Full Paper

Potent Biphalin Analogs with μ/δ Mixed Opioid Activity: *In Vivo* and *In Vitro* Biological Evaluation

Roberto Costante[1], Francesco Pinnen[1], Azzurra Stefanucci[2], and Adriano Mollica[1]

[1] Dipartimento di Farmacia, Università di Chieti-Pescara "G. d'Annunzio", Chieti, Italy
[2] Dipartimento di Chimica, "Sapienza" Università di Roma, Roma, Italy

Biphalin [(Tyr-D-Ala-Gly-Phe-NH-)$_2$] is an octapeptide with mixed μ/δ opioid activity. Its structure is based on two identical enkephalin-like portions linked "tail-to-tail" by a hydrazine bridge. This study presents the synthesis and *in vitro* and *in vivo* bioassays of two biphalin analogs that do not present the toxicity connected with the presence of the hydrazine moiety and are able to elicit a higher antinociceptive effect than biphalin.

Keywords: Analgesic / Antinociception / Biphalin / Intrathecal administration / Opioids

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Introduction

Biphalin is a potent opioid peptide agonist with a palindromic structure, composed of two enkephalin-like active fragments connected "tail-to-tail" by a hydrazine linker (Tyr-D-Ala-Gly-Phe-NH-NH<-Phe<-Gly<-D-Ala<-Tyr) (Fig. 1). Biphalin has been synthesized for the first time by Lipkowski et al. [1] and widely investigated by Hruby and coworkers [2–8]. Its dimeric structure greatly enhances the analgesic activity and the duration of the antinociceptive effect with respect to enkephalins probably due to a cooperative binding and a better enzymatic stability [9, 10].

After intrathecal (i.t) injection in mice biphalin produces intense analgesia [11] without showing toxicity [12]. Although only a small fraction of biphalin crosses the blood–brain barrier after intraperitoneal (i.p.) administration [13–15], it shows similar potency to that of morphine [16].

The tendency to induce less physical dependence than other opioid agonists [17–19] such as morphine represents an important feature of this dimeric neuropeptide, probably due to receptor selectivity and cooperative interactions among different opioid receptors [7]. Several chemical modifications have been performed in order to investigate the biphalin structure–activity relationships (SARs) and with the aim to obtain more potent and stable analogs [2–8, 10, 20–22].

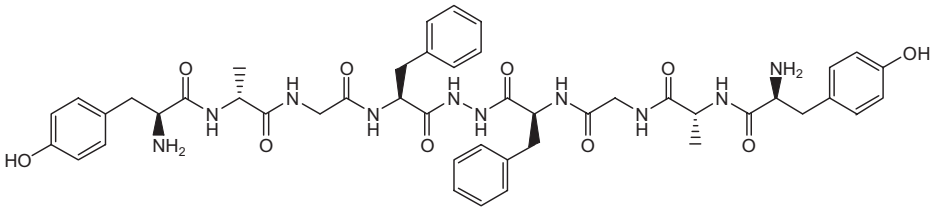
Different molecular fragments, such as piperazine, 1,2-phenylenediamine, and 1,4-phenylenediamine, were previously introduced as alternative linkers in place of hydrazine. The introduction of tertiary amide bonds, obtained by using a piperazine moiety as linker, and the 1,2-phenylenediamine, resulted to be the most favorable substitution and led to analogs with higher affinity and stability [4–6].

Encouraged by these results, as continuation of our works, we further investigated the *in vivo* and *in vitro* properties of **9** and **10**, two of the more promising compounds of the series (Fig. 2).

The peptides synthesis was improved with respect to the original work, obtaining better overall yields and avoiding the saponification step, which may lead to racemization as discussed below. The biological evaluation of the compounds has been enriched with *in vitro* [^{35}S]GTP-γ-*S* binding studies in presence of pertussin toxin (PTX) and *in vivo* through the tail-flick test, carried out in rats following intrathecal administration (i.t.).

Correspondence: Dr. Adriano Mollica, Dipartimento di Farmacia, Università di Chieti-Pescara "G. d'Annunzio", Via dei Vestini 31, 66100 Chieti, Italy
E-mail: a.mollica@unich.it
Fax: +39 0871 3554476

Abbreviations: DOR, δ-opioid receptor; **EDC,** 1-ethyl-(3-dimethylaminopropyl)-carbodiimide; **GPI,** guinea pig ileum; **GTP,** guanosine triphosphate; **[^{3}H]DAMGO,** [^{3}H]-[D-Ala(2), *N*-Me-Phe-(4), Gly-ol(5)] enkephalin; **[^{3}H]-DPDPE,** [^{3}H]-c[2-D-penicillamine,5-D-penicillamine] enkephalin; **HOBt,** 1-hydroxybenzotriazole; **i.p.,** intraperitoneal; **i.t.,** intrathecal; **MOR,** μ-opioid receptor; **MPE,** percentage of maximum effect; **MVD,** mouse vas deferens; **NMM,** *N*-methylmorpholine; **PTX,** pertussis toxin; **RP-HPLC,** reversed phase high performance liquid chromatography; **SEM,** standard error of measurement; **DMF,** *N,N*-dimethylformamide; **TFA,** trifluoroacetic acid.



