

New potent biphalin analogues containing *p*-fluoro-L-phenylalanine at the 4,4' positions and non-hydrazine linkers

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Received: 23 July 2010 / Accepted: 21 September 2010 / Published online: 6 October 2010
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Abstract We report the synthesis and the biological evaluation of two new analogues of the potent dimeric opioid peptide biphalin. The performed modification is based on the replacement of two key structural elements of the native biphalin, namely: the hydrazine bridge which joins the two palindromic moieties and the phenylalanine residues at the 4,4' positions of the backbone. The new analogues **9** and **10** contain 1,2-phenylenediamine and piperazine, respectively, in place of the hydrazidic linker and *p*-fluoro-L-phenylalanine residues at 4 and 4' positions. Binding values are: $K_i^\mu = 0.51$ nM and $K_i^\delta = 12.8$ nM for compound **9**, $K_i^\mu = 0.09$ nM and $K_i^\delta = 0.11$ nM for analogue **10**.

Keywords Activity · Biphalin · Dimeric peptide ligands · Opioid peptides · Synthesis

Abbreviations

Boc	<i>tert</i> -Butyloxycarbonyl
[³ H]DAMGO	[D-Ala(2), <i>N</i> -Me-Phe(4), Gly-ol(5)] enkephalin
[³ H]DPDPE	[³ H]-c[D-Pen ² , D-Pen ⁵]enkephalin
DCM	Dichloromethane
DIPEA	<i>N,N</i> -Diisopropylethylamine
DMAP	4-(Dimethylamino)pyridine
DMF	<i>N,N</i> -Dimethyl formamide
DMSO	Dimethylsulfoxide
EDC	1-Ethyl-(3-dimethylaminopropyl)carbodiimide
GPI/LMMP	Guinea pig ileum/longitudinal muscle myenteric plexus (μ -opioid receptors)
hMOR	Human μ -opioid receptor
HOBt	1-Hydroxybenzotriazole
MVD	Mouse vas deferens (δ -opioid receptors)
NMM	<i>N</i> -Methyl morpholine
rDOR	Rat δ -opioid receptor
TEA	Triethylamine
TFA	Trifluoroacetic acid

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Introduction

The opioid pentapeptide enkephalins (Tyr-Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe-Leu) were originally isolated from pig and cow brain (Hughes et al. 1975). Enkephalins are endogenous δ ligands of the opioid multiple receptor family located in neuronal cell membranes which express the extensively studied μ -, δ - and κ -opioid receptors. As expected for simple linear oligopeptides, enkephalins are highly sensitive to enzymatic degradation and, in addition

to this, exhibit low receptor binding selectivity and minimal capacity to cross the phospholipid bilayer (Hruby and Gehrig 1989). Generally unfit as therapeutic agents, native enkephalins continue to represent the reference structural model for studies on recognition and binding mechanisms of peptide ligands as well as for the development of secondary effect-free analgesics to be used in place of morphine.

Based on extensive SAR studies, a variety of synthetic strategies has been adopted to modify the enkephalin native structure and most of the relevant opioid peptide ligands so far described are enkephalin-based. Among consolidated findings in this field is the role exerted on enkephalin activity by the phenylalanine residue in position 4. It has been shown that the aromatic residue at position 4 can significantly modulate binding to the μ - and δ -opioid receptors (Morgan et al. 1976; Chang et al. 1976; Schiller et al. 1978). Modification of this residue, by introducing substituents at the *para* position of the aromatic ring, significantly enhances the activity with the highest effect shown by the introduction of electron-drawing groups (Schiller et al. 1983). Moreover, it has been found that the positive consequences of the modification performed at the Phe⁴ aromatic ring of enkephalins are maintained in the case of the highly active synthetic octapeptide biphalin (Tyr-D-Ala-Gly-Phe-NH-NH<-Phe<-Gly<-D-Ala<-Tyr; Fig. 1; Abbruscato et al. 1996; Li et al. 1998; Misicka et al. 1999).

Biphalin represents one of the most successful applications of the dimeric ligand approach to the design of native bioactive peptide analogues (Coy et al. 1976; Lipkowski et al. 1982; Shimohigashi et al. 1982; Horan et al. 1993). Its structure is characterized by two enkephalin-derived bioactive fragments joined “tail to tail” by a hydrazide bridge. The presence of two pharmacophores, combined with an appropriate linker, confers on biphalin highly affinity for both δ - and μ -receptors, a potency significantly higher than

morphine after i.c.v. and i.t. administration (Lipkowski et al. 1987; Horan et al. 1993) and less physical dependence than morphine (Horan et al. 1993; Yamazaki et al. 2001). Consequently, biphalin continues to be the object of chemical modifications and structural studies aimed at further improving the activity as well as to better understand the molecular bases of its interaction with opioid receptors.

Modification of the 4,4' residues of biphalin, by symmetrical incorporation of *para*-fluoro or *para*-nitro phenylalanine residues (see Fig. 1), leads to analogues which show, although to different degrees, enhancement of affinity towards δ - and μ -opioid receptors accompanied by an increase of δ/μ selectivity (Misicka et al. 1997; Li et al. 1998). Recently, Hruby and co-workers reported on a series of biphalin analogues in which the -NH-NH- linker is replaced by different non-hydrazidic bridges, namely the residues of 1,4-phenylenediamine, 1,2-phenylenediamine and piperazine (see Fig. 1, compounds **9a** and **10a**) (Mollica et al. 2005). These new analogues show better affinity and in vitro bioactivity than biphalin itself, thus suggesting that the high activity of biphalin is not critically related to the structural and conformational properties of the hydrazide bridge nor to its capability to form hydrogen bonds with the opioid receptors.

With the aim to investigate the effects of the *para* substitution at the Phe⁴ aromatic ring combined with hydrazine bridge replacements, we report here synthesis and in vitro assays of two new fluorinated biphalin analogues **9** and **10** containing non-hydrazine linkers.

