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Conformational analysis and μ-opioid receptor affinity of short peptides, endomorphin models in a low polarity solvent

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Received 30th May 2003, Accepted 10th July 2003
First published as an Advance Article on the web 28th July 2003

Peptide carbamates containing the sequence H-Pro-Trp-PheNH_2 showed in $CDCl_3$ restricted conformations stabilized by the presence of a γ-turn. To test the reliability of the peptides as endomorphin conformational models, we measured the affinities for μ-receptors labelled with [^{3}H]-DAMGO. In particular, Cbz-Pro-Trp-PheNH_2 displayed a nanomolar affinity.

Introduction

Endomorphin-1 (H-Tyr-Pro-Trp-PheNH_2) and endomorphin-2 (H-Tyr-Pro-Phe-PheNH_2) are endogenous opioid peptides with high affinity for the μ-receptor.[1] Their discovery[2] in the mammalian brain encouraged the application of natural and synthetic peptides as analgesics instead of morphine.[2,3]

They both show in the second position the presence of proline, which is known to play a key role in the formation of secondary structures in peptides. Indeed, the *cis–trans* conformational equilibrium of the peptide bond preceding the prolyl residue in solution is an important process in protein folding.[4] This equilibrium strongly depends on the amino acid sequence and the solvent.

The study of structure–activity relationships of μ-receptor agonists and antagonists is the subject of increasing interest, as it could clarify the structural and conformational requirements for ligand–receptor interaction.[5] Several papers reported investigations on the bioactive conformation of endomorphins and endomorphin derivatives. On the basis of spectroscopic analysis in polar solvents such as DMSO or water, or in membrane-mimetic environments, such as SDS micelles or AOT reverse micelles, predominant reverse conformations have been proposed,[6] not revealing the presence of intramolecular hydrogen-bonds.

As a part of our ongoing interest in this field,[7] we have investigated the possibility that tripeptide carbamates, designed as endomorphin analogues, could assume a compact bioactive conformation stabilized by the presence of a hydrogen bond. Herein we report the synthesis of peptide **1** (Cbz-Pro-Trp-PheNH_2), where the tyrosine present in endomorphin-1 was changed with a benzyl carbamate group. This functionality was introduced with the aim to mimic the aromatic group of tyrosine and to increase the peptide solubility in $CDCl_3$. We supposed that this non-competitive solvent could be considered a suitable environment to simulate the hydrophobic core of a receptor.[8] It is expected that folded structures stabilized by hydrogen bonds may be favored in this apolar solvent.[9] Our purpose was to verify if a compact structure, stabilized by a hydrogen bond, could interact with the opioid receptor, although lacking a cationic amine and a phenolic function,[10] which are commonly considered necessary to manifest biological activity through interaction with this kind of receptor.

In order to evaluate the role of the Cbz group, compound **2** (Boc-Pro-Trp-PheNH_2), with *tert*-butyl carbamate replacing benzyl carbamate, was synthesized. We also studied the effect of the introduction of a glycinol residue at the C-terminus in compound **3** (Boc-Pro-Trp-Phe-Glyol), the same residue present in DAMGO (Tyr-D-Ala-Gly-*N*-methyl-Phe-Gly-ol),[11] a prototypic μ-opioid agonist. To test the reliability of the peptide analogues as endomorphin-1 models, we evaluated the affinity towards μ-opioid receptors.

Finally, the influence on both conformation and affinity of the carbamate substituent was investigated by comparison of **1**–**3** with the N^{α}-benzyl derivative **4** ($PhCH_2$-Pro-Trp-PheNH_2), which maintains an aromatic hydrophobic group in the N-terminal region, but does not present a carbonyl moiety to be involved in a hydrogen bond.

Results and discussion

Peptides **1**, **2**, **3**, and endomorphin-1, have been synthesized in solution following the conventional Boc chemistry, using EDCI/HOBt as condensing agents.[12] Boc deprotection with trifluoroacetic acid (TFA) in CH_2Cl_2 gave TFA peptide salts, which were used without purification. After each coupling, peptides were purified by flash-chromatography over silica-gel.