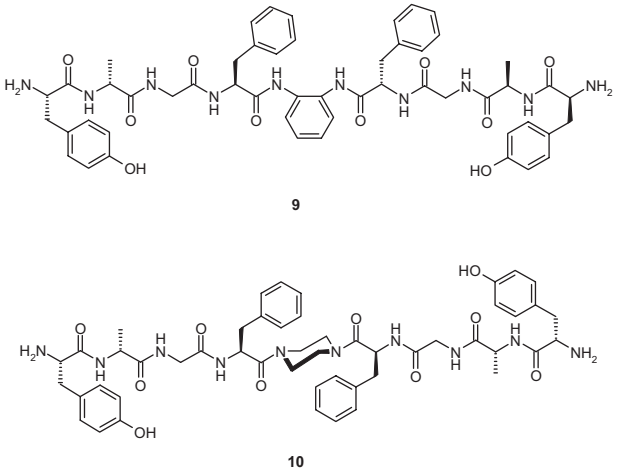
Biphalin

Figure 1. Representation of the biphalin molecular structure.

Results and discussion

Synthesis

The previous reported synthesis of compounds **9**–**10** involved the preparation of the tripeptide N^{α}-Boc-D-Ala-Gly-OEt by the asymmetric anhydride method [23] obtained with isobutyl chloroformate and TEA at −15°C [2]. In the next step, the tripeptide ethyl ester was hydrolyzed by 6 N NaOH in MeOH and coupled with 2TFA·$(Phe)_2$-1,2-phenylenediamine or 2TFA·$(Phe)_2$-1,2-piperazine, respectively, in order to obtain the Boc protected final products **8** and **7**. In order to avoid the possible partial loss of chirality due to the saponification step, and the low-yield reaction between the tripeptide and the bivalent portions, a different strategy was adopted, by using the standard coupling method HOBt/EDC/TEA in DMF to obtain the N^{α}-Boc protected octapeptides **7** and **8**. The synthesis was achieved by reacting 1,2-phenylenediamine or piperidine with repeated steps of coupling/purification/deprotection with the single amino acids, until the final products **9** and **10** were obtained as TFA salts (Scheme 1). This alternative method avoids the potential loss of chirality during the saponification step and allows an easier purification of the intermediates (i.e., by trituration in EtOAc) with high overall yields [24, 25].



9

10

Figure 2. Representation of the molecular structures of **9** and **10**.

