Research Article

Journal of PeptideScience

Received: 26 April 2012 Revised: 12 June 2012 Accepted: 19 June 2012 Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI 10.1002/psc.2433

TAPP analogs containing β^3-*homo*-amino acids: synthesis and receptor binding

D. Podwysocka,[a†] P. Kosson,[b] A. W. Lipkowski[b] and A. Olma[a]*

β-Amino acids containing α,β-hybrid peptides show great potential as peptidomimetics. In this paper, we describe the synthesis and affinity to μ-opioid and δ-opioid receptors of α,β-hybrids, analogs of the tetrapeptide Tyr-D-Ala-Phe-Phe-NH_2 (TAPP). Each amino acid was replaced with an L- or D-β^3-*h*-amino acid. All α,β-hybrids of TAPP analogs were synthesized in solution and tested for affinity to μ-opioid and δ-opioid receptors. The analog Tyr-β^3*h*-D-Ala-Phe-PheNH_2 was found to be as active as the native tetrapeptide. Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: β^3-*homo*-amino acid; tetrapeptide TAPP analogs; μ-opioid agonist peptides; radioligand binding; α,β-hybrids of opioid peptides

Introduction

Over the past three decades, many new bioactive opioid peptides have been discovered. Opioid peptides are widely known for their analgesic and psychoactive properties [1–3]. These compounds can be used as effective medical drugs in anticancer therapy [4,5], in Alzheimer disease treatment [6] and as painkillers [7,8]. Their limitations are related to their high sensitivity to endogenous enzymes, short period of action and low selectivity. These peptides interact with different subtypes of opioid receptors, such as μ, δ and κ. Among the three main opioid receptors, μ-opioid receptors (MORs) are perhaps of the greatest clinical importance. They are involved in pain signal transmission and perception, and therefore, compounds showing selectivity to MORs can be used in clinical practice as analgesic drugs. The dissociation of analgesia from tolerance using MOR selective agonists is nearly impossible. Indeed, investigations using MOR knockout mice have demonstrated that both the antinociception and tolerance effects are MOR-mediated [9].

In search for more potent μ-selective ligands, the tetrapeptide Tyr-D-Ala-Phe-Phe-NH_2 (TAPP; Figure 1) with high affinity to the μ-opioid receptor has been synthesized [10].

In spite of a D-amino acid residue and amide in the *C*-terminus of the peptide, it is not resistant to proteolytic enzymes [11,12]. In 1997 Zadina's group [13] isolated two tetrapeptides from bovine frontal cortex: endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2). Endomorphins show high affinity and the highest selectivity to the μ-opioid receptor among the peptides found in the mammalian nervous system.

In recent years, much attention has been focused on various β-amino acids and β-peptides. Investigations include the synthesis of α,β-hybrids and β-peptides [14]. The substitution of α-amino acids for their β-isomers in biologically active peptides may result in increased enzymatic stability and also in a strong influence on peptide conformation.

Previously, we described the conformational and metabolic consequences of the substitution of Phe with β^3-*homo*-Phe in position 3 or 4 and *N*-Me-β^3-*h*-Phe or β^3-*h*-Tic in position 3 of TAPP [15]. A radioreceptor binding assay showed that in this series of analogs, only [β^3-*h*-Phe]^{4}TAPP displayed significant affinity to the μ-receptor. All analogs were almost fully resistant to α-chymotrypsin.

In the present paper, we examined the receptor binding in TAPP analogs containing L- and D-β^3-*homo*-amino acids (Figure 2) in each position (**I–IX**, Table 2).

We also synthesized two tripeptides containing β^3-*homo*-amino acid residues in position 1 or 1 and 2, without the D-Ala residue, to check the influence of the distance between aromatic rings on bioactivity (**X–XI**).

Material and Methods

Synthesis of β^3-*Homo*-Amino Acids

Optically pure Boc-β^3-*homo*-amino acids **3** were prepared using two-step Arndt–Eistert homologation described in the literature. This methodology is useful for multifunctional amino acids (e.g. Tyr). The synthesis of Boc-α-diazo ketone intermediates was easily performed by applying the mixed anhydride methods [16]. Wolff's rearrangement of Boc-α-diazo ketones **2** was initiated by silver benzoate in the presence of silica gel in ethyl acetate [17] (Scheme 1).