Chemistry

In the present study, the previously adopted strategy for the synthesis of non-hydrazine biphalin analogues has been

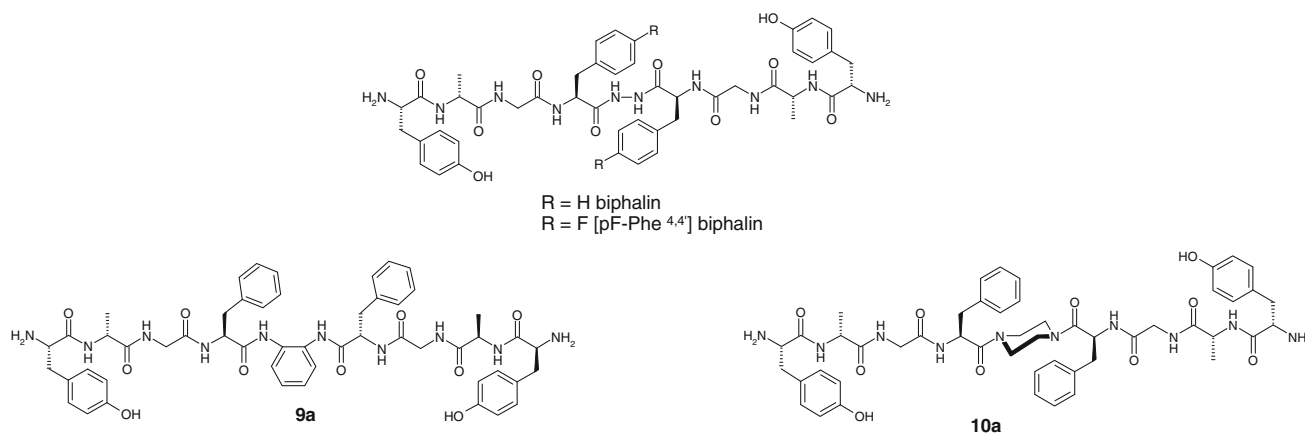


Fig. 1 Structure of biphalin (Bph) and its previously studied 4,4'-pF-Phe analogue (pF-Bph) (Misicka et al. 1997). For the sake of clarity, the structure of the non-hydrazine analogues **9a** and **10a** (Mollica et al. 2005) is also illustrated

modified. As illustrated in Scheme 1, the amino acid residues were symmetrically added one by one to the linkers. This procedure allows an easier isolation and characterization of the intermediate products and avoids possible racemization connected with alkaline hydrolysis of the intermediate tripeptide ester Boc-Tyr-D-Ala-Gly-OEt.

General procedures

All products were synthesized in solution using the EDC/HOBt/DIPEA coupling method (Scheme 1). The N^α terminal Boc-protected peptides were all deprotected by a mixture of TFA in DCM 1:1 at r.t. The intermediate TFA salts were used for subsequent reactions without further purification. Intermediate products **3** and **5** were purified by precipitation in EtOAc, the filtrate was washed on a Buchner funnel with 3 × 50 mL of NaHCO₃ s.s., 3 × 50 mL of 5% citric acid, 3 × 50 mL of deionized water, dried under vacuum, followed by trituration with diethyl ether and used in the next step without further purification. Products **1**, **2**, **4** and **6** were purified by silica gel column chromatography. Final products **7**, **8**, **9** and **10** were purified by RP-HPLC using a Waters XBridgeTM Prep BEH130 C₁₈, 5.0 μm, 250 mm × 10 mm column, HPLC solvent A, 0.1% TFA in water; solvent B, acetonitrile; gradient: 5–95% B in A over 45 min, flow rate 5.0 mL/min.

The purity of the N^α-Boc-protected products and the final TFA salts was assessed by analytical RP-HPLC, TLC confirmed by NMR analysis and mass spectrometry ESI-HRMS (see Table 1).

(Boc-pF-Phe)₂-piperazine (**1**)

EDC·HCl (2.2 eq.), HOBt (2.2 eq.) and DIPEA (6.6 eq.) were added to a solution of Boc-pF-Phe-OH (2.2 eq.) in DMF at 0°C. The reaction mixture was stirred for 10 min, piperazine (1 eq.) was added and the reaction was stirred for an additional 10 min at 0°C and then allowed to warm at r.t. overnight. The solvent was evaporated under reduced pressure, the residue was precipitated with EtOAc and the suspension was filtered through a Buchner funnel under reduced pressure. The solid residue was washed with three portions of 5% citric acid, NaHCO₃ s.s., brine and distilled water. The solid was dried under reduced pressure and triturated with diethyl ether to give the desired product **1** as a crude white solid. $R_f^I = 0.27$ (CH₂Cl₂/AcOEt 8:2), $R_f^{II} = 0.71$ (CH₂Cl₂/AcOEt 1:1). The product was purified by silica gel column chromatography (CH₂Cl₂/AcOEt 8:2 to CH₂Cl₂/EtOAc 3:7) to obtain the pure product **1** (92%). ¹H NMR (DMSO-*d*₆, 300 MHz) (δ, ppm): 1.32 [9H, s, (CH₃)₃-]; 2.78–2.70 [4H, m, Phe β-CH₂]; 3.41–3.26 [8H, m, -CH₂-CH₂-]; 4.55 [2H, m, Phe α-CH]; 7.30–7.07 [8H, m,