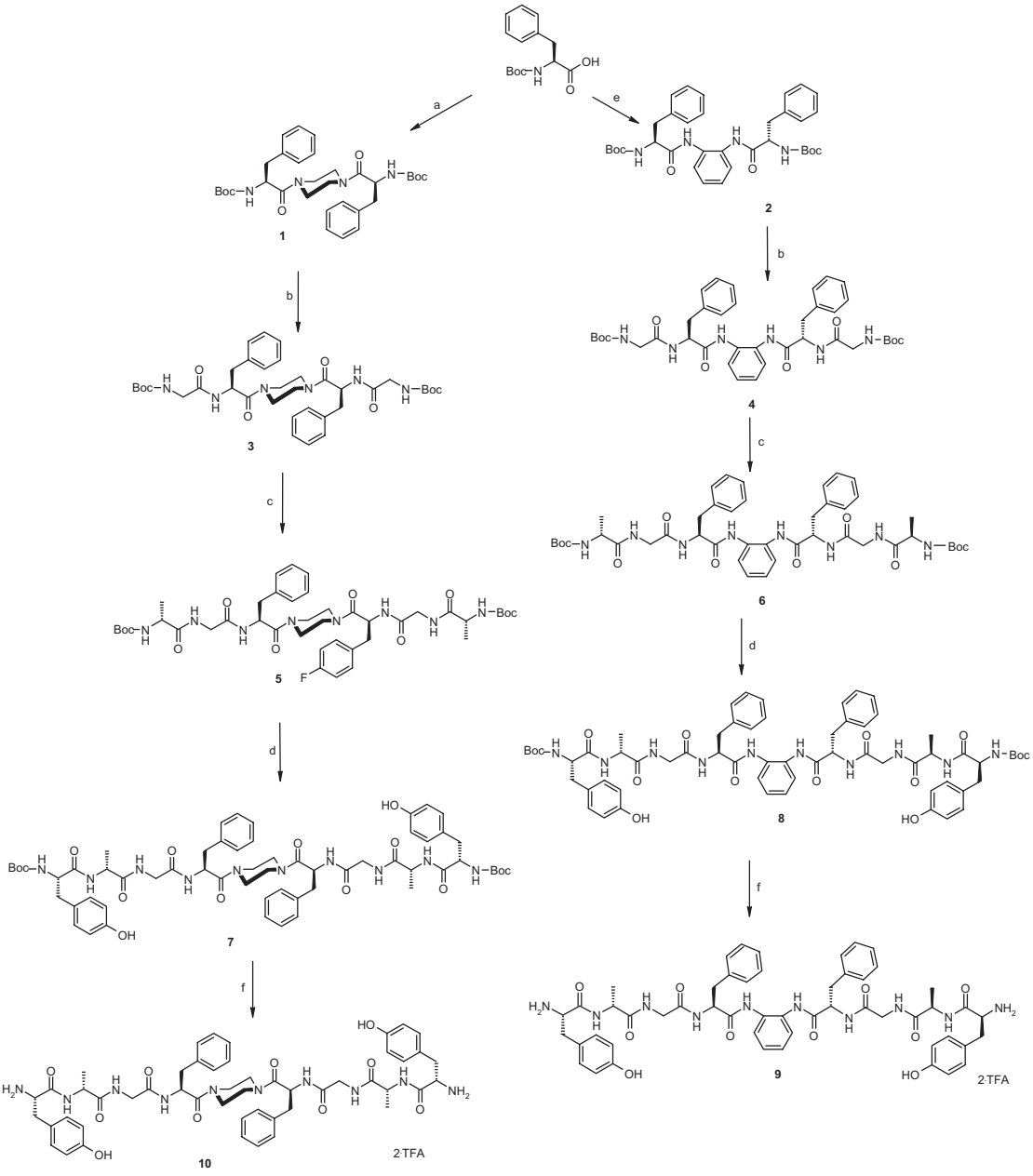
Biological results

In vitro evaluation

The binding affinity of compounds **9** and **10** and biphalin for μ and δ opioid receptors has been reported from [2] (Table 1).

Both analogs **9** and **10** show a good opioid receptor affinity, higher than biphalin, with subnanomolar affinity at the DOR and MOR. In particular, compound **10** has a high and similar affinity for DOR and MOR (0.65 and 0.48 nM, respectively), while **9** shows a discrete selectivity for DOR, with a K_i^{μ} 10-fold higher than K_i^{δ} (1.93 vs. 0.19 nM). Isolated tissue-based functional assay using guinea pig ileum/longitudinal muscle myenteric plexus (GPI) and mouse vas deferens (MVD) tissues previously reported by Mollica et al. [2] highlights that compound **9** is more efficacious toward δ opioid receptors than the μ receptor according to the binding assays pattern, whereas the compound **10** has the opposite behavior, in analogy with the parent peptide biphalin.

In order to complete *in vitro* evaluation, we have reported in Table 1 the [^{35}S]GTP-γ-*S* binding data from Mollica et al. [2], which display the capability of the two biphalin derivatives to induce the intracellular transduction of the signal in cells expressing hDOR (δ-receptor) or rMOR (μ-receptor) [4]. The GTP binding value (EC_{50}) reveals that **10** possesses a lower capacity to trigger the transduction mechanisms with respect to biphalin in particular at the DOR (44 vs. 2.5 nM), while analog **9** showed a transduction efficacy higher than parent compound in both opioid receptors (1.7 nM for DOR and 2.5 nM for MOR). The E_{max}% of both the analogs is higher than that of biphalin, in particular **9**, which may be considered as a full agonist while biphalin and **10** are partial agonists. To investigate the interaction of biphalin analogs with G_i-family G-proteins, the transduction of the signal induced by compounds **9** and **10** in MOR and DOR-expressing cells was evaluated after PTX overnight (18 h) treatment (100 ng/mL) [26]. This pretreatment strongly attenuated the inhibition of maximally effective concentrations of



Scheme 1. Synthesis of products **9** and **10**. Reagents and conditions: (a) EDC·HCl (2.2 eq.), HOBt (3.3 eq.), NMM (2.2 eq.), Boc-Phe-OH (2.2 eq.), piperazine (1 eq.) in DMF at r.t. overnight; (b) TFA/CH_2Cl_2 1:1, r.t. (1 h) then EDC·HCl (2.2 eq.), HOBt (2.2 eq.), NMM (3.3 eq.), Boc-Gly-OH (2.2 eq.), in DMF at r.t. overnight; (c) TFA/CH_2Cl_2 1:1, r.t. (1 h) then EDC·HCl (2.2 eq.), HOBt (2.2 eq.), NMM (3.3 eq.), Boc-D-Ala-OH (2.2 eq.) in DMF at r.t. overnight; (d) TFA/CH_2Cl_2 1:1, r.t. (1 h) then EDC·HCl (2.2 eq.), HOBt (2.2 eq.), NMM (3.3 eq.), Boc-Tyr-OH (2.2 eq.) in DMF at r.t. overnight; (e) EDC·HCl (2.2 eq.), HOBt (2.2 eq.), NMM (3.3 eq.), Boc-Phe-OH (2.2 eq.), 1,2-phenylenediamine (1 eq.) in DMF at r.t. overnight; (f) TFA/CH_2Cl_2 1:1, r.t. (1 h).