Enantiomerically pure *N*-Boc-protected β^3-*homo*-amino acids were obtained with high yield (69–95% in two steps; Table 1) without racemization.

* *Correspondence to: Aleksandra Olma, Faculty of Chemistry, Institute of Organic Chemistry, Lodz University of Technology, Żeromskiego 116, 90-924 Lodz, Poland. E-mail: aleksandra.olma@p.lodz.pl*

† *Current address at Polpharma SA, ul. Pelplińska 19, 83-200 Starogard Gdański, Poland.*

a *Institute of Organic Chemistry, Lodz University of Technology, Lodz, Poland*

b *Mossakowski Medical Research Centre, Polish Academy of Science, Warsaw, Poland*

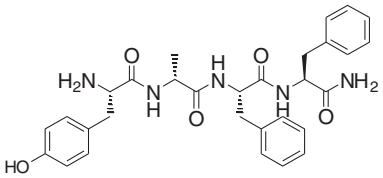


Figure 1. TAPP (Tyr-D-Ala-Phe-Phe-NH_2).

Peptide Synthesis

The α,β-hybrids of tetrapeptides (**I–XI**) were synthesized in solution, stepwise, starting from the *C*-terminal dipeptides, using TBTU (1.1 equivalent) as a coupling reagent in the presence of EDIA (2.2 equivalent). The deprotection of Boc-amino-protecting groups from dipeptides was accomplished with 2.5 N HCl/AcOEt while from tripeptides and tetrapeptides with 90% TFA/H_2O. Methyl esters of *N*-protected tetrapeptides were converted to amides by ammonolysis (liquid ammonia in methanol). The removal of the Bzl-protecting group was carried out by catalytic hydrogenation in acetic acid/methanol using 10% Pd/C as catalyst. The reactions were controlled using TLC or HPLC.

Peptide Purification and Characterization

Crude peptides were purified by preparative HPLC on a Vydac 218TP C_{18} column (25 × 2.2 cm; Grace Davison Discovery Sciences, Deerfield, IL, USA). The binary elution system was (A) 0.05% (v/v) trifluoroacetic acid in water and (B) 0.038% trifluoroacetic acid in acetonitrile/water 90 : 10. The linear gradient was 20–50% B over 25 min with a flow of 12 ml/min. UV detection was conducted at 220 nm. Each peptide was >97% pure as determined by analytical reverse-phase HPLC on a Vydac column (25 × 0.46 cm) using a

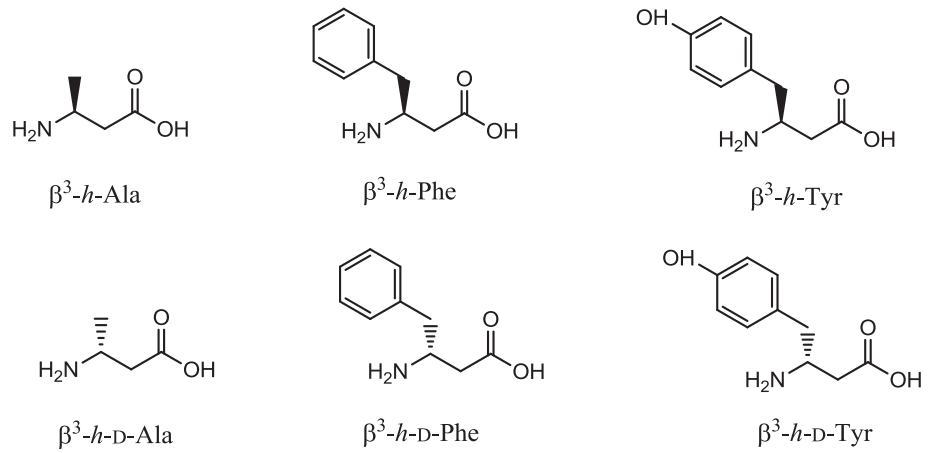
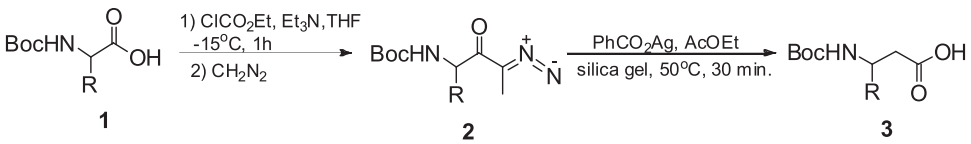


Figure 2. Structures of β^3-*homo*-amino acid.