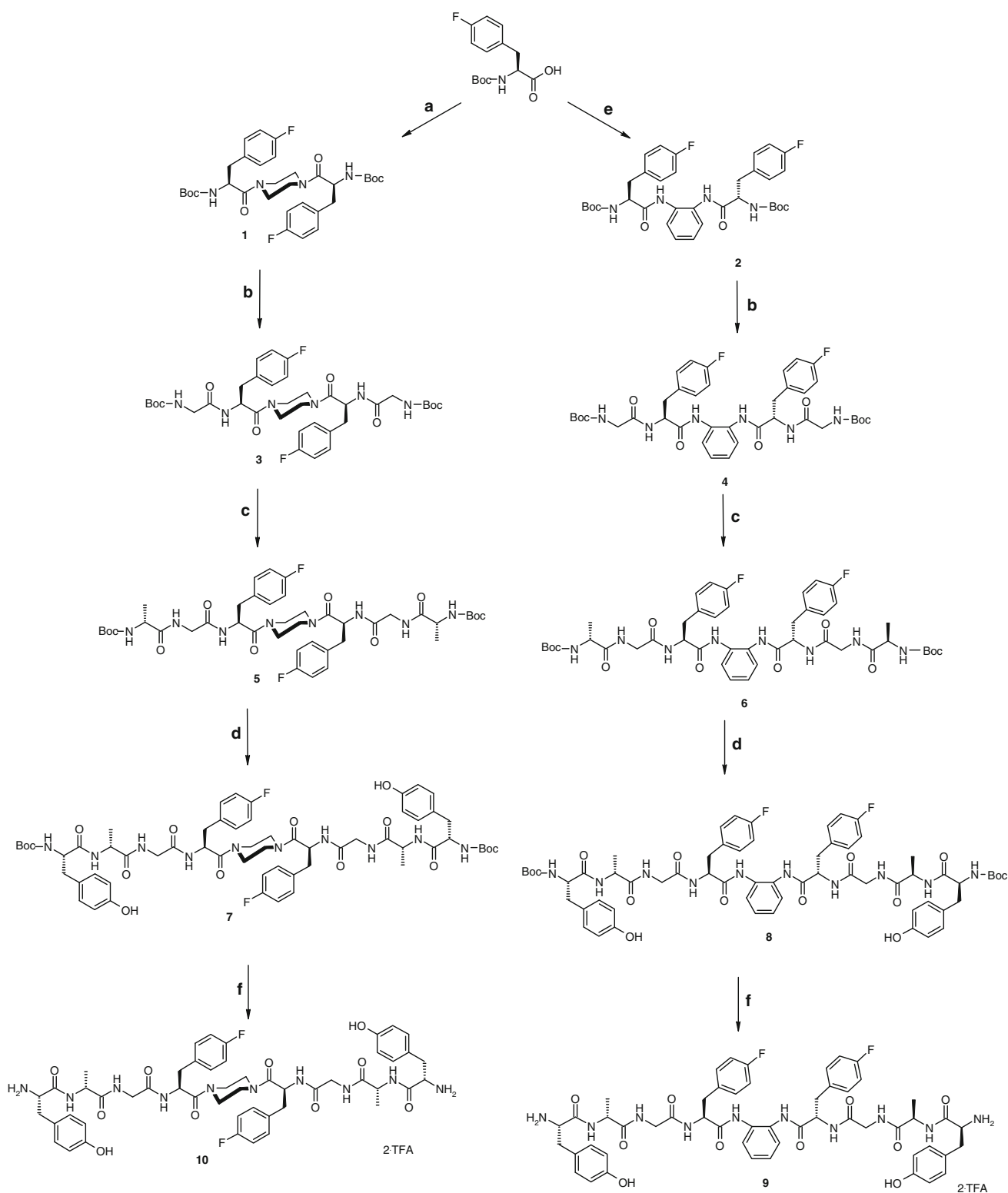
aromatics]; 7.25 [2H, d, Phe NH]. ¹³C NMR (CDCl₃, 300 MHz) (δ, ppm): 28.5, 39.6, 41.6, 45.2, 51.1, 80.3, 115.8, 131.2, 132.1, 155.2, 160.5, 170.3. HRMS (ESI) calcd. for C₃₂H₄₃F₂N₄O₆ *m/z*: 617.3151 [M + H]⁺; found 617.3155.

(Boc-pF-Phe)₂-1,2-phenylenediamine (**2**)

EDC·HCl (2.2 eq.), HOBt (2.2 eq.) and DIPEA (6.6 eq.) were added to a solution of Boc-pF-Phe-OH (2.2 eq.) in DMF at 0°C. The reaction mixture was stirred for 10 min, 1,2-phenylenediamine (1 eq.) was added, the reaction was stirred for an additional 10 min at 0°C and then allowed to warm at r.t. overnight. The solvent was evaporated under reduced pressure, the residue was precipitated with EtOAc, and the suspension was filtered through a Buchner funnel under reduced pressure. The solid residue was washed with three portions of 5% citric acid, NaHCO₃ s.s., brine and distilled water. Then the solid was dried under reduced pressure and triturated with diethyl ether to give the desired product **2** as a crude white solid. $R_f^I = 0.40$ (CH₂Cl₂/AcOEt 8:2), $R_f^{II} = 0.33$ (CHCl₃/CH₃OH 98:2). The product was purified by silica gel column chromatography (CH₂Cl₂/AcOEt 95:5 to CH₂Cl₂/AcOEt 7:3) to obtain the pure product **2** (41%). ¹H NMR (DMSO-*d*₆, 300 MHz) (δ, ppm): 1.31 [9H, s, (CH₃)₃-]; 2.97–2.76 [4H, m, Phe β-CH₂]; 4.24 [2H, m, Phe α-CH]; 7.40–6.50 [12H, m, aromatics]; 6.95 [2H, d, Phe NH]; 9.46 and 9.23 [2H, two singlets, Ar NH]. ¹³C NMR (CDCl₃, 300 MHz) (δ, ppm): 28.5, 37.4, 56.5, 80.8, 115.8, 126.8, 130.3, 131.1, 132.4, 155.8, 160.5, 170.7. HRMS (ESI) calcd. for C₃₄H₄₁F₂N₄O₆ *m/z*: 639.2994 [M + H]⁺; found 639.2991.