compounds **9** and **10** toward both receptors (Table 1), indicating that the *in vitro* activity of the analogs is receptor-mediated, dose-dependent, and involves substantially the $G_{i\alpha}/G_{o\alpha}$ subfamily of G-proteins [27, 28].

In vivo evaluation

The antinociceptive profile of compounds **9** and **10** was investigated using the rat tail-flick assay, following a local intrathecal (i.t.) administration. The potency of the analogs

Table 1. Binding affinity, GTP binding assay, E_{max} (%) (net total bound/basal binding × 100), and *in vitro* activity.

	Binding K_i[a),b),c)] (nM)		GTP binding[c),d)] (nM)						Bioassay[a),c),e)] IC_{50} (nM)	
Cpd	K_i^{δ}	K_i^{μ}	EC_{50}^{δ} (nM)[a)]	E_{max} (%)[a),f)]	E_{max}^{PTX} (%)[g)]	EC_{50}^{μ} (nM)[a)]	E_{max} (%)[a),f)]	E_{max}^{PTX} (%)[g)]	MVD	GPI
Bph[h)]	2.6 ± 0.4	1.4 ± 0.2	2.5 ± 0.5	27 ± 3	–	6.0 ± 0.02	25 ± 4	–	27 ± 1.5	8.8 ± 0.3
9	0.19 ± 0.04	1.93 ± 0.25	1.7 ± 0.4	85 ± 9	18 ± 6	2.5 ± 0.3	89 ± 8	14 ± 9	0.73 ± 0.2	39.5 ± 13
10	0.65 ± 0.35	0.48 ± 0.06	44 ± 5.9	56 ± 6	6 ± 2	13 ± 1.1	47 ± 4	7 ± 3	9.3 ± 0.4	2.5 ± 0.7

[a)] Previously reported [2, 4].
[b)] Displacement of [^{3}H]DAMGO (μ-receptor) and [^{3}H]DPDPE (δ-receptor).
[c)] ±SEM.
[d)] Reference compound [^{35}S]GTP-γ-S.
[e)] Concentration at 50% inhibition of muscle contraction in electrically stimulated isolated tissues.
[f)] Net total bound/basal binding × 100 ± SEM.
[g)] E_{max} ± SEM after 18 h cells pretreatment with PTX.
[h)] Ref. [10].

was then compared with biphalin and morphine. Co-administrations of compounds **9** and **10** together with morphine were also performed in order to investigate a possible synergic effect with non-peptide opioid drugs. The analgesic profile of the two derivatives and reference compounds is shown in Fig. 3.

Following i.t. administration in rats, **9** showed a very similar antinociceptive profile compared with biphalin and reached the maximum effect at 30 min after the injection.

In the same way, compound **10** showed in the first 20 min a similar potency with respect to biphalin, while it induced a higher and very persistent analgesia later: In particular, it reached the maximum efficacy from 30 to 45-min after the administration (99–100% MPE) and it showed the higher distance from biphalin at 45 min (99.3% vs. 48.6%). It is worth noting that, in this animal model, all peptides have a higher antinociceptive effect than morphine.

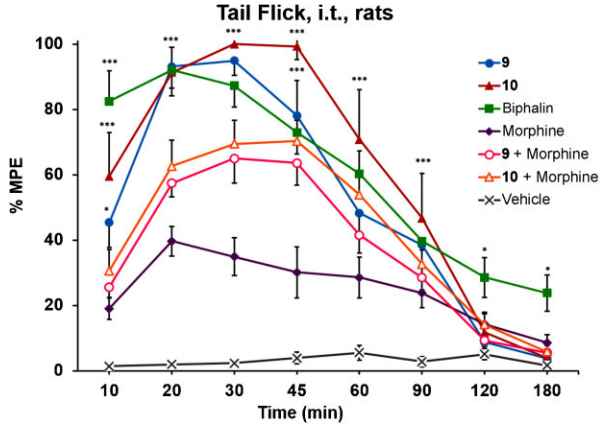


Figure 3. Time–response of the antinociceptive activity (tail-flick test) of morphine (7.8 nmol), biphalin (8.8 nmol), **9** (8.25 nmol), **10** (8.40 nmol), and co-administrations of **9** (4.1 nmol) and **10** (4.2 nmol) with morphine (3.9 nmol) following i.t. injection in rats ($n = 6$–8). The antinociceptive activity is expressed as percentage of the maximum possible effect (% MPE) ± SEM. *Statistical significance was assumed at $p < 0.05$ ($^*p < 0.05$; $^{***}p < 0.001$).