1a and **1b**	R=Me	(Boc-L- and D-Ala)
1c and **1d**	R= CH_2-Ph	(Boc-L- and D-Phe)
1e and **1f**	R = CH_2-C_6H_4(*o*-OBoc)	(Boc-D-Tyr(*O*Boc))

Scheme 1. Homologation of Boc-amino acids.

Table 1. Protected β^3-*homo*-amino acids

No.	Amino acid	$[\alpha]_D$ ($CDCl_3$)	Melting point [°C]
3a	Boc-β^3-*homo*-Ala	−16.2 (*c* 1) (−14.0 (*c* 1) [18])	95–98 (White solid [18])
3b	Boc-β^3-*homo*-D-Ala	+16.5 (*c* 1) (+ 16.0 (*c* 1) [18])	White solid (white solid [18])
3c	Boc-β^3-*homo*-Phe	−17.8 (*c* 1) (−17.5;*c*1, CH_2Cl_2 [16])	White solid (96 [19])
3d	Boc-β^3-*homo*-D-Phe	+17.6 (*c* 1)	White solid
3e	Boc-β^3-*homo*-Tyr(OBoc)	−6.5 (*c*1)	117–119
3f	Boc-β^3-*homo*-D-Tyr(OBoc)	+6.6 (*c*1)	116–119

Journal of PeptideScience

linear gradient of 30–50% B for 25 min at a flow rate of 1 ml/min, with UV detection at 220 nm. The mass spectra were recorded with the following spectrometers: a Finningan MAT (Finningan MAT GmbH, Bremen, Germany) for FAB analysis and a Voyager-Elite (PerSeptive Biosystem Inc., Framingham, MA, USA) for MALDI analysis.

Radioreceptor Binding

New α/β-peptides, analogs of tetrapeptides TAPP, were tested for μ-opioid and δ-opioid receptor affinity. The receptor binding assay was performed using the radioligands [^{3}H]-naltrexone and [^{3}H]-deltorphin II. Radioreceptor binding assays were performed by the method described previously [20] and were conducted as follows.

Radioligand displacement assays

A rat brain membrane preparation containing 0.2–0.5 mg of protein/ml was incubated for 60 min at 25.5 °C in 50 mM Tris–HCl buffer (pH 7.4) containing 0.1 mg/ml BSA, 30 μg/ml bacitracin, 30 μM bestatin, 10 μM captopril and 0.1 mM PMSF in the presence of 0.5 nM [^{3}H]-naltrexone or [^{3}H]DELT II, as well as increasing concentrations of the analyzed compound (10^{-11}–10^{-6}) in a total volume of 1 ml. Non-specific binding was determined by the addition of 10 μM naltrexone. All experiments were performed in triplicate. Incubation was terminated by rapid filtration with 6 mL of ice-cold 0.9% NaCl through glass microfiber GF/B Whatman filters (Whatman, Piscataway, NJ, USA) soaked up in 0.5% PEI using an M-24 cell harvester (Brandel, Gaithersburg, MD, USA). Filter disks were then placed on 24-well clear PET 24-well flexible microplates (PerkinElmer, Waltham, MA, USA) and submerged in 1 ml of OptiPhase Supermix Cocktail (PerkinElmer). Radioactivity was counted with a MicroBeta LS Trilux (PerkinElmer) scintillation counter. Binding curves were fitted using nonlinear regression. Compound potency was expressed as log IC_{50} values.

Results and Discussion

The new analogs of the opioid tetrapeptide TAPP were obtained in solution. *N*-Protected β^3-*homo*-amino acids were synthesized using two-step Arndt–Eistert homologation. After deprotection and HPLC purification, the purity of α/β-peptides was higher than 97% (analytical HPLC). The molecular weight of obtained analogs was confirmed by FAB MS or MALDI MS. The physicochemical properties of the new compound are presented in Table 2.

