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β-Casomorphins: Substitution of Phenylalanine with β-*homo* Phenylalanine Increases the μ-Type Opioid Receptor Affinity

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Abstract—Two analogues of bovine β-casomorphin-7 and β-casomorphin-5 containing a β-*homo* phenylalanine in substitution of the phenylalanine in position 3 were synthesised and tested for their μ-opioid receptor affinity. The modification enhanced the μ receptor affinity 5-fold in the case of modified β-CM-7 and 2-fold for modified β-CM-5 when compared to the natural peptides.

In addition to its well-known nutritive role, milk also releases chemical signals to the recipient organism. In this context, a number of peptides derived from milk may act as opioid receptor ligands. While casoxins and lactoferroxins display opioid antagonistic properties, other peptides such as β-casomorphin-7, H-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-OH (β-CM-7) and β-casomorphin-5 H-Tyr-Pro-Phe-Pro-Gly-OH, (β-CM-5) behave like opioid receptor agonists.[1] Most of the information available to date concerns β-CMs. These peptides can be released by enzymatic hydrolysis of β-casein in the gut of both adults and newborns, and may elicit opioid effects by acting as hormone-like substances. Recently, it has also been reported that β-CM-5 stimulates neurite outgrowth in a mouse neuroblastoma cell line, suggesting that β-CM-5 may play a role as a neurite elongation factor during the suckling period.[2] Several synthetic β-CM derivatives have been shown to be highly specific and potent μ-type opioid receptor ligands, and have been frequently used as pharmacological tools in opioid research. In particular, the effect on μ- and δ-opioid-like properties of the aromatic amino acid substitution[3] in the position-3 of some cyclic β-CM-5 analogues and the modification in the Tyr[1]-Phe[3] domain has been found to play a crucial role for biological activity.[4,5]

In recent years, β-*homo* amino acids (previously reported as *homo*-β[6]) have raised considerable interest as potentially active biological compounds. These novel amino acids have been the object of several studies[7] in which the focus was mainly on the structure and pharmacological properties compared to the natural α-amino acids counterparts. β-Peptides, made entirely with β-amino acids, have been so far synthesised[8] in order to understand what properties can be acquired following β-amino acid insertion.

The availability of several β-homologues of protein amino acids by a new, fast and efficient procedure,[6] developed to prepare β-*homo* amino acids in any protected form by direct homologation of their corresponding *N*-protected α-amino acid, allowed us to prepare β-*homo* analogues of bioactive β-CMs.

We have synthesised and tested, in a μ-type opioid receptor binding assay, two new analogues of β-CM-7 and β-CM-5, containing the natural amino acid phenylalanine substituted by the β-*homo* counterpart, H-Tyr-Pro-β*h*Phe-Pro-Gly-Pro-Ile-OH and H-Tyr-Pro-β*h*Phe-Pro-Gly-OH.

With this type of substitution, the sequence Tyr[1]-Phe,[3] commonly found in β-CMs as well as in other opioid peptides, such as dermophin and deltorphin, contain an extra methylene group in the direction of the carbonyl of the phenylalanine residue. The natural and modified peptides were tested and compared for their capability to displace the potent radioligand μ-opioid receptor agonist [^{3}H]-DAMGO[9] from rat brain membrane preparations.

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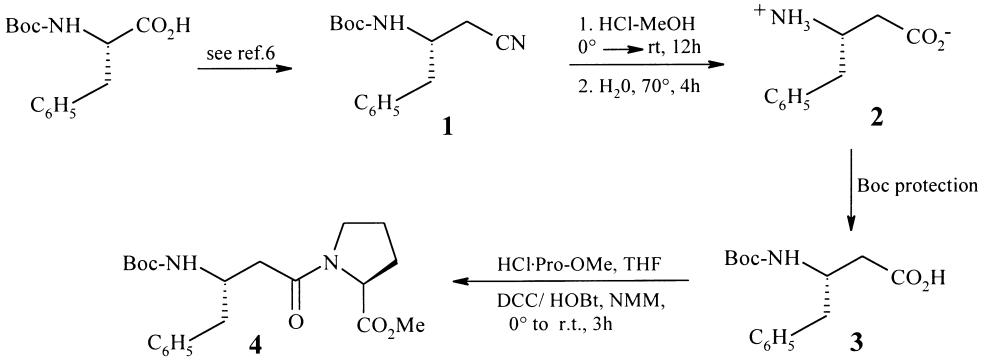
Synthesis