Although **9** reported the best results for δ-receptor in *in vitro* evaluation (K_i binding, MVD IC_{50}, and GTP EC_{50}), the **10**, which shows a good *in vitro* activity/affinity for both the receptors, resulted more efficacious in eliciting analgesia in thermal *in vivo* antinociception test. These results seem to confirm the role of the μ-receptor in eliciting analgesia and confirm that a well-balanced affinity for both the opioid receptors produces a greater antinociceptive response. In co-administration experiments, half the previous doses of compounds **9**–**10** were injected i.t. together with half the previous dose of morphine. No substantial additive effect was observed, with a weak increasing of the antinociceptive effect only 30 and 45 min after the administration.

Conclusions

This paper represents the continuation of our previous works [2, 4], with the aim to complete characterization of the antinociceptive profile of the **9** and **10**. Previously reported interaction with human opioid receptors (MOR and DOR) has been improved by *in vitro* PTX-[^{35}S]GTP-γ-S binding assay, **9** and **10** have been tested alone and in co-administration with morphine, through *in vivo* tail-flick test following i.t. administration in rats and compared with the parent peptide biphalin.

This work confirms the importance of mixed μ/δ agonists as a future alternative in treatment of severe pain, due to the synergistic activity of δ-receptor activation on μ opioid functional activities [29, 30].

The *in vivo* hot plate test reveals that products **9** and **10** are capable of eliciting a similar or improved antinociceptive

effect than the parent compound biphalin. While analogue **9** has a very similar *in vivo* profile in comparison with biphalin, compound **10** showed a higher efficacy. This is probably due to the presence of the piperazine linker, which strongly influences the conformational rigidity of the molecule, in particular with an increased affinity for μ receptor. The absence of an *in vivo* synergic effect after combination of half-doses of **9** and **10** with morphine and the loss of *in vitro* activity after cells pretreatment with PTX confirm that the analgesic activity of these compound is due to μ/δ receptors activation through G protein pathway [31].

In conclusion, compounds **9** and **10** are good candidates for further studies, since they have shown some improvements versus native biphalin: (i) a better capability to trigger the transduction mechanisms; (ii) an increased antinociceptive effect, and (iii) reduced toxicity connected with the absence of the hydrazine linker [32–34].

Experimental

Chemistry

Synthesis of all new analogs was performed in solution phase, using the N^{α}-Boc strategy. All synthesis began with the appropriate diamine, performing repeated steps of coupling/purification/deprotection of the intermediate products, until the final products were obtained as TFA salts (Scheme 1). All coupling reactions were performed with the standard method of HOBt/EDC/NMM in DMF [8]. Deprotection of N^{α}-*tert*-butyloxycarbonyl group was performed using TFA/CH_2Cl_2 1:1 for 1 h, under nitrogen atmosphere. The intermediate TFA salts were used for subsequent reactions without further purification. Boc protected intermediate products were purified by silica gel column chromatography, or in case of scarcely soluble products, the purification was performed by trituration in EtOAc. Final products **9** and **10** were purified by RP-HPLC using a Waters XBridge Prep BEH130 C_{18}, 5.0 μm, 250 mm × 10 mm column at a flow rate of 4 mL/min on a Waters Binary pump 1525, using as eluent a linear gradient of H_2O/acetonitrile 0.1% TFA ranging from 5% acetonitrile to 90% acetonitrile in 45 min. The purity of the N^{α}-Boc-protected products was confirmed by NMR analysis on a Varian VXR 300 MHz and mass spectrometry ESI-HRMS. The purity of final TFA salts was confirmed by NMR analysis, ESI-HRMS and by analytical RP-HPLC (C_{18}-bonded 4.6 × 150 mm) at a flow rate of 1 mL/min using as eluent a gradient of H_2O/acetonitrile 0.1% TFA ranging from 5% acetonitrile to 95% acetonitrile in 50 min and was found to be not <95%.