(Boc-Gly-pF-Phe)₂-piperazine (**3**)

Product **1** was deprotected at the N^α terminal by TFA in DCM 1:1 using 1 mL of mixture per 100 mg of Boc-protected product for 1.5 h at r.t. The mixture was then evaporated under high vacuum and the TFA salt was used for the next step without further purification. EDC·HCl (2.2 eq.), HOBt (2.2 eq.) and DIPEA (6.6 eq.) were added to a solution of Boc-Gly-OH (2.2 eq.) in DMF at 0°C. The reaction mixture was stirred for 10 min, TFA-pF-Phe-Pip-pF-Phe-TFA (1 eq.) was added, the reaction was stirred for an additional 10 min at 0°C then allowed to warm at r.t. overnight. The solvent was evaporated under reduced pressure, the residue was precipitated with EtOAc and the suspension was filtered through a Buchner funnel under reduced pressure. The solid residue was washed with three portions of 5% citric acid, NaHCO₃ s.s., brine and distilled water. The solid was dried under reduced pressure and triturated with diethyl ether to yield the desired product **3** as pure white solid (82%). $R_f^I = 0.52$ (AcOEt), $R_f^{II} = 0.61$



Scheme 1 Synthesis of the fluorinated biphalin analogues with non-hydrazine linker **9** and **10**. Reagents and conditions: **a** EDC·HCl (2.2 eq.), HOBT (2.2 eq.), DIPEA (6.6 eq.), Boc-pF-Phe-OH (2.2 eq.), piperazine (1 eq.) in DMF at r.t. overnight; **b** TFA/CH₂Cl₂ 1:1, r.t. (1.5 h) then EDC·HCl (2.2 eq.), HOBT (2.2 eq.), DIPEA (6.6 eq.), Boc-Gly-OH (2.2 eq.), in DMF at r.t. overnight; **c** TFA/CH₂Cl₂ 1:1, r.t.

(1.5 h) then EDC·HCl (2.2 eq.), HOBT (2.2 eq.), DIPEA (6.6 eq.), Boc-D-Ala-OH (2.2 eq.) in DMF at r.t. overnight; **d** TFA/CH₂Cl₂ 1:1, r.t. (1.5 h) then EDC·HCl (2.2 eq.), HOBT (2.2 eq.), DIPEA (6.6 eq.), Boc-Tyr-OH (2.2 eq.) in DMF at r.t. overnight; **e** EDC·HCl (2.2 eq.), HOBT (2.2 eq.), DIPEA (6.6 eq.), Boc-pF-Phe-OH (2.2 eq.), 1,2-phenylenediamine (1 eq.) in DMF at r.t. overnight. **f** TFA/CH₂Cl₂ 1:1, r.t. (1.5 h)

Table 1 Structures and the physicochemical properties of the biphalin analogues **9–10** and *N*-Boc derivatives **1–8**

No.	Structures	<i>m/z</i> [M + H] ⁺ (ESI)		RP-HPLC ^a , retention time (min)
		Calcd	Found	
1	(Boc-pF-Phe) ₂ -piperazine	617.3151	617.3155	39.50
2	(Boc-pF-Phe) ₂ -1,2-phenylenediamine	639.2994	639.2991	43.79
3	(Boc-Gly-pF-Phe) ₂ -piperazine	731.3580	731.3588	32.91
4	(Boc-Gly-pF-Phe) ₂ -1,2-phenylenediamine	753.3426	753.3425	39.49
5	(Boc-D-Ala-Gly-pF-Phe) ₂ -piperazine	873.4322	873.4318	30.74
6	(Boc-D-Ala-Gly-pF-Phe) ₂ -1,2-phenylenediamine	895.4166	895.4160	37.46
7	(Boc-Tyr-D-Ala-Gly-pF-Phe) ₂ -piperazine	1,199.5589	1,199.5586	29.35
8	(Boc-Tyr-D-Ala-Gly-pF-Phe) ₂ -1,2-phenylenediamine	1,221.5432	1,221.5435	34.15
9	2 TFA·(Tyr-D-Ala-Gly-pF-Phe) ₂ -1,2-phenylenediamine	1,021.4384	1,021.4381	22.73
10	2 TFA·(Tyr-D-Ala-Gly-pF-Phe) ₂ -piperazine	999.4540	999.4542	20.98

^a HPLC column: waters XBridgeTM BEH130 C18, 250 × 4.6 mm, 5.0 μm; HPLC solvent A, 0.1% TFA in water; solvent B, acetonitrile; gradient: 5–95% B in A over 45 min, flow rate 1.0 mL/min, 50 μL injection of a solution containing 1 mg/mL in acetonitrile/water 2:1

(AcOEt/CH₃OH 99:1). ¹H NMR (DMSO-*d*₆, 300 MHz) (δ, ppm): 1.35 [9H, s, (CH₃)₃–]; 2.96–2.75 [4H, m, Phe β-CH₂]; 3.41–3.26 [8H, m, –CH₂–CH₂–]; 3.49 [4H, m, Gly CH₂]; 4.89 [2H, m, Phe α-CH]; 6.65 [2H, br, Gly NH]; 7.22–6.98 [8H, m, aromatics]; 7.90 [2H, d, Phe NH]. ¹³C NMR (CDCl₃, 300 MHz) (δ, ppm): 28.5, 38.7, 41.7, 45.2, 49.8, 60.6, 80.6, 115.9, 131.2, 131.7, 156.1, 160.5, 169.7, 171.4. HRMS (ESI) calcd. for C₃₆H₄₆F₂N₆O₈ *m/z*: 731.3580 [M + H]⁺; found 731.3588.

(Boc-Gly-pF-Phe)₂-1,2-phenylenediamine (4)

Product **2** was deprotected at the N^z terminal by TFA in DCM 1:1 using 1 mL of mixture per 100 mg of the Boc-protected product for 1.5 h at r.t. The mixture was then evaporated under high vacuum and the TFA salt was used for the next step without further purification. EDC·HCl (2.2 eq.), HOBT (2.2 eq.), DIPEA (6.6 eq.) were added to a solution of Boc-Gly-OH (2.2 eq.) in DMF at 0°C and reaction mixture was stirred for 10 min, TFA·pF-Phe-1,2-phenylenediamine-pF-Phe·TFA (1 eq.) was added, the reaction was stirred for an additional 10 min at 0°C then allowed to warm at r.t. overnight. The solvent was evaporated under reduced pressure, the residue was precipitated with EtOAc, and the suspension was filtered through a Buchner funnel under reduced pressure. The solid residue was washed with three portions of 5% citric acid, NaHCO₃ s.s., brine and distilled water, then the solid dried under reduced pressure and triturated with diethyl ether to give the desired product **4** as a crude white solid. The product was purified by silica gel column chromatography (AcOEt/CH₂Cl₂ 1:1 to AcOEt/CH₂Cl₂ 8:2) to obtain the pure product **4** (77%). *R*_f^I = 0.21 (AcOEt/CH₂Cl₂ 1:1), *R*_f^{II} = 0.80 (AcOEt). ¹H NMR (DMSO-*d*₆, 300 MHz) (δ, ppm): 3.22–2.86 [4H, m, Phe β-CH₂]; 3.55 [4H, m, Gly NH]; 4.77

[2H, m, Phe α-CH]; 7.22–6.59 [12H, m, aromatics]; 7.99 [4H, br, Gly NH]; 8.88 [2H, br, Phe NH]; 9.70 [2H, s, Ar NH]. ¹³C NMR (CDCl₃, 300 MHz) (δ, ppm): 28.4, 37.1, 55.3, 60.6, 80.7, 115.9, 125.5, 126.8, 131.1, 132.1, 132.2, 156.7, 160.5, 170.2, 170.6. HRMS (ESI) calcd. for C₃₈H₄₇F₂N₆O₃ *m/z*: 753.3426 [M + H]⁺; found 753.3425.

