Letter

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Novel cyclic biphalin analogues by Ruthenium-catalysed ring closing metathesis: *in vivo* and *in vitro* biological profile

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ABSTRACT: In this work we report the application of the Ring-Closing Metathesis (RCM) to the preparation of two cyclic olefin bridged analogues of biphalin (Tyr-D-Ala-Gly-Phe-NH-NH<-Phe<-Gly<-D-Ala<-Tyr), using the second generation Grubbs' catalyst. The resulting *cis* and *trans* cyclic isomers were identified, fully characterized and tested *in vitro* at μ (MOR), δ (DOR) and κ (KOR) opioid receptors and *in vivo* for antinociceptive activity. Both were shown to be full agonists at MOR and potential partial antagonists at DOR, with low potency KOR agonism. They also share a strong antinociceptive effect after intracerebroventricular (i.c.v.) and intravenous (i.v.) administration, higher than that of the cyclic biphalin analogues containing a disulfide bridge between the side chains of two D-Cys or D-Pen residues, previously described by our group.

Peptide cyclization represents a common strategy for the development of compounds with enhanced conformational stability, compared to their linear analogues. Cyclic peptides may act as mimetics of protein secondary structures, and are often able to freeze the molecule in the bioactive conformation, leading to the optimization of ligand's activity in terms of binding affinity, potency, selectivity and protease stability.[1,2] Disulfide-bridge incorporation into a linear peptide allows the design of synthetic models characterized by the limitation of conformational flexibility to form a well-defined secondary structure, but the disulfide bond itself is labile and sensitive to the activity of reductases, and can be cleaved by various nucleophiles. Moreover, the stereoelectronic hindrance of sulfur atoms' lone pairs guide the geometry of the bond fixing the dihedral angle C-S-S-C at a value of ±90°.[3]

In searching for more stable and versatile products, the replacement of the labile S-S junction in cyclic peptides with an uncleavable C-C bond appears to be a promising strategy. Dicarba-analogues of oxytocin incorporating a 1,6-α,α'-diamino-suberic acid residue, in place of the cysteine residue,[4,5] and cyclic olefin analogues prepared starting from linear D-allylglycine containing peptides,[6] are notable applications of this strategy. In the field of opioid peptide analogues, the incorporation of a C-C bond into a cyclic structure resulted in highly active and potent δ/μ analogues of H-Tyr-*c*[D-Cys-Gly-Phe-Cys]-OH, with increased chemical and biological stability compared to the disulfide counterparts.[7] The cyclization reaction utilized ruthenium-catalyzed ring-closing olefin metathesis (RCM), affording *cis* and *trans* olefin analogues of enkephalins, that resulted in metabolic stability and rigid conformation.[8]

Despite the amount of literature reporting the use of RCM in peptide chemistry,[9] and a great variety of enkephalin derivatives,[10] this cyclization strategy has not yet been proven on bivalent analogues such as biphalin. Biphalin is an octapeptide characterized by two enkephalin-derived pharmacophores joined together by a hydrazine bridge; biphalin is able to bind μ and δ-opioid receptors with an EC_{50} of 1-5 nM, with a strong antinociceptive effect associated with few side-effects and low dependence upon chronic use.[11] This profile makes biphalin a strong lead compound for further modification. The combination of the entropic factors related to the two pharmacophore branches in biphalin with the favorable properties of a cyclic compound, could result in the design of more stable and potent opioid peptide analogues.

Indeed, our research group described a series of cyclic biphalin analogues containing a disulfide bridge between the side chains of Cys and D-Pen residues in place of the D-Ala amino acid,[12] different xylene isomers,[13] and a fluorescein-maleimide moiety (Figure 1).[14] The first two classes of compounds are characterized by mixed μ/δ-opioid agonism, while the analogue containing the *o*-xylene moiety shows an improved human plasma stability compared to biphalin. While an improvement, these compounds may still be

limited in their pharmacokinetic (PK) properties by the labile sulfur bonds. We then hypothesized that cyclic biphalin analogues with a covalent C-C bond would further improve the PK properties of our earlier generations of cyclic biphalin analogues. Thus in this work we describe the synthesis of cyclic analogues of biphalin in which the D-Ala amino acids in position 2,2' have been replaced with D-allylglycine (D-allyl-Gly) residues to promote cyclization mediated by the RCM reaction.