The ^{1}H NMR spectrum of **1**, **2**, and **3** in $CDCl_3$ solution (0.01 M)[13] resulted as a sharp set of unique resonances.

Strong evidence for the intramolecular hydrogen-bond stabilization in chloroform was provided by variable temperature (VT) NMR, and IR experiments.

The unambiguous assignment of the resonance sets of Phe and Trp in each compound was performed by HMBC-NMR analysis (heteronuclear multiple bond correlation). The correlation observed between Hα and Hβ with the quaternary indole or with the quaternary phenyl carbons, allowed attribution of the side chain ^{1}H-NMR signals to Trp or Phe, respectively.

The preferential conformation assumed by **1** in $CDCl_3$ was analyzed by IR, CD and ^{1}H NMR spectroscopy.

The IR absorption spectrum of **1** in $CDCl_3$ (0.01 M) showed comparable intense bands at 3479 cm^{-1}, at 3384 cm^{-1} and at 3325 cm^{-1} in the NH stretching region. The first one is clearly associated with a non hydrogen-bonded group, while the other two bands can be ascribed to NH amide protons hydrogen-bonded in a folded conformation (Fig. 1).[9d,14] The above conclusion is supported by the spectrum in the C=O stretching region, which showed a smooth, broad band at 1692–1672 cm^{-1}, consisting of a non hydrogen-bonded amide and a

DOI: 10.1039/b306161m

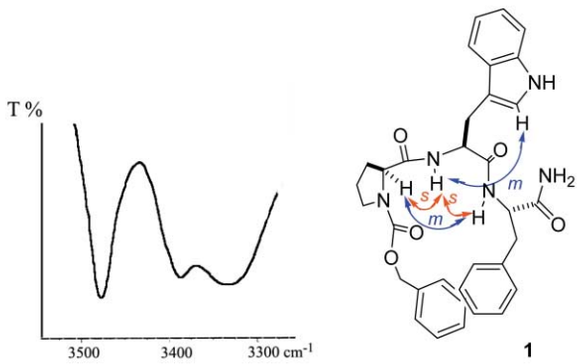

Fig. 1 NH region of the infrared absorption spectrum of peptide **1**, and conformation in $CDCl_3$.

urethane carbonyl. The noticeable lowering of the frequency of the C=O stretching mode of the urethane group is an unequivocal point in favor of the involvement of this carbonyl in the intramolecular bond.[14b]

The CD spectrum of **1** measured in CH_3OH could be indicative of a reverse turn, for the presence of the typical negative band at 220 nm;[15] however, no definitive conclusions could be deduced for the conformation of **1** in $CDCl_3$, for the different behaviour of short and flexible peptides in the different solvents.

The down field ^1H NMR chemical shifts of αNH-Trp (6.63 ppm) and NH-Phe (6.98 ppm) suggest their involvement in intramolecular hydrogen bonds.[16] Furthermore, the ^{13}C chemical shifts of C(β) and C(γ) of the Pro residue are very sensitive to the conformation of the preceding peptide bond.[17] A difference of 4–6 ppm is indicative of a *trans* conformation while a difference of 8-10 ppm is expected for a *cis* conformation. Peptide **1** showed a difference of 5.2 ppm between C(β) and C(γ) of Pro, confirming a *trans* conformation of the Pro-amide bond.

The VT (variable temperature) NMR analysis was in agreement with the formation of hydrogen bonds.[14a,18] In a 0.01 M solution in $CDCl_3$, over the range of 296–326 K, both NH-Phe and αNH-Trp resonances were scarcely sensitive to increasing temperature, typical behaviour of protons involved in hydrogen bonds (Fig. 2, for NH-Phe $\Delta\delta/\Delta T = 0.45$ ppb K^{-1}, for NH-Trp $\Delta\delta/\Delta T = -0.55$ ppb K^{-1}). In contrast, the resonances of the two protons of the primary amide group were strongly sensitive (Fig. 2, $\Delta\delta/\Delta T$ around -5 ppb K^{-1}), indicating that they do not participate in hydrogen bonds.