(Boc-D-Ala-Gly-pF-Phe)₂-piperazine (5)

Product **3** was deprotected at the N^z terminal by TFA in DCM 1:1 using 1 mL of the mixture per 100 mg of Boc-protected product for 1.5 h at r.t. The mixture was then evaporated under high vacuum and the TFA salt was used for the next step without further purification. EDC·HCl (2.2 eq.), HOBT (2.2 eq.) and DIPEA (6.6 eq.) were added to a solution of Boc-D-Ala-OH (2.2 eq.) in DMF at 0°C. The reaction mixture was stirred for 10 min, TFA·Gly-pF-Phe-Pip-pF-Phe-Gly·TFA (1 eq.) was added, the reaction was stirred for an additional 10 min at 0°C then allowed to warm at r.t. overnight. The solvent was evaporated under reduced pressure, the residue was precipitated with EtOAc and the suspension was filtered through a Buchner funnel under reduced pressure. The solid residue was washed with three portions of 5% citric acid, NaHCO₃ s.s., brine and distilled water, then dried under reduced pressure and triturated with diethyl ether to give the desired product **5** as a pure white solid (60%). *R*_f^I = 0.16 (AcOEt/CH₃OH 9:1), *R*_f^{II} = 0.80 (AcOEt/CH₃OH 8:2). ¹H NMR (DMSO-*d*₆, 300 MHz) (δ, ppm): 1.16 [6H, m, Ala CH₃]; 1.34 [9H, s, (CH₃)₃–]; 2.89–2.74 [4H, m, Phe β-CH₂]; 3.41–3.10 [8H, m, –CH₂–CH₂–]; 3.67–3.55 [4H, m, Gly CH₂]; 3.98 [2H, m, D-Ala α-CH]; 4.83 [2H, m, Phe α-CH]; 6.98 [2H, d, D-Ala NH]; 7.23–7.04 [8H, m, aromatics]; 7.96 [2H, t, Gly NH]; 8.21 [2H, d, Phe NH]. ¹³C NMR (DMSO-*d*₆, 300 MHz) (δ, ppm): 18.7, 28.8, 37.3, 42.1, 42.3, 50.2, 50.3,

78.7, 115.6, 131.9, 133.9, 155.7, 160.1, 168.8, 169.6, 173.6. HRMS (ESI) calcd. for $C_{42}H_{59}F_2N_8O_{10}$ m/z : 873.4322 $[M + H]^+$; found 873.4318.

(Boc-D-Ala-Gly-pF-Phe)₂-1,2-phenylenediamine (6)

Product **4** was deprotected at the N^z terminal by TFA in DCM 1:1 using 1 mL of mixture per 100 mg of Boc-protected product for 1.5 h at r.t. The mixture was then evaporated under high vacuum and the TFA salt was used for the next step without further purification. EDC·HCl (2.2 eq.), HOBT (2.2 eq.) and DIPEA (6.6 eq.) were added to a solution of Boc-D-Ala-OH (2.2 eq.) in DMF at 0°C and stirred for 10 min. TFA·Gly-pF-Phe-1,2-phenylenediamine-pF-Phe-Gly·TFA (1 eq.) was added, the reaction was stirred for an additional 10 min at 0°C, then allowed to warm at r.t. overnight. The solvent was evaporated under reduced pressure, the residue was precipitated with EtOAc and the suspension was filtered through a Buchner funnel under reduced pressure. The solid residue was washed with three portions of 5% citric acid, $NaHCO_3$ s.s., brine and distilled water, dried under reduced pressure and triturated with diethyl ether to give the desired product **6** as a crude white solid. $R_f^I = 0.34$ (AcOEt), $R_f^{II} = 0.50$ (AcOEt/ CH_3OH 99:1). The product was purified by silica gel column chromatography (AcOEt/ CH_2Cl_2 1:1 to AcOEt/ CH_2Cl_2 94:6) to obtain the pure product **6** (35%). 1H NMR (DMSO- d_6 , 300 MHz) (δ , ppm): 1.16 [6H, m, Ala CH_3]; 1.34 [9H, s, $(CH_3)_3$]; 3.18–2.74 [4H, m, Phe β - CH_2]; 3.81–3.61 [4H, m, Gly CH_2]; 3.95 [2H, m, D-Ala α -CH]; 4.60 [2H, m, Phe α -CH]; 7.35–6.50 [12H, m, aromatics]; 7.50 [2H, br, D-Ala NH]; 8.05 [2H, t, Gly NH]; 8.21 [2H, d, Phe NH]; 9.50 [2H, s, Ar NH]. ^{13}C NMR ($CDCl_3$, 300 MHz) (δ , ppm): 18.7, 28.5, 37.1, 43.1, 50.6, 55.9, 80.4, 115.7, 125.4, 126.6, 130.4, 131.1, 132.5, 156.0, 160.4, 169.9, 170.4, 174.3. HRMS (ESI) calcd. for $C_{44}H_{57}F_2N_8O_{10}$ m/z : 895.4166 $[M + H]^+$; found 895.4160.