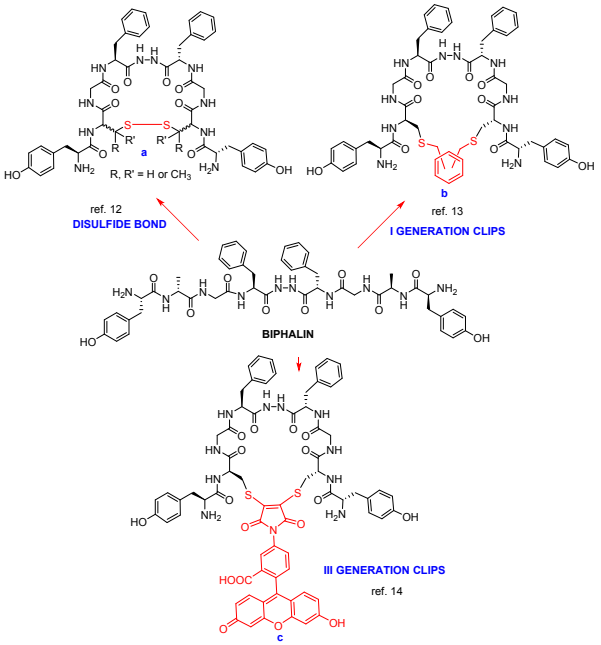


Figure 1. Cyclic biphalin analogues prepared via different strategies: (a) disulfide bond containing analogues,[12] (b) xylene moiety containing analogue via I generation CLIPS technology,[13] (c) fluorescein-maleimide containing analogue via III generation CLIPS technology.[14]

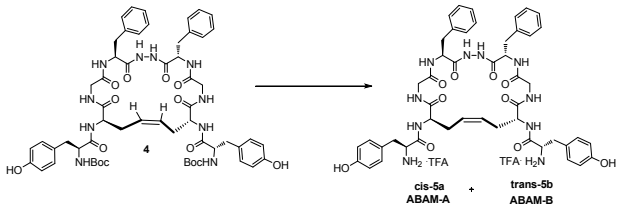


Figure 2. Structures of **5a (ABAM-A), 5b (ABAM-B)** and Boc-protected precursor **4.**

Two cyclic geometric isomers of biphalin *cis*-5a and *trans*-5b (Figure 2) were separated and purified by RP-HPLC, then characterized by NMR analysis and identified by HRMS. The *in vitro* binding affinity and G protein stimulation on μ (MOR), δ (DOR) and κ (KOR) opioid receptors combined with *in vivo* antinociceptive efficacy were then evaluated.

RESULTS AND DISCUSSION

The linear intermediate peptides were prepared in solution using N^{α}-Boc strategy, EDC·HCl/anhydrous HOBt/NMM in DMF for coupling reactions and a solution of TFA/DCM = 1:1 for Boc deprotection (Scheme 1S, see SI).[15] All the intermediate products were purified by trituration in diethyl ether and then characterized by LRMS and NMR spectroscopy (see SI). For the preparation of the cyclic Boc-protected analogue **4** via RCM, the reaction conditions were extensively optimized; Ruthenium carbene complexes are easier to prepare and handle than molybdenum complexes. Their remarkable stability to air, water, acid and functional group tolerance render these group of catalysts suitable for applications in peptide chemistry.[16,17] We first explored RCM using Zhan-1B catalyst and Grubbs I generation under several reaction conditions,[18] leading to major recovery of the starting material and undetermined byproducts, probably due to the low solubility of the linear compound in the reaction solvent. The desired cyclic product **4** was finally obtained in good yield as a mixture of *cis*/*trans* isomers, using the more stable and reactive Grubbs II generation catalyst in DCE under reflux for 78 h. The ring closure was achieved with a catalytic amount of catalyst (10%) to a solution of peptide **3** in DCE at reflux, monitoring by TLC (AcOEt/MeOH = 97:3) until reaction completion.