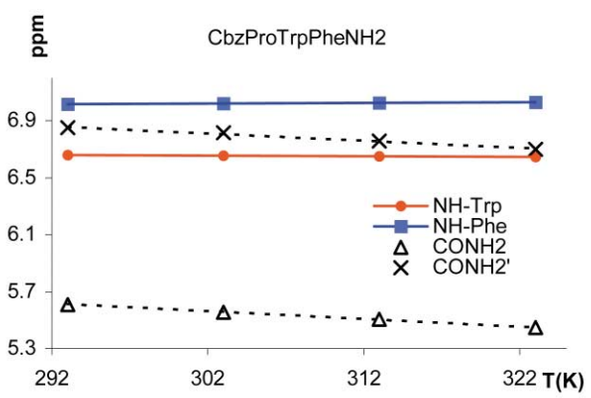

Fig. 2 VT NMR data for **1** in $CDCl_3$ solution.

Further clues as to the participation of αNH-Trp and NH-Phe in hydrogen bonds were obtained with the examination of the dependence of chemical shift of the ^1H NMR resonances in $CDCl_3$ upon the addition of a small amount of DMSO-d_6 as competitive solvent. The NH-Phe and αNH-Trp signals showed chemical shifts which were scarcely modified by the addition of DMSO-d_6 (0 to 2%, for NH-Phe $\Delta\delta = -0.003$ ppm, for NH-Trp $\Delta\delta = -0.014$ ppm; 2 to 5%, for NH-Phe $\Delta\delta = 0.006$ ppm, for NH-Trp $\Delta\delta = 0.039$ ppm).[9d,19]

Finally, DPFG-NOE (Double Pulse Field Gradient Nuclear Overhauser Effect) experiments showed strong signals due to the proximity of αNH-Trp and NH-Phe, αNH-Trp and Hα-Pro and a signal of moderate intensity between Hα-Pro and NH-Phe. Furthermore, a signal of moderate intensity was recorded between αNH-Trp and the hydrogen on C2 of the indole heterocycle.

These data provided evidence that αNH-Trp and NH-Phe in $CDCl_3$ are involved in a seven-membered ring γ-turn and a ten-membered ring β-turn, respectively, in equilibrium.[9d] A comprehensive evaluation of the spectroscopic data reported above indicates for peptide **1** a folded conformation, as reported in Fig. 1.

In an almost similar way, compound **2** (0.01 M solution in $CDCl_3$) showed in the IR absorption spectrum an intense band at 3477 cm^{-1}, and a comparatively less intense absorbance from 3390 to 3323 cm^{-1}, in the hydrogen-bonded NH region (Fig. 3).

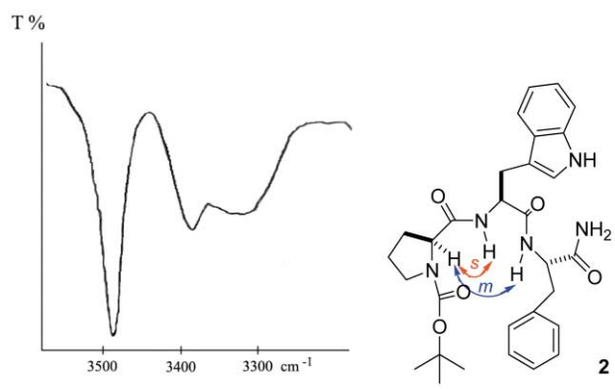

Fig. 3 NH region of the infrared absorption spectrum of peptide **2**, and conformation in $CDCl_3$.