(Boc-Tyr-D-Ala-Gly-pF-Phe)₂-piperazine (7)

Product **5** was deprotected at the N^z terminal by TFA in DCM 1:1 using 1 mL of mixture per 100 mg of Boc-protected product for 1.5 h at r.t. The mixture was then evaporated under high vacuum and the TFA salt was used for the next step without further purification. EDC·HCl (2.2 eq.), HOBT (2.2 eq.) and DIPEA (6.6 eq.) were added to a solution of Boc-Tyr-OH (2.2 eq.) in DMF at 0°C and stirred for 10 min. TFA·D-Ala-Gly-pF-Phe-Pip-pF-Phe-Gly-D-Ala-TFA (1 eq.) was added, the reaction was stirred for an additional 10 min at 0°C then allowed to warm at r.t. overnight. The solvent was evaporated under reduced pressure, the residue was precipitated with EtOAc and the suspension was filtered through a Buchner funnel under

reduced pressure. The solid residue was washed with three portions of 5% citric acid, $NaHCO_3$ s.s., brine and distilled water, dried under reduced pressure and triturated with diethyl ether to give the product **7** as crude white solid. The product was purified on RP-HPLC to give the pure product **7** (90%). $R_f^I = 0.20$ (AcOEt/MeOH 9:1), $R_f^{II} = 0.73$ ($CHCl_3$ /MeOH 8:2). 1H NMR (DMSO- d_6 , 300 MHz) (δ , ppm): 1.15 [6H, m, Ala CH_3]; 1.32 [9H, s, $(CH_3)_3$]; 2.98–2.54 [8H, m, Phe β - CH_2 and Tyr β - CH_2]; 3.45–3.10 [8H, m, $-CH_2-CH_2-$ superimposed to the water signal]; 3.65 [4H, m, Gly CH_2]; 4.05 [2H, m, D-Ala α -CH]; 4.22 [2H, m, Tyr α -CH]; 4.85 [2H, m, Phe α -CH]; 6.90 [2H, d, Tyr NH]; 7.25–6.64 [16H, m, aromatics]; 8.05 [4H, br, Phe NH and D-Ala]; 8.22 [2H, t, Gly NH]; 9.20 [2H, s, Tyr OH]. ^{13}C NMR (DMSO- d_6 , 300 MHz) (δ , ppm): 18.7, 28.8, 37.4, 38.4, 45.2, 48.8, 50.1, 56.7, 60.4, 78.8, 115.4, 128.5, 128.9, 130.0, 130.8, 137.8, 155.9, 156.4, 168.6, 169.8, 172.0, 173.0. HRMS (ESI) calcd. for $C_{60}H_{77}F_2N_{10}O_{14}$ m/z : 1,199.5589 $[M + H]^+$; found 1,199.5586.

(Boc-Tyr-D-Ala-Gly-pF-Phe)₂-1,2-phenylenediamine (8)

Product **6** was deprotected at the N^z terminal by TFA in DCM 1:1 using 1 mL of mixture per 100 mg of Boc-protected product for 1.5 h at r.t. The mixture was then evaporated under high vacuum and the TFA salt was used for the next step without further purification. EDC·HCl (2.2 eq.), HOBT (2.2 eq.) and DIPEA (6.6 eq.) were added to a solution of Boc-Tyr-OH (2.2 eq.) in DMF at 0°C. The reaction mixture was stirred for 10 min. TFA·D-Ala-Gly-pF-Phe-1,2-phenylenediamine-pF-Phe-Gly-D-Ala-TFA (1 eq.) was added, the reaction was stirred for an additional 10 min at 0°C, then allowed to warm at r.t. overnight. The solvent was evaporated under reduced pressure, the residue was precipitated with EtOAc and suspension was filtered through a Buchner funnel under reduced pressure. The solid residue was washed with three portions of 5% citric acid, $NaHCO_3$ s.s., brine and distilled water, dried under reduced pressure and triturated with diethyl ether to give the desired product **8** as a crude white solid. $R_f^I = 0.46$ (AcOEt/MeOH 9:1), $R_f^{II} = 0.37$ ($CHCl_3$ /MeOH 9:1). The product was purified on RP-HPLC to give the pure product **8** (30%). 1H NMR (DMSO- d_6 , 300 MHz) (δ , ppm): 1.16 [6H, m, Ala CH_3]; 1.32 [9H, s, $(CH_3)_3$]; 3.15–2.42 [8H, m, Tyr β - CH_2 and Phe β - CH_2]; 3.70 [4H, m, Gly CH_2]; 4.03 [2H, m, D-Ala α -CH]; 4.23 [2H, m, Tyr α -CH]; 4.65 [2H, m, Phe α -CH]; 7.42–6.59 [20H, m, aromatics]; 6.85 [2H, d, Tyr NH]; 8.22–8.05 [6H, br, D-Ala NH, Gly NH and Phe NH]; 9.50 and 9.15 [4H, two singlets, Tyr OH and Ar NH]. ^{13}C NMR (DMSO- d_6 , 300 MHz) (δ , ppm): 18.7, 28.7, 37.1, 37.2, 42.5, 48.7, 56.8, 56.9, 78.8, 115.4, 115.6, 120.7, 128.5, 130.7, 130.8, 131.8, 133.3,

134.3, 156.0, 156.4, 160.1, 169.6, 170.6, 172.2, 173.1. HRMS (ESI) calcd. for $C_{62}H_{75}F_2N_{10}O_{14}$ m/z : 1,221.5432 $[M + H]^+$; found 1,221.5435.