The modified peptides were prepared by solution methods using the *t*-Boc and methyl ester protection, dicyclohexylcarbodiimide and 1-hydroxybenzotriazole as coupling agents and *N*-methymorpholine as the base. As for the natural peptides, commercially available ones were used. The β-*homo*-(*S*)-phenylalanine was prepared with a new homologation procedure,[6] with a slight modification in the hydrolytic steps involved in the reported method. The final acid hydrolysis of the *N*-Boc-protected β-amino nitriles **1** into free β-*homo*-amino acid was actually performed as a three-step, one pot reaction in which, after the removal of the Boc protection, the nitrile was converted into the methyl ester. The latter was then hydrolyzed by the addition of water.[10]

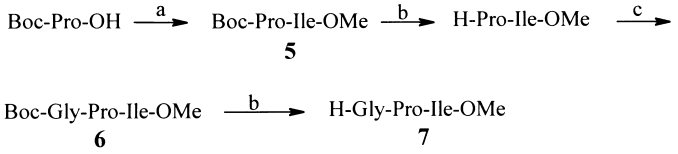
Subsequently, the free β-*homo*-phenylalanine **2** obtained in this way was reprotected with a Boc group to yield **3**. The latter compound was used in the coupling reaction with proline methyl ester in THF, which provided the Boc dipeptide methyl ester **4** (in 96% yield), as illustrated in Scheme 1.

The heptapeptide analogue was assembled via *C*-elongation of **4**, Boc-β*h*Phe-Pro-OMe, by coupling it with the *C*-protected tripeptide **7**, H-Gly-Pro-Ile-OMe, followed by *N*-elongation via the coupling of the resulting pentapeptide with the *N*-protected dipeptide **9**, Boc-Tyr(*t*Bu)-Pro-OH. The *C*-protected tripeptide **7**, H-Gly-Pro-Ile-OMe was synthesized step-wise beginning with the coupling of Boc-Pro-OH with H-Ile-OMe. After the usual work-up, the product **5**, Boc-Pro-Ile-OMe, was *N*-deprotected with 50% of TFA in CH_2Cl_2 for 30′, and then used in the reaction with Boc-Gly-OH. The subsequent *N*-deprotection of the new coupling product **6**, Boc-Gly-Pro-Ile-OMe, resulted in fragment **7** (Scheme 2).

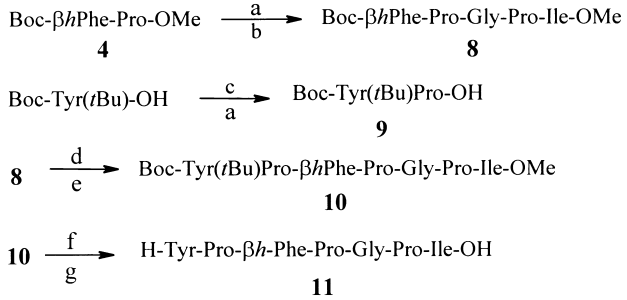
The *C*-deprotection of dipeptide **4** and its coupling with fragment **7** yielded the full protected pentapeptide **8** (Scheme 3). The pentapeptide **8** was *N*-deprotected and used in the coupling reaction with the *N*-protected dipeptide Boc-Tyr(*t*Bu)-Pro-OH **9**, based on the reaction of the commercially available *N*-Boc-Tyr(*t*Bu)-OH with proline methyl ester followed by ester hydrolysis. The coupling produced the fully protected heptapeptide **10**. To obtain free heptapeptides, ester hydrolysis at the isoleucine



Scheme 1. Synthesis of Boc-β*h*Phe-Pro-OMe.