The ^1H NMR spectrum of **2** in $CDCl_3$ displayed the same trend as **1**, and exhibited a set of unique signals. The resonance of αNH-Trp was scarcely sensitive to increasing temperature, showing a strong hydrogen bond involvement (Fig. 4, $\Delta\delta/\Delta T = -0.5$ ppb K^{-1}), while NH-Phe was more sensitive, indicating a not completely bonded proton (Fig. 4, $\Delta\delta/\Delta T = -2.4$ ppb K^{-1}). Also in this case NOE experiments showed, besides the obvious signals, a strong interaction of αNH-Trp and NH-Phe with Hα-Pro (Fig. 3).

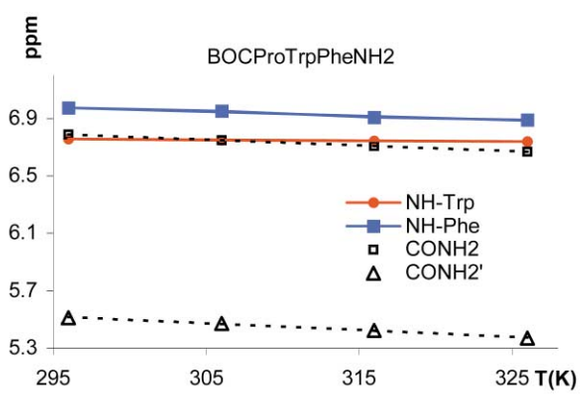

Fig. 4 VT NMR data for **2** in $CDCl_3$ solution.

The last compound **3** showed a similar behaviour in solution as **2**. The IR absorption spectrum of **3** in $CDCl_3$ (0.01 M) confirmed the formation of a compact structure, exhibiting a narrow absorbance at 3475 cm^{-1}, a more intense broad band at 3385 cm^{-1} and a weak third broad signal around 3350 cm^{-1} (Fig. 5). Under identical conditions the same peaks were present

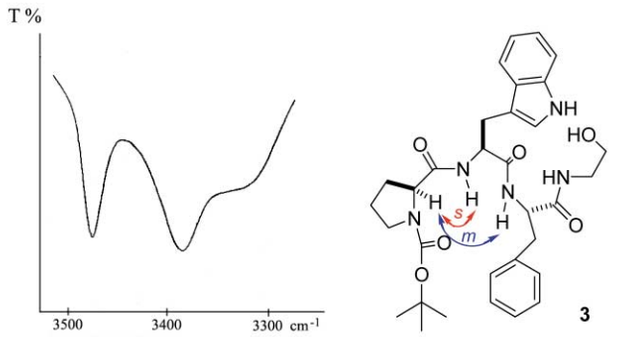

Fig. 5 NH region of the infrared absorption spectrum of peptide **3**, and conformation in $CDCl_3$.

in the Glyol *O*-acetylated derivative of **3**, confirming that none of the above peaks could be attributed to the OH stretch.

The VT (variable temperature) NMR analysis was in agreement with the formation of a hydrogen bond. The chemical shifts of αNH-Trp showed a small temperature dependence (Fig. 6, $\Delta\delta/\Delta T$ = 0.64 ppb K^{-1}), while NH-Phe (Fig. 4, $\Delta\delta/\Delta T = -1.9$ ppb K^{-1}), and NH-Glyol (Fig. 6, $\Delta\delta/\Delta T = -1.8$ ppb K^{-1}), were more sensitive to increasing temperature. The latter amide protons participate in hydrogen bonds more weakly than those involving NH-Trp.[20] In addition, the narrow IR absorbance at 3475 cm^{-1} could be attributed to the non hydrogen-bonded amide NH-Glyol group.