Affinities of α,β-peptides, analogs of TAPP, for μ-receptors and δ-receptors were determined by the radioreceptor binding assay method described previously using the radioligands [^{3}H]-naltrexone and [^{3}H]-deltorphin II as μ-receptor-specific and δ-receptor-specific ligands, respectively.

Table 3 shows the binding affinity of α,β-peptide analogs to δ-opioid and μ-opioid receptors in comparison with Tyr-D-Ala-Phe-$PheNH_2$.

The binding affinity results for the synthetic analogs revealed the following properties: (i) α,β-Hybrids **I**, **II** and **IV–XI** exhibit the absence of affinity to δ-receptors. Only analog **III** has some,

Table 3. Receptor binding of α/β-peptides

	Peptide	IC_{50} [nM]	
		μ[a]	δ[b]
	Tyr-D-Ala-Phe-$PheNH_2$ (TAPP)	15.5 ± 6.33	616–797 ± 7.15
I	β^3-*h*-Tyr-D-Ala-Phe-$PheNH_2$	Not active	Not active
II	β^3-*h*-D-Tyr-D-Ala-Phe-$PheNH_2$	Not active	Not active
III	Tyr-β^3-*h*-D-Ala-Phe-$PheNH_2$	9.5 ± 2.9	794 ± 5.53
IV	Tyr-β^3-*h*-Ala-Phe-$PheNH_2$	524 ± 2.95	Not active
V	Tyr-D-Ala-β^3-*h*-Phe-$PheNH_2$[c]	>1000	Not active
VI	Tyr-D-Ala-β^3-*h*-D-Phe-$PheNH_2$	>1000	Not active
VII	Tyr-D-Ala-Phe-β^3-*h*-$PheNH_2$[c]	91.2 (±5.54)	Not active
VIII	Tyr-D-Ala-Phe-β^3-*h*-D-$PheNH_2$	95.4 ±3.72	Not active
IX	β^3-*h*-Tyr-D-Ala-β^3-*h*-Phe-$PheNH_2$	>1000	Not active
X	β^3-*h*-Tyr- Phe-$PheNH_2$ (X)	602.5 ± 3.52	Not active
XI	β^3-*h*-Tyr-β^3-*h*-Phe-$PheNH_2$ (XI)	>1000	Not active

[a]*Versus* [^{3}H]-naltrexone.
[b]*Versus* [^{3}H]-DT II.
[c][13].

Table 2. Physicochemical properties of α/β-peptides, analogs of TAPP

No	Peptide	HPLC[c]		MS[a]	
		t_R [min]	Purity [%]	M_r	$[M+H]^+$
I	β^3-*h*-Tyr-D-Ala-Phe-$PheNH_2$	13.07	97	559. 67	560.4[a]
II	β^3-*h*-D-Tyr-D-Ala-Phe-$PheNH_2$	11.82	98	559.67	560[a]
III	Tyr-β^3-*h*-D-Ala-Phe-$PheNH_2$	16.33	98	559. 67	560.3[a]
IV	Tyr-β^3-*h*-Ala-Phe-$PheNH_2$	7.93	98	559. 67	560.3[a]
V	Tyr-D-Ala-β^3-*h*-Phe-$PheNH_2$[e]	10.39	99	559. 67	560[a]
VI	Tyr-D-Ala-β^3-*h*-D-Phe-$PheNH_2$	13.18	100	559. 67	560[a]
VII	Tyr-D-Ala-Phe-β^3-*h*-$PheNH_2$[e]	10.39	97	559. 67	560[a]
VIII	Tyr-D-Ala-Phe-β^3-*h*-D-$PheNH_2$	12.35	98	559. 67	560[a]
IX	β^3-*h*-Tyr-D-Ala-β^3-*h*-Phe-$PheNH_2$	13.04	99	488.89	489/596.3[M + Na][b]
X	β^3-*h*-Tyr- Phe-$PheNH_2$	8.34	98	488. 6	489[a]
XI	β^3-*h*-Tyr-*h*-β^3-*h*-Phe-$PheNH_2$	14.23	100	502.6	503,4[a]

[a]FAB MS.
[b]MALDI MS.
[c]Linear gradient 30–50% B, 25 min flow rate 1 ml/min.
[d]Linear gradient 20–80% B.
[e][15].