*(Boc-Phe)$_2$-piperazine (**1**)*

EDC·HCl (2.2 eq.), HOBt (2.2 eq.), and NMM (3.3 eq.) were added to a solution of Boc-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, $NaHCO_3$ 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, in 95% yield. R_f 0.56 (EtOAc). The product was used for the next step without further purification. ^{1}H NMR (DMSO-d_6, 300 MHz) (δ, ppm): 1.32 [18H, s, $(CH_3)_3$–]; 2.75–2.89 [4H, m, Phe β-CH_2]; 2.86–2.93 [8H, m, –CH_2–CH_2– Pip]; 4.57 [2H, m, Phe α-CH]; 7.15 [2H, br, Phe NH]; 7.18–7.26 [10H, m, aromatics]. HR-MS (ESI) calcd. for $C_{32}H_{44}N_4O_6$ *m/z*: 581.334 $[M+H]^+$; found 581.342.

*(Boc-Phe)$_2$-1,2-phenylenediamine (**2**)*

EDC·HCl (2.2 eq.), HOBt (2.2 eq.), and NMM (3.3 eq.) were added to a solution of Boc-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, $NaHCO_3$ 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 0.54 (CH_2Cl_2/EtOAc 2:1). The product was purified by silica gel column chromatography (CH_2Cl_2/EtOAc 98:2 to $CHCl_3$/EtOAc 90:10) to obtain the pure product **2** (88%). ^{1}H NMR (DMSO-d_6, 300 MHz) (δ, ppm): 1.30 [18H, s, $(CH_3)_3$–]; 2.88–2.95 [4H, m, Phe β-CH_2]; 4.40 [2H, m, Phe α-CH]; 7.05 [2H, d, Phe NH]; 7.15–7.55 [14H, m, aromatics]; 9.46 [2H, s, Ar NH]. HR-MS (ESI) calcd. for $C_{34}H_{42}N_4O_6$ *m/z*: 603.318 $[M+H]^+$; found 603.330.

*(Boc-Gly-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 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 NMM (3.3 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, then TFA·Phe-Pip-Phe·TFA (1 eq.) was added, and 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. The solid was dried under reduced pressure and triturated with diethyl ether to yield the desired product **3** as pure white solid (93%). R_f 0.63 (EtOAc/MeOH 9:1). ^{1}H NMR (DMSO-d_6, 300 MHz) (δ, ppm): ^{1}H NMR (DMSO-d_6, 300 MHz) (δ, ppm): 1.32 [18H, s, $(CH_3)_3$–]; 2.75–2.90 [4H, m, Phe β-CH_2]; 2.86–2.93 [8H, m, –CH_2–CH_2– Pip]; 3.55 [4H, m, Gly β-CH_2]; 4.50 [2H, m, Phe α-CH]; 6.90 [2H, t, Gly NH]; 7.20–7.32 [10H, m, aromatics]; 8.25 [2H, br, Phe NH]. HR-MS (ESI) calcd. for $C_{36}H_{50}N_6O_8$ *m/z*: 695.377 $[M+H]^+$; found 695.381.

*(Boc-Gly-Phe)$_2$-1,2-phenylenediamine (**4**)*

Product **2** was deprotected at the N^{α} terminal by TFA in DCM 1:1 using 1 mL of mixture per 100 mg of the Boc protected product for 1 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.), NNM (3.3 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·Phe-1,2-phenylenediamine-Phe·TFA (1 eq.) was added, and 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, and then the solid dried under reduced pressure and triturated with diethyl ether to give the desired product **4** as a pure white solid. (97%). R_f 0.66 (EtOAc). ^{1}H NMR (DMSO-d_6, 300 MHz) (δ, ppm): 1.32 [18H, s, $(CH_3)_3$–]; 2.88–2.95 [4H, m, Phe β-CH_2]; 3.50 [4H, m, Gly β-CH_2]; 4.65 [2H, m, Phe α-CH]; 6.85 [2H, t, Gly NH]; 7.15–7.55 [14H, m, aromatics]; 8.40 [2H, d, Phe NH]; 9.46 [2H, s, Ar NH]. HR-MS (ESI) calcd. for $C_{38}H_{48}N_6O_8$ *m/z*: 717.361 $[M+H]^+$; found 717.369.

*(Boc-D-Ala-Gly-Phe)$_2$-piperazine (**5**)*

Product **3** was deprotected at the N^{α} terminal by TFA in DCM 1:1 using 1 mL of the mixture per 100 mg of Boc protected product for 1 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 NMM (3.3 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-Phe-Pip-Phe-Gly·TFA (1 eq.) was added, and 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, and then dried under reduced pressure and triturated with diethyl ether to give the desired product **5** as a pure white solid (80%). R_f 0.22 (EtOAc/CH_3OH 9:1). ^{1}H NMR (DMSO-d_6, 300 MHz) (δ, ppm): 1.32 [18H, s, $(CH_3)_3$–]; 2.75–2.90 [4H, m, Phe β-CH_2]; 2.86–2.93 [8H, m, $-CH_2-CH_2-$ Pip]; 3.65 [4H, m, Gly β-CH_2]; 4.52 [2H, m, Phe α-CH]; 6.90 [2H, br, D-Ala α-CH]; 7.20–7.32 [10H, m, aromatics]; 8.15 [2H, t, Gly NH]; 8.24 [2H, br, Phe NH]. HR-MS (ESI) calcd. for $C_{42}H_{60}N_8O_{10}$ *m/z*: 837.451 $[M+H]^+$; found 837.460.