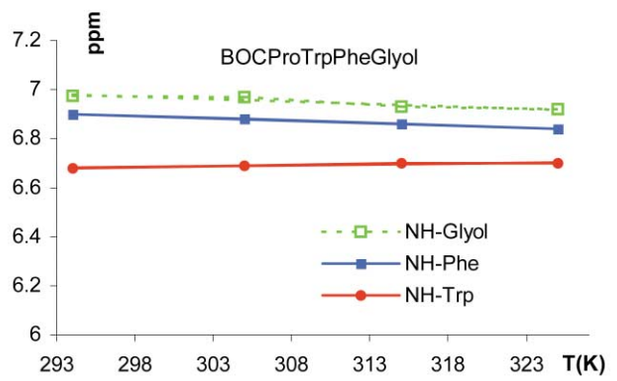

Fig. 6 Chemical shift data for peptide **3** as a function of temperature in $CDCl_3$.

The NH-Phe and NH-Trp signals showed chemical shifts which were scarcely modified by the addition of a small amount of a competitive solvent (DMSO, 0 to 2%, for NH-Phe $\Delta\delta = -0.056$ ppm, for NH-Trp $\Delta\delta = -0.036$ ppm; 2 to 5%, for NH-Phe $\Delta\delta = 0.031$ ppm, for NH-Trp $\Delta\delta = 0.075$ ppm), while the NH-Glyol signal was more sensitive (DMSO, 0 to 2%, $\Delta\delta = -0.095$ ppm; 2 to 5%, $\Delta\delta = 0.080$ ppm).

Further useful information was deduced from NOE experiments, that showed a strong interaction between αNH-Trp and Hα-Pro and a medium interaction between NH-Phe and Hα-Pro (Fig. 5).

All these data collectively indicate for compounds **2** and **3** a strong γ-turn between αNH-Trp and carbamate C=O, and a weaker β-turn between NH-Phe and carbamate C=O, in equilibrium. Therefore, it can be deduced that the conformation of **2** and **3** shows a stronger preference for the γ-turn with respect to the β-turn.

Apparently, trimers **2** and **3**, containing the bulky *t*-butyl carbamate, showed a less compact structure than benzyl carbamate **1**.

Finally, peptide **4** ($PhCH_2$-Pro-Trp-PheNH_2) was designed to evaluate the importance of the effect of carbamate carbonyl on conformations. It was prepared by coupling N^{α}-benzyl-proline[21] and the TFA salt of the dipeptide H-Trp-PheNH_2 under the usual conditions. The unambiguous assignment of the resonance sets was performed by HMBC-NMR analysis.

Table 1 Affinities and Hill slopes of ligands **1**–**4**, DAMGO, and endomorphin-1 for [^{3}H]-DAMGO binding sites in rat brain membranes

Compounds	K_i/nM	IC_{50}/nM	n_H
DAMGO	1.64 (±0.33)	9.89 (±0.67)	0.9 (±0.1)
Endomorphin-1	0.14 (±0.05)	4.80 (±0.21)	0.8 (±0.1)
1	3.44 (±0.11)	103 (±10)	0.6 (±0.2)
2	250 (±13)	500 (±50)	0.7 (±0.2)
3	> 10^3	nd	nd
4	200 (±15)	13800 (±200)	1.05 (±0.1)

nd = not determined. Means ± S.E. of three experiments performed in triplicate.

As could be expected, on the basis of VT NMR, no hydrogen bond was deduced for **4**. A series of NOE experiments indicated a conformation which strongly differed from that of peptides **1**–**3**. Besides the obvious signals, a strong NOE between a proline N^{α}-benzylic proton and a Trp methylene proton allowed placement of the indolyl group in proximity to the N^{α}-benzylic substituent of proline.

Since the information obtained by NMR analysis was not sufficient to determine the preferred conformation assumed by **4**, we calculated its more stable geometry (Fig. 7) by means of molecular mechanics computations.[22] The minimum-energy conformation compatible with NOEs was calculated by means of AMBER minimization of a conformation set generated by a Monte Carlo procedure, introducing the estimated distances furnished by NOEs as restraints.