2 TFA·(Tyr-D-Ala-Gly-pF-Phe)₂-1,2-phenylenediamine (**9**)

Product **8** was deprotected at the N^z terminal by TFA in DCM 1:1 using 1 mL of mixture per 100 mg of Boc-protected product for 1.5 h at r.t. The mixture was then evaporated under high vacuum and the TFA salt was purified on RP-HPLC to give the pure product **9** (78%). $R_f^I = 0.69$ (*n*-Bu-OH/CH₃COOH/H₂O 4:1:1), $R_f^{II} = 0.65$ (*n*-Bu-OH/CH₃COOH/AcOEt/H₂O 5:1:3:1). ¹H NMR (DMSO-*d*₆, 500 MHz) (δ, ppm): 1.05 [6H, m, Ala CH₃]; 3.20–2.90 [8H, m, Tyr β-CH₂ and Phe β-CH₂]; 3.60 [4H, m, Gly CH₂]; 4.00 [2H, m, Tyr α-CH]; 4.32 [2H, m, D-Ala α-CH]; 4.65 [2H, m, Phe α-CH]; 7.50–6.70 [20H, m, aromatics]; 8.06 [2H, br, Tyr NH]; 8.15 [4H, br, Gly NH and Phe NH]; 8.55 [2H, br, D-Ala NH]; 9.56 and 9.35 [4H, two singlets, Tyr OH and Ar NH]. ¹³C NMR (DMSO-*d*₆, 300 MHz) (δ, ppm): 18.9, 36.9, 37.2, 48.8, 54.2, 55.4, 55.5, 115.9, 125.4, 126.0, 131.1, 131.8, 134.3, 157.2, 158.6, 159.0, 160.1, 163.3, 168.3, 169.5, 170.7, 172.5. HRMS (ESI) calcd. for $C_{52}H_{59}F_2N_{10}O_{10}$ m/z : 1,021.4384 $[M + H]^+$; found 1,021.4381.

2 TFA·(Tyr-D-Ala-Gly-pF-Phe)₂-piperazine (**10**)

Product **7** was deprotected at the N^z terminal by TFA in DCM 1:1 using 1 mL of mixture per 100 mg of Boc-protected product for 1.5 h at r.t. The mixture was then evaporated under high vacuum and the TFA salt was purified on RP-HPLC to give the pure product **10** (90%). $R_f^I = 0.46$ (*n*-Bu-OH/CH₃COOH/H₂O 4:1:1), $R_f^{II} = 0.12$ (*n*-Bu-OH/CH₃COOH/AcOEt/H₂O 5:1:3:1). ¹H NMR (DMSO-*d*₆, 500 MHz) (δ, ppm): 1.08 [6H, m, Ala CH₃]; 2.98–2.77 [8H, m, Tyr β-CH₂ and Phe β-CH₂]; 3.35 [8H, m, piperazine protons under the water signal]; 3.68 [4H, m, Gly CH₂]; 3.99 [2H, m, Tyr α-CH]; 4.35 [2H, m, D-Ala α-CH]; 4.90 [2H, m, Phe α-CH]; 7.25–6.67 [16H, m, aromatics]; 8.15 [2H, br, Tyr NH]; 8.25 [4H, br, Gly NH and Phe NH]; 8.58 [2H, br, D-Ala NH]; 9.34 [2H, s, Tyr OH]. ¹³C NMR (DMSO-*d*₆, 300 MHz) (δ, ppm): 18.9, 36.9, 42.1, 48.9, 50.2, 50.9, 54.2, 58.4, 115.6, 115.9, 125.4, 131.1, 132.0, 133.8, 157.2, 158.5, 158.9, 168.7, 169.9, 172.5, 172.6. HRMS (ESI) calcd. for $C_{50}H_{61}F_2N_{10}O_{10}$ m/z : 999.4540 $[M + H]^+$; found 999.4542.

Biological assays

Receptor binding affinities to the δ- and μ-opioid receptors were performed using cell membrane preparations from

transfected cells that stably express the respective receptor type and were evaluated as previously described (Polt et al. 1994; Misicka et al. 1992). The ligands used were [³H]DPDPE and [³H]DAMGO for δ- and μ-receptors, respectively.

We used [³⁵S]GTP-γ-S binding to examine opioid agonist efficacy and functional characterization of the ligands at the δ- and μ-opioid receptors. Agonist efficacy can be determined at the level of receptor G-protein interaction by measuring agonist-stimulated binding with a non-hydrolyzable GTP analogue. The ability of μ- and δ-opioid agonists to activate G-proteins has been demonstrated by studying the binding of the GTP analogue guanosine-5'-O-(3-[³⁵S]thio)triphosphate ([³⁵S]GTP-γ-S). The opioid receptor-mediated assay was performed as previously described (Szekeres and Traynor 1997). Cells expressing hDOR for δ-receptor (or rMOR for μ-receptor) were incubated with increasing concentrations of the test compounds in the presence of 0.1 nM [³⁵S]GTP-γ-S (1,000–1,500 Ci/mmol, MEN, Boston, MA) in assay buffer (total volume of 1 mL, duplicate samples) as a measure of agonist-mediated G-protein activation. After incubation (90 min, 30°C), the reaction was terminated by rapid filtration under vacuum through Whatman GF/B glass fibre filters, followed by four washes with ice-cold 15 mM Tris/120 mM NaCl, pH 7.4. Filters were pre-treated with assay buffer prior to filtration to reduce non-specific binding. Bound reactivity was measured by liquid scintillation spectrophotometry after an overnight extraction with EcoLite (ICN, Biomedicals, Costa Mesa, CA) scintillation cocktail. The data were analysed using GraphPad Prism Software (San Diego, CA).

The in vitro tissue bioassays (MVD and GPI/LMMP) were performed as described previously (Kramer et al. 1993). IC₅₀ values represent means of no less than four experiments. IC₅₀ 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. All biological data are summarized in Table 2.

Results and discussion

In order to better discuss the consequences of the structural modifications, Table 2 reports, in addition to the activity data of the new analogues **9** and **10** and native biphalin, the biological evaluations previously found for pF-Bph, the pF-Phe analogue of biphalin (Misicka et al. 1997) and the analogues **9a** and **10a** (Mollica et al. 2005). The latter, as compared with **9** and **10**, maintain the non-hydrazine linker but lack the *para*-fluoro (pF) substitution at the 4,4'-Phe residues.