*(Boc-D-Ala-Gly-Phe)$_2$-1,2-phenylenediamine (**6**)*

Product **4** 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 NMM (3.3 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-Phe-1,2-phenylenediamine-Phe-Gly·TFA (1 eq.) was added, and 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 pure white solid (92%). R_f 0.70 (EtOAc/MeOH 9:1). ^{1}H NMR (DMSO-d_6, 300 MHz) (δ, ppm): 1.16 [6H, m, Ala CH_3]; 1.34 [18H, s, $(CH_3)_3$–]; 2.78–3.05 [4H, m, Phe β-CH_2]; 3.70 [4H, m, Gly β-CH_2]; 4.00 [2H, m, D-Ala α-CH]; 4.60 [2H, m, Phe α-CH]; 6.80 [2H, d, D-Ala NH]; 7.15–7.55 [14H, m, aromatics]; 8.15 [2H, t, Gly NH]; 8.35 [2H, d, Phe NH]; 9.50 [2H, s, Ar NH]. HR-MS (ESI) calcd. for $C_{44}H_{58}N_8O_{10}$ *m/z*: 859.435 $[M+H]^+$; found 859.443.

*(Boc-Tyr-D-Ala-Gly-Phe)$_2$-piperazine (**7**)*

Product **5** 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 NMM (3.3 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-Phe-Pip-Phe-Gly-D-Ala·TFA (1 eq.) was added, and 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 by silica gel column chromatography (CH_2Cl_2/EtOAc 20:80 to EtOAc/MeOH 86:14) to obtain the pure product **7** (82%). R_f 0.3 (EtOAc/MeOH 85:15). ^{1}H NMR (DMSO-d_6, 300 MHz) (δ, ppm): 1.08 [6H, d, Ala CH_3]; 1.38 [9H, s, $(CH_3)_3$–]; 2.84–3.29 [8H, m, Phe β-CH_2 and Tyr β-CH_2]; 3.65–3.89 [8H, m, $-CH_2-CH_2-$]; 3.71 [4H, m, Gly CH_2]; 4.15 [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]; 7.28–6.68 [18H, m, aromatics]; 8.05 [4H, br, Phe NH and D-Ala]; 8.22 [2H, t, Gly NH]; 9.20 [2H, s, Tyr OH]. HR-MS (ESI) calcd. for $C_{60}H_{78}N_{10}O_{14}$ *m/z*: 1163.578 $[M+H]^+$; found 1163.585.

*(Boc-Tyr-D-Ala-Gly-Phe)$_2$-1,2-phenylenediamine (**8**)*

Product **6** 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 NMM (3.3 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-Phe-1,2-phenylenediamine-Phe-Gly-D-Ala·TFA (1 eq.) was added, and 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. The product was purified by silica gel column chromatography (EtOAc/$CHCl_3$ 80:20 to EtOAc/MeOH 95:5) to obtain the pure product **8** (79%). R_f 0.4 (EtOAc/MeOH 9:1). ^{1}H NMR (DMSO-d_6, 300 MHz) (δ, ppm): 1.22 [6H, d, Ala CH_3]; 1.30 [18H, s, $(CH_3)_3$–]; 2.65 and 2.90 [2H, m, Tyr β-CH_2]; 2.70 and 3.18 [4H, m, Phe β-CH_2]; 3.75 [4H, m, Gly CH_2]; 4.10 [2H, m, Tyr α-CH]; 4.28 [2H, m, D-Ala α-CH]; 4.68 [2H, m, Phe α-CH]; 6.62 and 7.01 [8H, dd, Tyr aromatics]; 6.78 [2H, d, Tyr NH]; 7.15–7.35 [14H, m, aromatics]; 8.05 [2H, d, D-Ala NH]; 8.19 [2H, t, Gly NH]; 8.21 [2H, d, Phe NH]; 9.15 and 9.45 [4H, two singlets, Tyr OH and Ar NH]. HR-MS (ESI) calcd. for $C_{62}H_{76}N_{10}O_{14}$ *m/z*: 1185.562 $[M+H]^+$; found 1185.575.