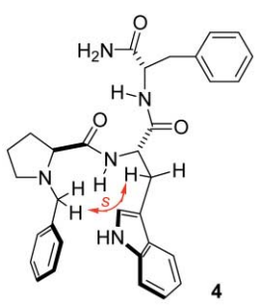

Fig. 7 Conformation of **4** in $CDCl_3$ solution.

To evaluate if the carbamate-peptides might be suitable endomorphin-1 models for conformational analysis, we tested the biological relevance of **1**, **2**, **3** and **4** and we compared their affinities for μ-opioid receptors with that of endomorphin-1 and DAMGO.

The affinities for μ-receptors labelled with [^{3}H]-DAMGO were measured through binding assays performed on rat brain membranes (Table 1).[7c,23] The K_i values measured for DAMGO and for endomorphin-1 were in agreement with the literature.[1,10,11]

The results in Table 1 showed that, while the Cbz-tripeptide **1** had a K_i value in the nanomolar scale, peptides **2** and **3** maintained only a poor affinity for μ-receptors, even though the three peptides displayed very similar conformations. These results suggest that the substitution of Tyr for Cbz group gave a peptide which still retained a certain ability to bind receptors. The low affinity measured for **2** and **3** could be attributed to the presence of the bulky *tert*-butyl group in place of the aromatic substituent which is present in endomorphin-1 and in **1**.

The affinity of **4** for μ-opioid receptors, measured under the same conditions as reported for **1**–**3**, is rather poor (Table 1). Since **4** carries an aromatic substituent at the proline, the low affinity measured could be attributed to the different conformation adopted with respect to **1**–**3**, which can be ascribed for the most part to the absence of the intramolecular hydrogen-bonds.

Conclusion

In conclusion, we have prepared the carbamate-tripeptides **1**, **2**, **3** and N^{α}-benzyl tripeptide **4**, and we have examined their conformational behaviour in $CDCl_3$. By way of the analysis reported above, we observed for **1**, **2**, and **3**, a similar preferred conformation, showing the Cbz or Boc group close to the Phe aromatic substituent. Peptide **4** showed a completely different conformation, mainly because of the absence of the intramolecular hydrogen bond. In compounds **1**, **2** and **3**, a *trans* Pro-amide bond configuration was stabilized by the formation of a γ-turn involving αTrp-NH and Boc or Cbz carbonyl. For the Cbz peptide **1**, a β-turn involving Phe-NH and the same carbonyl was also possible.

Interestingly, peptide **1** showed a good affinity for the μ-receptors, giving the opportunity to probe the biologically active conformation of peptides that potentially adopt a reverse turn conformation.

Experimental section

General remarks

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 Mercury spectrometer (Oxford magnet) at 400 (^{1}H NMR) and at 75 MHz (^{13}C NMR). Chemical shifts are reported as δ values relative to the solvent peak of $CDCl_3$ set at $\delta = 7.27$ (^{1}H NMR) or $\delta = 77.0$ (^{13}C NMR). Infrared absorptions were recorded with an FT-IR Nicolet 210 spectrophotometer. Optical activity measurements were performed with a Perkin-Elmer 343 polarimeter. DPFG-NOE spectra were recorded in $CDCl_3$ at r.t. HMBC spectra were recorded in $CDCl_3$ at r.t., selecting a spin coupling constant of 8 Hz. 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. Desolvation temperature was set at 200 °C and capillary voltage at 3.0 kV. Analytical HPLC was performed on an HP Series 1100, with an HP Hypersil ODS column (4.6-μm particle size, 100 Å pore diameter, 250 mm), DAD 215.8 nm. Homogenates were centrifuged in Beckman J6B and Beckman J2-21 centrifuges. Radioactivity was measured by liquid scintillation spectrometry using a Beckman apparatus. N^{α}-Benzylproline was prepared according to the literature (see below).

