth, who attributed this behaviour to the presence of DPP IV impurities.

29 O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 1951, **193**, 265–275.