The mixture of the Boc protected cyclic products is inseparable by C18-HPLC, thus the two isomeric peptides *cis*-**5a** (**ABAM-A**) and *trans*-**5b** (**ABAM-B**) were obtained by HPLC separation after Boc removal, in about 1.4:1 ratio, respectively. The *cis* and *trans* isomers of the TFA salts were clearly distinguishable in preparative C-18 column showing different retention times, thus they were separated and characterized. The two novel compounds were obtained in good overall yields (52% for *cis*-**5a** and 36% for *trans*-**5b**) and ≥ 95% purity following analytical RP-HPLC. Because of the symmetry of the two isomers, the analysis of the J coupling constant of the olefin group was not possible. Thus in this case the assignment was based on the chemical shift of the carbon in alpha position to the double bond (Cβ, Table 1S see SI). Their chemical shifts differ markedly by around 5 ppm, due to the gauche γ-substituent effect and permit identification of **ABAM-B** as the *trans* isomer and **ABAM-A** as the *cis* isomer.[19,20]

Competition radioligand binding tests were then performed against the non-selective opioid antagonist [^{3}H]-Diprenorphine to measure compound affinity for opioid receptors: K_i values for **ABAM-A** and **ABAM-B** are 75.8 and 53.6 nM respectively at MOR and 7.3 and 5.2 nM for DOR, while both have low affinity for KOR (495 and 686 nM) (Figure 3S, Table 1). The compounds are modestly selective for DOR>MOR>>>KOR, and there is no apparent difference between the two **ABAM** compounds in their binding profile.

We then tested the **ABAM** compounds for their functional activity at the opioid receptors using ^{35}S-GTPγS coupling (Figure 4S, Table 2). Both compounds showed full efficacy and high potency at the MOR, with a very similar profile to the prototypical high efficacy full agonist DAMGO. The compounds further showed a higher potency than binding affinity (Table 1), suggesting that the compounds possess high intrinsic efficacy at the MOR. Interestingly, the compounds showed no detectable agonist activity at the DOR, despite the high binding affinity detected in Table 1. The prototypical DOR agonist SNC80 exerted the expected activity, validating the assay. Lastly, the compounds showed full efficacy but low potency agonist activity at the KOR, in line with the low binding affinity of the compounds for the KOR.

Both **ABAM** compounds showed high DOR affinity (Table 1) but no DOR agonist activity (Table 2), suggesting that the compounds could be antagonists for the DOR. We thus tested the antagonist activity of the **ABAM** compounds vs. 100 nM SNC80 at the DOR using ^{35}S-GTPγS coupling (Figure 3). Intriguingly, both compounds showed potent (44.8 nM for **ABAM-A** and 0.36 nM for **ABAM-B**) but partial (25.6% and 27.3%) antagonist activity at the DOR. The positive control antagonist naloxone displayed full (100%) antagonist activity, validating the assay. These

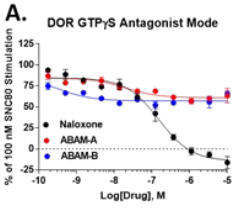
results are unexpected; as weak partial antagonism would classically be correlated with relatively high efficacy partial agonism. However, the ABAM compounds showed no detectable partial agonist activity (Table 2). We further confirmed the lack of **ABAM** antagonist activity at the DOR using a forskolin-induced cAMP assay; neither **ABAM** compound produced antagonism, while the selective DOR antagonist naltrindole did (Figure 5S, SI).