N^{α}-Benzylproline[21]

A suspension of proline (0.87 g, 7.56 mmol) and KOH (1.27 g, 27.0 mmol) in isopropanol (15 mL) was heated at 40 °C while stirring. After 10 min, BnCl (1.0 mL, 8.7 mmol) was added, and the mixture was stirred at 40 °C for 8 h. The mixture was filtered, and the solid was washed twice with $CHCl_3$, twice with acetone, and the filtrate was concentrated at reduced pressure. The resulting solid was re-crystallized from MeOH and ether, giving benzylproline (1.40 g, 90%) as a white solid. Spectroscopic analysis was in agreement with the literature.[21]

Synthesis and characterization of the peptides

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

N-tert-Butyloxycarbonyl group deprotection was performed by treatment with 30% TFA in CH_2Cl_2 at r.t. After 45 min the solvent was evaporated at reduced pressure and the resulting TFA peptide salt, obtained in quantitative yield, was used without purification for the next coupling.

Cbz-Pro-Trp-PheNH$_2$ (1). IR ($CDCl_3$) *v* 3695, 3595, 3479, 3384, 3325, 3155, 1818, 1792, 1680 br., 1460, 1387, 1301, 1255, 1215, 1162, 1096 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.60–1.80 (m, 2H), 1.85–2.20 (m, 2H), 2.86 (dd, J = 11.1, 14.7 Hz, 1H), 2.85–3.07 (m, 1H), 3.13 (dd, J = 6.6, 14.4 Hz, 1H), 3.18–3.27 (m, 2H), 3.44 (dd, J = 4.0, 15.0 Hz, 1H), 4.03 (dd, J = 4.8, 9.3 Hz, 1H), 4.47–4.55 (m, 1H), 4.56 (d, J = 12.9 Hz, 1H), 4.88–4.98 (m, 1H), 5.01 (d, J = 12.9 Hz, 1H), 5.45 (s, 1H), 6.30 (s, 1H), 6.63 (d, J = 6.3 Hz), 6.87 (s, 1H), 6.98 (d, J = 8.1 Hz, 1H), 7.09–7.48 (m, 14H), 7.69 (s, 1H); ^{13}C-NMR ($CDCl_3$) δ 24.8, 29.6, 36.7, 46.8, 53.7, 55.7, 67.3, 111.5, 116.4, 116.7, 116.8, 117.1, 120.0, 122.4, 126.4, 127.1, 128.3, 128.6, 129.1, 135.7, 138.3, 158.1, 165.2, 171.3, 173.9; FAB-MS [M+1]: 580.4; calcd. for **1**: 579.2. $[a]_D^{20} = -86.9$ (c 0.4, $CHCl_3$).

Boc-Pro-Trp-PheNH$_2$ (2). IR ($CDCl_3$) *v* 3477, 3390, 3323, 3158, 1816, 1793, 1671, 1470, 1382, 1095 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.40 (s, 9H), 1.60–1.80 (m, 2H), 1.87–2.20 (m, 2H), 2.78 (dd, J = 11.1, 14.7 Hz, 1H), 2.95–3.05 (m, 1H), (dd, J = 6.6, 14.4 Hz, 1H), 3.21–3.35 (m, 2H), 3.41 (dd, J = 4.0, 14.7 Hz, 1H), 3.98 (dd, J = 5.0, 9.3 Hz, 1H), 4.54 (q, J = 5.1 Hz, 1H), 5.00 (m, 1H), 5.44 (s, 1H), 6.46 (s, 1H,), 6.72 (d, J = 6.6 Hz, 1H), 6.82 (s, 1H), 6.97 (d, J = 8.0 Hz, 1H), 7.10–7.50 (m, 9H), 8.11 (s, 1H); ^{13}C-NMR ($CDCl_3$) δ 24.5, 27.5, 28.1, 29.7, 36.6, 47.1, 55.8, 61.0, 80.7, 109.5, 111.7, 117.7, 119.9, 122.3, 123.7, 126.8, 128.2, 128.8, 135.9, 137.8, 156.2, 173.7, 173.8. FAB-MS [M+1]: 546.6; calcd. for **2**: 545.3. $[a]_D^{20} = -86.4$ (c 0.3, $CHCl_3$).