Scheme 2. Synthesis of *C*-protected tripeptide **7**: (a) HCl·Ile-OMe, NMM, DCC/HOBt, THF, 0° to rt, 3 h; (b) 50% TFA in CH_2Cl_2, rt, 30′ (c) Boc-Gly-OH, NMM, DCC/HOBt, THF, 0° to rt, 3 h.



Scheme 3. Synthesis of the heptapeptide analogue: (a) 1 M LiOH (aq), MeOH, rt, 1 h; (b) **7**, NMM, DCC/HOBt, THF, 0° to rt, 3 h. (c): HCl·Pro-OMe, NMM, DCC/HOBt, THF, 0° to rt, 3 h; (d) 50% TFA in CH_2Cl_2 rt, 30′; (e) **9**, NMM, DCC/HOBt, THF, 0° to rt, 3 h; (f) 1 M LiOH (aq), MeOH, rt, 24 h; (g) 50% TFA in CH_2Cl_2, rt, 1 h.

residue of **10** was performed and a subsequent removing of the acid-labile protecting group, with 50% TFA in CH_2Cl_2 for 1 h produced the free heptapeptide **11**. The HPLC analysis established that the ester hydrolysis of **10** was completed in 24 h, and showed that hydrolysis occurred without racemization.

The pentapeptide analogue was assembled via *N*-elongation of **4** by its coupling with **9**, followed by the coupling with glycine methyl ester (Scheme 4). The modified pentapeptide, H-Tyr-Pro-β*h*Phe-Pro-Gly-OH, was synthesised in a similar way to the heptapeptide **11**, but the key dipeptide **4** was first deprotected at the Boc group and then coupled with the *N*-protected dipeptide **9**, thereby producing the full protected tetrapeptide **12**. The ester hydrolysis of **12** and a new coupling with H-Gly-OMe yielded the protected pentapeptide **13**. Ultimately, the removal of the protecting group yielded the free pentapeptide **14** (Scheme 4).

Scheme 5 reports the overall synthetic pathway of [β*h*Phe]^3-β-CM-7 and [β*h*Phe]^3-β-CM-5.

The purity and the chemical structure of the synthesised peptides were verified by RP-HPLC followed by electrospray mass spectrometry analyses. Results are summarised in Table 1. The peptides were more than 95% pure and there was a excellent agreement between the expected and the experimental mass values, thus confirming the putative peptide structures.

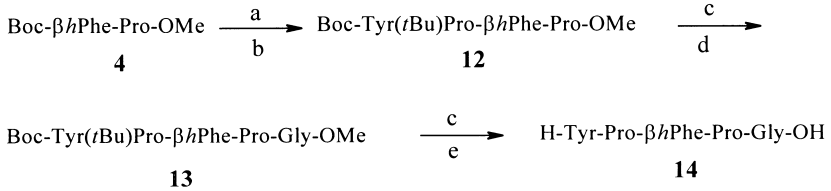
Table 1. Analytical data for natural and modified (β-CM-7) and (β-CM-5)

Peptide	Retention time[11] (min)	Molecular Weight[12]	
		Experimental	Theoretical
Boc-β*h*Phe-Pro-OMe	—	390.3	390.23
Tyr-Pro-Phe-Pro-Gly	17.73	579.3	579.25
Tyr-Pro-β*h*Phe-Pro-Gly	18.21	593.3	593.25
Tyr-Pro-Phe-Pro-Gly-Pro-Ile	21.10	789.5	789.38
Tyr-Pro-β*h*Phe-Pro-Gly-Pro-Ile	21.68	803.4	803.38