albeit weak, δ-affinity; similar to TAPP. (ii) In all analogs, the substitution of Tyr-1 with L- or D-β^3-*homo*-Tyr reduces their activity (**X**) or gives inactive peptides (**I**, **II**, **IX** and **XI**). Surprisingly, the tripeptide without D-Ala (**X**) binds to μ-receptors only 40 times less than native TAPP whereas the tripeptide containing two β^3-*homo*-amino acids in positions 1 and 2 (**XI**) is not active. (iii) The replacement of D-Ala with β-*homo*-D-Ala (**III**) results in the most interesting analog. This modification gives an analog with μ-binding affinity (IC_{50}; 9.5 ± 2.9) comparable with TAPP (IC_{50}; 15.5 ± 6.33). Also the analog containing β^3-*homo*-Ala in position 2 shows affinity (33 times lower as compared with TAPP) to the μ-receptor (**IV**). The configuration of β^3-*homo*-alanine in position 2 has a strong impact on binding affinity. (iv) The incorporation of β^3-*homo*-Phe in position 3 (**V**, **VI**) causes a loss of affinity to μ-receptors, whereas the presence of L- or D-β^3-*homo*-Phe in the *C*-terminus position (**VII**, **VIII**) decreases affinity to μ-receptors only sixfold. The chirality of β^3-*homo*-Phe in position 3 or 4 has no influence on binding affinity. It is worth to mention that homologation of Phe^4 residue is detrimental to μ-affinity in endomorphin-1 analogs [21].

The present results suggest that an increased distance between two aromatic rings, or increased flexibility (an additional CH_2 group in the main chain of the peptide), can facilitate fitting to the μ-opioid receptor. The analog Tyr-β^3*h*-D-Ala-Phe-PheNH_2 may adopt a conformation well tolerated by the μ-opioid receptor during the peptide ligand–receptor interaction. This result can be compared with the one previously achieved with the synthesis of the endomorphin-1 and 2 analogs containing alicyclic β-amino acids [22] and piperidine-2-, 3- or 4-carboxylic acids [23] in position 2. It confirms that homologation of residue 2 in μ-selective tetrapeptides is generally well tolerated and does not cause significant changes in activity and selectivity.

Acknowledgement

This work was supported partially by the Lodz University of Technology grant DS I-18/501/15.