The positive influence of the introduction of a pF-Phe residue in position 4,4' of biphalin has already been

Table 2 Binding affinity, GTP binding assay, $E_{\max}$ (%) (net total bound/basal binding $\times$ 100), and in vitro activity

Compound	Binding $K_i^{a,b}$ (nM)		GTP binding ^{b,c} (nM)				Functional bioactivity ^b (nM)	
	K_i^{δ}	K_i^{μ}	EC ₅₀ (nM), hDOR	$E_{\max}$ (%) ^d	EC ₅₀ (nM), hMOR	$E_{\max}$ (%) ^d	MVD	GPI/LMMP
Bph ^e	2.6 $\pm$ 0.4	1.4 $\pm$ 0.2	2.5 $\pm$ 0.5	27 $\pm$ 3	6.0 $\pm$ 0.2	25 $\pm$ 4	27 $\pm$ 1.5	8.8 $\pm$ 0.3
pF-Bph ^f	0.31 $\pm$ 0.1	0.64 $\pm$ 0.3	— ^g	— ^g	— ^g	— ^g	1.3 $\pm$ 0.1	2.14 $\pm$ 0.6
9a ^h	0.19 $\pm$ 0.04	1.9 $\pm$ 0.2	1.7 $\pm$ 0.2	83 $\pm$ 7	2.5 $\pm$ 0.7	89 $\pm$ 10	0.72 $\pm$ 0.21	40 $\pm$ 13
9	13 $\pm$ 1	0.51 $\pm$ 0.07	0.56 $\pm$ 0.04	72 $\pm$ 6	2.9 $\pm$ 0.5	44 $\pm$ 3	0.66 $\pm$ 0.17	3.7 $\pm$ 1.0
10a ^h	0.65 $\pm$ 0.3	0.48 $\pm$ 0.06	44 $\pm$ 2	56.9 $\pm$ 3	13 $\pm$ 1	47 $\pm$ 5	9.3 $\pm$ 0.4	2.5 $\pm$ 0.7
10	0.09 $\pm$ 0.01	0.11 $\pm$ 0.01	0.80 $\pm$ 0.05	94 $\pm$ 8	1.0 $\pm$ 0.02	77 $\pm$ 8	2.7 $\pm$ 1.7	0.48 $\pm$ 0.07

^a Displacement of [³H]DAMGO (μ -selective) and [³H]DPDPE (δ -selective) using membrane preparations from transfected cells expressing rat μ -opioid receptor and human δ -opioid receptor, respectively

^b $\pm$ SEM

^c Reference compound: [³⁵S]GTP- γ -S

^d Net total bound/basal binding $\times$ 100 $\pm$ SEM

^e Data from Lipkowski et al. (1999)

^f Data from Misicka et al. (1997)

^g Data not present in the original paper

^h Radiolabeled binding and MVD GPI functional bioassays from Mollica et al. (2005)

reported and is analogous to that previously observed in the field of enkephalins (Toth et al. 1990). In particular, the pF-Phe containing biphalin analogue (see pF-Bph in Table 2), when compared to the parent, exhibits ten to two times higher affinity to δ - and μ -receptor types, respectively (Misicka et al. 1997).

Examination of the biological data reported in Table 2 confirms that, as already demonstrated (Mollica et al. 2005), the hydrazine bridge is not essential for binding and activity and that the pF substitution on the phenylalanine side chain maintains or improves the interaction of the ligands with both δ - and μ -opioid receptors.

When compared to biphalin, the fluorinated piperazinic derivative **10** shows improved binding values for both μ - and δ -receptors (0.11 vs. 1.4 nM and 0.09 vs. 2.6 nM, respectively). Furthermore, the GTP binding value (EC₅₀; 1.0 nM) reveals an extremely high capacity to trigger the transduction mechanisms with an efficacy three to six times higher than biphalin for both receptors (0.8 vs. 2.5 and 1.0 vs. 6.0, for δ - and μ -receptors, respectively). In particular, the $E_{\max}$ exhibited by **10** (94.1%) is, to the best of our knowledge, the highest value so far reported for a non-cyclic biphalin analogue. Table 2 shows that the new analogues **9** and **10** are both more active than biphalin: the IC₅₀ (nM) of **10** is in fact 1:10 for μ /GPI receptors (2.7 vs. 27) and ca. 1:20 for δ /MVD (0.48 vs. 8.8). The corresponding values of **9** are ca. 1:30 for μ /GPI (0.7 vs. 27) and ca. 1:2 for δ /MVD (3.7 vs. 8.8).

It should be noted that, when compared with native biphalin, the activity data of the non-fluorinated analogues **9a** and **10a** (Fig. 1; Table 2) are in general comparable to those of corresponding analogues **9** and **10**. The non-

fluorinated analogues, which share with **9** and **10** a non-hydrazine linker, show in fact greater binding affinities to both δ - and μ -receptors with respect to biphalin (Mollica et al. 2005). In analogue **10a**, which possesses a piperazine linker, the pF substitution improves all of the measured parameters and efficacy (see in vitro bioassays, binding assays, and GTP binding in Table 2). On the contrary, in the 1,2-phenylenediamine containing analogue **9a**, the pF substitution was detrimental for the δ binding assays (0.19 vs. 13) and three to four times better for μ binding assays (1.9 vs. 0.51). Concerning the GTP- γ -³⁵S binding assays, the pF substitution leads to a 1.2–2-fold drop in efficacy for both receptors ($E_{\max}$ (%) is 83 and 89 for **9a** and 71.6 and 43.8 for **9**).

In summary, the reported results indicate that the improvement of the activity due to the replacement of the native hydrazine linker and those derived from the pF substitution on Phe aromatic ring at positions 4,4' are in part additive and synergistic, in particular for the stimulation of the second messenger system. As data in Table 2 show, the piperazine linker, as compared to the 1,2-phenylenediamine linker, confirms more favourable properties in bridging the two palindromic arms of biphalin. The absence in the piperazine moiety of the two CO–NH groups, available for H-bond donor interactions as well as its more flexible and less planar structure, is probably at the basis of the different behaviours observed. The influence on the activity of the analogue **10** appears remarkable as the introduction of the piperazine link and the pF substitution leads to the most potent non-cyclic biphalin analogue so far described. This result can be only compared to that previously achieved with the synthesis of the cyclic

biphalin analogue containing a disulphide Cys-Cys bridge (Mollica et al. 2006) which shows a $E_{\max}$ (%) value of 100. In the case of the opioid peptide **10** described here, the synthesis of a linear potent full agonist which escapes the problems and low yields often associated with the cyclization reactions may be a valuable advantage.

Acknowledgments This study was supported in part by a grant from the USDHS, NIDA (DA06284).

Conflict of interest The authors declare that they have no conflict of interest.

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