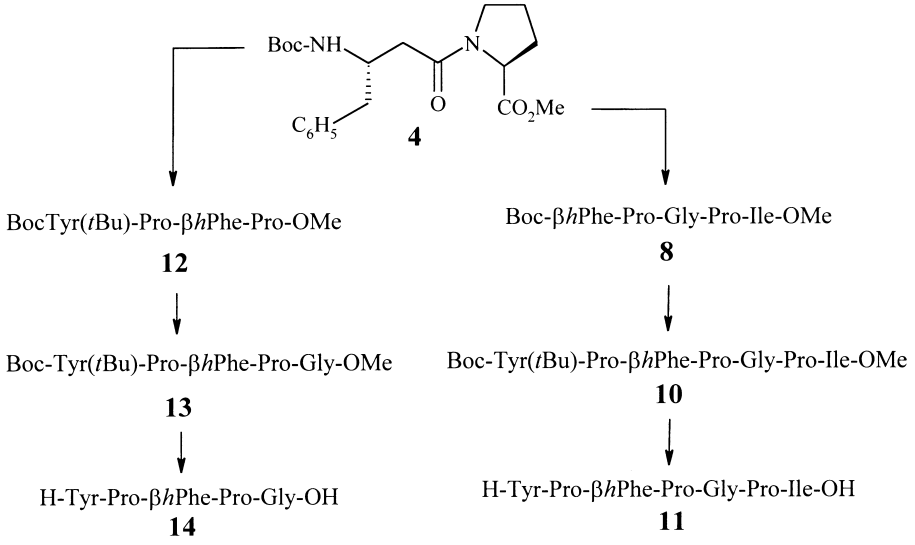
Receptor Binding Assay

The natural β-casomorphins are μ-selective opioid agonist with moderate potency and they exhibit only low δ- and almost no κ-receptor affinities.[13] The binding affinities of the natural and modified peptides for μ-receptors were determined by measuring the displacement of [^{3}H]-DAMGO from rat brain membrane binding sites.[14,15] Incubations were performed for 90 min at 25 °C with 1 nM labeled ligand in a final volume of 1 mL.

IC_{50} values were obtained from log dose-displacement curves, and K_i values were calculated from the IC_{50} values by means of Chang and Prusoff equation,[16] by using the value of 0.53±0.06 nM for the dissociation constant of [^{3}H]-DAMGO (B_{max} 120,7±3,67 fmol/mg). The results are shown in Table 2.



Scheme 4. Synthesis of the pentapeptide analogue: (a) 50% TFA in CH_2Cl_2, rt, 30′; (b) **9**, NMM, DCC/HOBt, THF, 0° to rt, 3 h; (c) 1 M LiOH (aq) in MeOH, 1 h; (d) HCl·Gly-OMe, NMM, DCC/HOBt, THF, 0° to rt, 3 h; (e) 50% TFA in CH_2Cl_2, rt, 1 h.



Scheme 5. [β*h*Phe]^3-β-Casomorphins synthesis.

Table 2. IC_{50} and K_i values for natural and modified β-casomorphins

Peptides	IC_{50} (μM)	K_i (μM)
β-CM-7	20.0±2.5	7.54±0.89
[β*h*Phe]^3-β-CM-7	4.0±1.5	1.44±0.54
β-CM-5	4.5±1.2	1.62±0.43
[β*h*Phe]^3-β-CM-5	2.0±0.8	0.72±0.20

The evaluation of μ-receptor binding activity of the modified β-CMs showed that the synthetic analogues not only retained binding activity but were even more potent: 5-fold in the case of modified β-CM-7 and 2-fold for modified β-CM-5 in comparison with the natural peptides.