References

1 Ciccone PE, O'Brien CP, Khatami M. Psychotropic agents in opiate addiction: a brief review. *Int. J. Addict.* 1980; **15**: 499–513.
2 Zubieta JK, Smith YR, Bueller JA, Xu Y, Kilbourn MR, Jewett DM, Meyer CR, Koeppe RA, Stohler CS. Regional mu opioid receptor regulation of sensory and affective dimensions of pain. *Science* 2001; **293**: 311–315.
3 Moles A, Kieffer BL, D'Amato FR. Deficit in attachment behavior in mice lacking the [mu]-opioid receptor gene. *Science* 2004; **304**: 1983–1986.
4 Jaglowski JR, Zagon IS, Stack Jr. BC, Verderame MF, Aeure-duPree AE, Manning JD, McLaughlin PJ. Opioid growth factor enhances tumor growth inhibition and increases the survival of paclitaxel-treated mice with squamous cell carcinoma of the head and neck. *Cancer Chemother. Pharmacol.* 2005; **56**: 97–104.
5 Lazarczyk M, Matyja E, Lipkowski AW. A comparative study of morphine stimulation and biphalin inhibition of human glioblastoma T98G cell proliferation in vitro. *Peptides* 2010; **31**: 1606–1612.
6 Yakovleva T, Marinova Z, Kuzmin A, Seidah NG, Haroutunian V, Terenius L, Bakalkin G. Dysregulation of dynorphins in Alzheimer disease. *Neurobiol. Aging* 2007; **28**: 1700–1708.
7 Varrassi G, Angeletti C, Guetti C, Marinangeli F, Paladini A. Systemic opioid and chronic pain. *Eur. J. Pain Suppl.* 2009; **3**: 77–83.
8 Moulédous L, Mollereau C, Zajac J-M. Opioid-modulating properties of the neuropeptide FF system. *Biofactors* 2010; **36**: 423–429.
9 Kieffer BL, Gavériaux-Ruff C. Exploring the opioid system by gene knockout. *Prog. Neurobiol.* 2002; **66**: 285–306.
10 Schiller PW, Nguyen TM-D, Chung NN, Lemieux C. Dermorphin analogues carrying an increased positive net charge in their 'message' domain display extremely high mu opioid receptor selectivity. *J. Med. Chem.* 1989; **32**: 698–703.
11 Cardillo G, Gentilucci L, Tolomelli A, Calienni M, Qasem AR, Spampinato S. Stability against enzymatic hydrolysis of endomorphin-1 analogues containing β-proline. *Org. Biomol. Chem.* 2003; **1**: 1498–1502.
12 Krondahl E, Orzechowski A, Ekström G, Lennernäs H. Rat jejunal permeability and metabolism of mu-selective tetrapeptides in gastrointestinal fluids from humans and rats. *Pharm. Res.* 1997; **14**: 1780–1785.
13 Zadina JE, Hackler L, Ge L-J, Kastin AJ. A potent and selective endogenous agonist for the mu-opiate receptor. *Nature* 1997; **386**: 499–502.
14 For example, see Ebert M-O, Gardinera J, Balletb S, Abell AD, Seebach D. Synthesis and high-resolution NMR structure of a β^3-octapeptide with and without a tether introduced by olefin metathesis. *Helv. Chim. Acta* 2009; **92**: 2643–2658; Wilczyńska D, Kosson P, Kwasiborska M, Ejchart A, Olma A. Synthesis and receptor binding of opioid peptide analogues containing β^3-*homo*-amino acids. *J. Pept. Sci.* 2009; **15**: 777–782.
15 Wilczyńska D, Olma A, Kosson P, Lipkowski AW. Conformational and metabolic consequences of substitution of phenylalanine with β^3-homophenylalanine. In *Peptides* Rolka K, Rekowski P, Silbering J. (eds.) Kenes International: Geneva, Switzerland, 2007; 364–365.
16 Linder MR, Steurer S, Podlech J. (*S*)-3-(*tert*-Butyloxycarbonylamino)-4-phenylbutanoic acid. *Org. Synth.* 2002; **79**: 154–159.
17 Koch K, Podlech J. Exceptionally simple homologation of protected α- to β-amino acids in the presence of silica gel. *Synth. Comm.* 2005; **35**: 2789–2794.
18 Seebach D, Ciceri P E, Overhand M, Jaw B, Rigo D. Probing the helical secondary structure of short-chain-peptides. *Helv. Chim. Acta* 1996; **79**: 2043–2066.
19 Gademann K, Ernst M, Seebach D. The cyclo-β-tetrapeptide (β-HPhe-β-HThr-β-HLys-β-HTrp): synthesis, NMR structure in methanol solution, and affinity for human somatostatin receptors. *Helv. Chim. Acta* 2000; **83**: 16–33.
20 Misicka A, Lipkowski AW, Horvat R, Davis P, Kramer TH, Yamamura HI, Hruby VJ. Topographical requirements for delta opioid ligands: common structural features of dermenkephalin and deltorphin. *Life Sci.* 1992; **51**: 1025–1032.
21 Cardillo G, Gentilucci L, Qasem A R, Sgarzi F, Spampinato S. Endomorphin-1 analogues containing β-proline are μ-opioid receptor agonists and display enhanced enzymatic hydrolysis resistance. *J. Med. Chem.* 2002; **45**: 2571–2578.
22 Keresztes A, Szucs M, Borics A, Kövér KE, Forró E, Fülöp F, Tömböly C, Péter A, Páhi A, Fábián G, Murányi M, Tóth G. New endomorphin analogues containing alicyclic beta-amino acids: influence on bioactive conformation and pharmacological profile. *J. Med. Chem.* 2008; **51**: 4270–4279.
23 Staniszewska R, Fichna J, Gach K, Toth G, Poels J, Vanden Broeck J, Janecka A. Synthesis and biological activity of endomorphin-2 analogs incorporating piperidine-2-, 3- or 4-carboxylic acids instead of proline in position 2. *Chem. Bio. Drug Des.* 2008; **72**: 91–94.