Ligands	Compounds K_i (nM)		
	MOR	DOR	KOR
Naloxone	51.2 ± 2.5	70.0 ± 2.9	-
U50,488	-	-	23.9 ± 4.2
ABAM-A	75.8 ± 11.9	7.3 ± 0.7	495 ± 43
ABAM-B	53.6 ± 2.7	5.2 ± 2.7	686 ± 53

Table 1. Opioid receptor affinity of **ABAM-A** and **ABAM-B**. **ABAM** compounds or naloxone (MOR, DOR) or U50,488 (KOR) positive control were competed against ^{3}H-diprenorphine in membranes from CHO cells expressing one of the human opioid receptors. Data reported as the mean ± SEM of N = 3 independent experiments. The affinity values (K_i) are shown for all compounds, calculated separately from each experiment and reported as the mean ± SEM. The naloxone and U50,488 positive controls showed expected values at the receptors, validating the assays.

Ligands	MOR		DOR		KOR	
	EC_{50} (nM)	E_{MAX} (%)	EC_{50} (nM)	E_{MAX} (%)	EC_{50} (nM)	E_{MAX} (%)
DAMGO	19.7 ± 3.4	100	-	-	-	-
SNC80	-	-	210 ± 69	100	-	-
U50,488	-	-	-	-	7.8 ± 0.4	100
ABAM-A	19.6 ± 4.7	91.0 ± 3.0	NC	NC	1843 ± 169	69.0 ± 3.6
ABAM-B	17.7 ± 2.6	85.7 ± 2.6	NC	NC	969 ± 64	73.3 ± 0.3

Table 2. Opioid receptor functional activity of **ABAM-A** and **ABAM-B**. The functional agonist activity of the compounds was measured using ^{35}S-GTPγS coupling in the membranes of CHO cells expressing one of the human opioid receptors. Data reported as the mean ± SEM of N = 3 independent experiments. The data for each set of assays was normalized to the maximum stimulation (100%) caused by positive control agonist (DAMGO for MOR; SNC80 for DOR; U50,488 for KOR) or vehicle (0%). The potency (EC_{50}) and efficacy (E_{MAX}) of each compound from each receptor calculated separately from each experiment and reported as the mean ± SEM. Positive control agonists behaved as expected, validating the assays. The E_{MAX} was normalized to the maximum stimulation (100%) caused by positive control agonist.



B.

Ligands	GTPγS Coupling at DOR - Antagonist	
	IC_{50} (nM)	I_{Max} (%)
Naloxone	146 ± 12	100
ABAM-A	44.8 ± 27.9	25.6 ± 2.7
ABAM-B	0.36 ± 0.13	27.3 ± 2.2