These results suggest that also non cyclic analogues of bioactive peptides can be used in the search for new opioid ligands, although the two analogues presented in this paper are less potent at μ-opioid receptors than cyclic analogues of β-CM-5 (e.g., the Tyr-c[-D-Orn-2-Nal-D-Pro-Gly-]) which contain, however, more substantial modifications[3] with respect to the natural peptide. Further studies are in progress in our laboratory in order to evaluate if the affinities at δ-and κ-opioid receptor and the agonist activity of the modified peptides is different from the natural ones. However, the results presented here clearly indicate that a slight chemical modification, a-CH_2- group in the phenylalanine, induces an appreciable variation in the μ-type binding affinities in the studied β-CMs peptides. In the case of β-casomorphin, the β-homo amino acid containing analogues may represent a useful pharmacological tool, particularly in consideration of the enhanced stability to enzymatic hydrolysis of the β–*homo* residues.[17]

Acknowledgements

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References and Notes

1. Teschemacher, H.; Koch, G.; Brantl, V. *Biopolymers* **1997**, *43*, 99.
2. Sakaguchi, M.; Murayama, K.; Satoh, M.; Takeuchi, M.; Matsumura, E. *Neurosci. Lett*. **1998**, *251*, 97.
3. Schmidt, R.; Wilkes, B. C.; Chung, N.; Lemieux, C.; Schiller, P. W. *Int. J. Pept. Protein Res*. **1996**, *48*, 411.
4. Kostetsky, P. V.; Arkhipova, S. F. *Biochemistry (Mosc.)* **1999**, *64*, 1005.
5. Brandt, W.; Stoldt, M.; Schinke, H. J. *Computer Aided Mol. Des*. **1996**, *10*, 201.
6. Caputo, R.; Cassano, E.; Longobardo, L.; Palumbo, G. *Tetrahedron* **1995**, *51*, 12337.
7. Enantioselective Synthesis of β-Amino Acids; Juaristi E. Ed.; Wiley-VCH. 1997.
8. Abele, S.; Vögtli, K.; Seebach, D. *Helv. Chim. Acta* **1999**, *82*, 1539 and literature cited therein.
9. Kosterlitz, H. W.; Paterson, S. J. *Br. J. Pharmacol*. **1980**, *73*, 299.
10. Longobardo, L.; Cassano, E.; Cammarota, G.; Melk, D. Manuscript in preparation.
11. Analyses of the synthetic peptides were carried out by RP-HPLC on a Vydac C18 column (250×4.6 mm, 5 μm) using a Waters HPLC System (Datasystem Millenium, HPLC pumps Waters 510, Detector Waters 486). Eluents were: 0.1% trifluoroacetic acid (solvent A) and 0.07% trifluoroacetic acid in 95% acetonitrile (solvent B). The elution was performed by means of a linear gradient from 10 to 60% solvent B over 25 min at a flow rate of 1 mL/min. The elution was monitored at 220 nm.
12. HPLC fractions were submitted to electrospray mass spectrometry (ESMS) analysis using a platform single quadrupole mass spectrometer (Micromass). Samples were dissolved in 1% acetic acid in 50% acetonitrile and 2–10 μL were injected into the mass spectrometer at a flow rate of 10 μL/min. The quadrupole was scanned from *m*/*z* 300 to 1600 at 10 s/scan and the spectra were acquired and elaborated using the MassLynx software. All mass values are reported as monoisotopic masses.
13. Brantl, V.; Teschemacher, H.; Bläsig, J.; Henschen, A.; Lottspeich, F. *Life Sci*. **1981**, *28*, 1903.
14. Srucs, M.; Belkeva, M.; Simon, J.; Benyhe, S.; Toth, G.; Hepp, J.; Wolemamn, M.; Medzihardzhy, K. *Life Sci*. **1987**, *41*, 177.
15. Oktem, H. A.; Varga, E.; Hepp, J.; Medzihardzhy, K.; Lajtha, A.; Borsodi, A. *Life Sci*. **1991**, *48*, 1757.
16. Cheng, Y. C.; Prusoff, W. H. *Biochem. Pharmacol*. **1973**, *22*, 3099.
17. Seebach, D.; Overhand, M.; Kühnle, F. N. M.; Martinoni, B.; Oberer, L.; Hommel, U.; Widmer, H. *Helv. Chim. Acta* **1996**, *79*, 913.