Boc-Pro-Trp-Phe-Glyol (3). IR ($CDCl_3$) *v* 3689, 3602, 3475, 3385, 3350 br., 1818, 1800, 1680, 1534, 1467 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.33 (s, 9H), 1.58–1.94 (m, 4H), 2.15–2.23 (m, 1H), 2.70 (dd, J = 11.4, 14.0 Hz, 1H), 3.08–3.18 (m, 2H), 3.18–3.34 (m, 2H), 3.59 (dd, J = 3.2, 14.0 Hz, 1H), 3.62–3.73 (m, 4H), 3.99 (dd, J = 4.8, 8.8 Hz, 1H), 4.56 (q, J = 4.6 Hz, 1H), 5.18 (m, 1H), 6.22 (s, 1H), 6.60 (d, J = 6.8 Hz, 1H), 6.85 (d, J = 8.8 Hz, 1H), 7.00 (t, J = 4.4 Hz, 1H), 7.11–7.56 (m, 5H), 7.81 (s, 1H); ^{13}C-NMR ($CDCl_3$) δ 24.5, 25.2, 28.1, 29.9, 36.9, 43.2, 47.1, 52.7, 56.2, 61.2, 61.5, 81.1, 108,1, 111.8, 116.8, 120.1, 122.3, 123.8, 126.4, 127.5, 128.2, 128.8, 135.8, 138.0, 155.7, 171.1, 171.3, 174.5; FAB-MS [M+1]: 592.2; calcd. for **3**: 591.3. $[a]_D^{20} = -111$ (c 0.4, $CHCl_3$).

PhCH$_2$-Pro-Trp-PheNH$_2$ (4). ^{1}H-NMR ($CDCl_3$) δ 1.49–1.62 (m, 2H), 1.89–2.12 (m, 2H), 2.45–2.55 (m, 1H), 2.64 (t, J = 8.0 Hz, 1H), 2.77 (dd, J = 7.2, 10.0 Hz, 1H), 2.85 (dd, J = 6.4, 10.0 Hz, 1H), 2.98 (dd, J = 4.2, 10.2 Hz, 1H), 3.00–3.12 (m, 2H), 3.14 (d, J = 12.9 Hz, 1H), 3.51 (d, J = 12.9 Hz, 1H), 4.50 (q, J = 6.9 Hz, 1H), 4.56 (q, J = 6.3 Hz, 1H), 5.64 (s, 1H), 6.19 (s, 1H), 6.63 (d, J = 7.8 Hz, 1H), 6.86–7.18 (m, 11H), 7.29 (d, J = 7.8 Hz, 2H), 7.53 (d, J = 7.8 Hz, 2H), 7.94 (d, J = 7.5 Hz, 1H), 9.42 (s, 1H); ^{13}C-NMR ($CDCl_3$) δ 24.7, 28.8, 30.7, 39.0, 54.2, 54.7, 55.5, 60.5, 68.1, 110.2, 112.4, 119.9, 122.5, 124.8, 126.0, 127.6, 128.1, 128.9, 129.3, 130.2, 137.9, 138.2, 139.5, 173.1, 175.4, 176.8. FAB-MS [M+1]: 536.4; calcd. for **4**: 535.3. $[a]_D^{20} = -63$ (c 0.4, $CHCl_3$).

Acknowledgements

We thank ISOF-CNR, MURST (Cofin 2002 and 60%), and the University of Bologna (funds for selected topics ″Sintesi e Caratterizzazione di Biomolecole per la Salute Umana″) for financial support.

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23 [^{3}H]-DAMGO as μ-specific radioligand, 1 nM, specific activity 64 Ci mmol^{-1}, K_d = 0.485 nM, B_{max} = 48 fmol mg^{-1} protein; incubation buffer: 50 mM TRIS, 0.1% BSA pH 7.4 at 4 °C.