*TFA·(Tyr-D-Ala-Gly-Phe)$_2$-1,2-phenylenediamine (**9**)*

Product **8** 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 purified on RP-HPLC to give the pure product **9** (quantitative). R_f 0.7 (*n*-Bu-OH/CH_3COOH/H_2O 8:1:1). ^{1}H NMR (DMSO-d_6, 500 MHz) (δ, ppm): 1.05 [6H, m, Ala CH_3]; 2.80–3.10 [8H, m, Tyr β-CH_2 and Phe β-CH_2]; 3.75 [4H, m, Gly CH_2]; 4.00 [2H, m, Tyr α-CH]; 4.32 [2H, m, D-Ala, α-CH]; 4.68 [2H, m, Phe α-CH]; 6.70–7.50 [22H, m, aromatics]; 8.06 [6H, br, Tyr NH_3^+]; 8.20 [4H, br, Gly NH and Phe NH]; 8.50 [2H, br, D-Ala NH]; 9.30 and 9.55 [4H, two singlets, Tyr OH and Ar NH. HR-MS (ESI) calcd. for $C_{52}H_{60}N_{10}O_{10}$ *m/z*: 985.457 $[M+H]^+$; found 985.462.

*TFA·(Tyr-D-Ala-Gly-Phe)$_2$-piperazine (**10**)*

Product **7** 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 purified on RP-HPLC to give the pure product **10** (quantitative). R_f 0.5 (*n*-Bu-OH/CH_3COOH/H_2O 8:1:1). ^{1}H NMR (DMSO-d_6, 500 MHz) (δ, ppm): 1.08 [6H, m, Ala CH_3]; 2.98–2.77 [8H, m, Tyr β-CH_2 and Phe β-CH_2]; 3.35 [8H, m, piperazine protons under the water signal]; 3.68 [4H, m, Gly CH_2]; 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 [18H, 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]. HR-MS (ESI) calcd. for $C_{50}H_{62}N_{10}O_{10}$ *m/z*: 963.473 $[M+H]^+$; found 963.477.

In vitro biological assays

GTP binding assay

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 [^{35}*S*]GTP-γ-*S* (1000–1500 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 fiber filters, followed by four washes with ice-cold 15 mM Tris/120 mM NaCl, pH 7.4. Filters were pretreated 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 analyzed using GraphPad Prism Software (San Diego, CA). For PTX control test, cells were treated overnight (18 h) with PTX at concentration of 100 ng/mL and were washed twice with ice-cold phosphate-buffered saline following the procedure described by Clark et al. [35] before incubation with test compounds as described above.

In vivo nociception tests

Animals

Male adult Wistar rats (200–250 g) were used for all experiments. Animals were housed in plexiglass cages, (40 cm × 25 cm × 15 cm), in climatized colony rooms (22 ± 1°C; 60% humidity), on a 12 h/12 h light/dark cycle (light phase: 07:00–19:00 h), with free access to tap water and food. Housing conditions and experimental procedures were strictly in accordance with the European Community ethical regulations for the use and care of animals for scientific research (European Economic Community Council 89/609; Italian D.L. 22-1-92 No. 116), and were approved by the Ethical Committee on Animal Health and Care of G. d'Annunzio University (Project n. 35, prot. N. 2913, 17/09/2007).

Surgery for i.t. injections

Rats were implanted with chronic indwelling intrathecal catheters using a modification [36] of the method of Yaksh and Rudy [37]. Rats were anesthetized with 10% isoflurane. Catheters were made of silastic tubing (ID = 0.30 mm; OD = 0.64 mm) and had a dead volume of 10 μL. Catheters measured a total of 12 cm with 7.5 cm inserted into the intrathecal space to the level of T13-L1. The catheter was inserted through the atlanto-occipital membrane and into the intrathecal space using a guide wire. Sutures were used to secure the placement of the catheter. All rats were allowed to recover from the surgery for 3 days. Rats exhibiting any sign of neurological or motor impairment (paralysis, abnormal gait, weight loss, or negligent grooming) were excluded from the experiment. Seventy hours after surgery, peptides solutions were freshly prepared using saline containing 0.9% NaCl and rats (6–8 per group) were injected i.t. with biphalin (8.8 nmol), **9** (8.25 nmol), **10** (8.40 nmol), morphine sulfate (7.8 nmol), saline (control rats), and **9** (4.10 nmol) and **10** (4.20 nmol) together with morphine sulfate (3.9 nmol) in a volume of 5 μL. After completion of drug testing, the catheter position was verified in each animal by injection of 20 μg of biphalin. Animals that did not show transient, opioid-induced rigidity of the hind paws were excluded from analyses.

Data analysis and statistics

Experimental data were expressed as mean ± SEM. The significance among groups was evaluated with the analysis of variance (one-way ANOVA) followed by Student's *t*-test or Bonferroni's post hoc comparisons using the statistical software SPSS. Statistical significance was assumed at $p < 0.05$ ($^*p < 0.05$; $^{***}p < 0.001$). Statistical analysis was performed using GraphPad Prism Software (GraphPad Software, San Diego, CA).

The authors have declared no conflict of interest.

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