Figure 3. DOR antagonist activity of **ABAM-A** and **ABAM-B**. **ABAM** compounds or naloxone positive control was competed against 100 nM of SNC80 at the DOR using ^{35}S-GTPγS coupling. Data was normalized to the stimulation caused by 100 nM SNC80 (100%) or vehicle (0%) and reported as the mean ± SEM of N = 3 independent experiments. **A)** The summary curves combined from each independent experiment are shown. **B)** The antagonist potency (IC_{50}) and efficacy (I_{MAX}) was calculated separately from each experiment and reported as the mean ± SEM. The I_{MAX} of each compound was normalized to the inhibition caused by the positive control antagonist naloxone (100%). It is unclear why **ABAM** compounds show this profile at DOR, which could include multiple explanations. The **ABAM** compounds also show their strongest potency difference in this assay, with **ABAM-B** showing a ~100-fold potency improvement over **ABAM-A**. This difference was not observed in the other assays, and the compounds should be further investigated for their unique molecular pharmacology at DOR. Also of note, these results show a strong difference from the profile of biphalin, which is a high potency full agonist at both MOR and DOR. These results suggest that the ligands exhibit high potency as MOR agonist/DOR partial antagonist compounds, with a binding affinity for DOR higher than that of MOR with very low binding affinity and potency for KOR. This profile is in contrast with the cyclic amide analogues of enkephalins described by Purington,[21] Prydzial,[22] and Schiller,[23] for which a high binding affinity has been demonstrated for all three opioid receptors. In particular, peptide Tyr-*c*[D-Cys-Phe-2-Nal-Cys]NH_2 exerted agonism at MOR and KOR, while displaying antagonism at DOR in the GTPγS binding assay;[21] our novel compounds have lost the high potency KOR agonism exhibiting a biological profile that closely resemble that of the KSK-103 compound.[24] Notably, the two geometrical isomers do not show any significant difference in their opioid profile with the exception of their DOR antagonist potency (Figure 3). Encouraged by these data, the antinociceptive effect of **ABAM-A** and **ABAM-B** was measured by the hot plate test in mice after i.v. and i.c.v. administration (Figure 4). Biphalin was used as a reference compound, with vehicle administration as a negative control. **ABAM-B** produced an MPE% ≥ 50% that lasted over 60 min after i.c.v. and 45 min after i.v. administration, implying a longer lasting analgesic effect than that of **ABAM-A**, which fell under the 50% mark at both of these timepoints. Both compounds exerted a strong analgesic effect higher than that of biphalin after i.c.v. and i.v. administration; this result suggests that both **ABAM** ligands are resistant to enzymatic degradation and are also able to cross the BBB to reach the CNS. Analogous behavior has been observed previously with other biphalin analogues published by our research group.[12,13] These results support our hypothesis that replacement of the labile disulfide link in the cyclic analogs further improves on the *in vivo* performance of the parent ligand biphalin. The cyclic MOR agonists/DOR antagonists DIPPΨNH_2 described by Schiller *et al*.[23] and KSK-103 reported by Purington *et al*.[24] showed poor BBB penetration and have poor bioavailability. In this case glycosylation of peptides has been demonstrated to improve metabolic stability and CNS activity following peripheral administration.[24-26] However, our compounds show high efficacy after i.v. administration, suggesting high BBB penetration. Our compounds may thus improve on these earlier generations of compounds.

In conclusion, the substitution of the disulfide bridge in the first cyclic analogue of biphalin[27] with an olefin bridge leads to geometrical isomer analogues with less affinity at MOR and DOR in respect to the previously reported analogues.[12,13,27] Surprisingly the novel compounds show an increased potency and efficacy in G protein stimulation at MOR, with no observed activation of the DOR. Indeed, our compounds show a slightly improved DOR binding selectivity and a strong analgesic activity higher than that of

the cyclic analogue containing D-Pen,[12] in the hot-plate test after i.v. and i.c.v. administration. These results indicate that the **ABAM** compounds are MOR agonists /DOR–partial antagonists, different from the mixed MOR/DOR agonist activity exerted by biphalin and its cyclic analogues reported in the literature so far.[12-14,27] In this work we have described a cyclic biphalin analogue endowed with MOR agonist /DOR partial antagonist activity, which retains a good binding affinity for MOR and DOR, avoiding KOR binding; this latest aspect could be crucial in the design of safer drugs without undesirable side effects, such as tolerance and dependence, that hamper the clinical use of opioid analgesics.

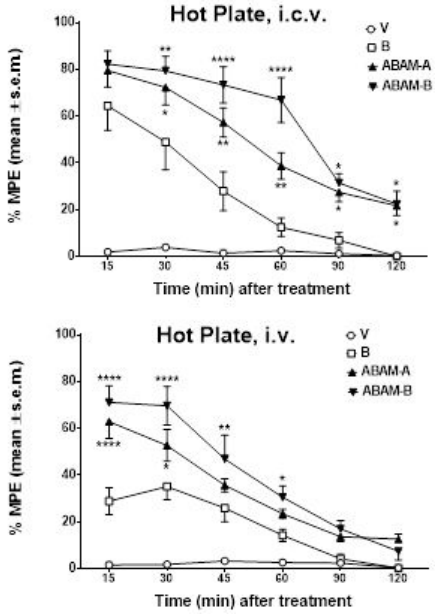


Figure 4. In vivo hot-plate tests on **ABAM-A** and **ABAM-B** after i.c.v. and i.v. administrations. In these experiments, peptides were administered at the dose of 0.15 nmol/mouse for i.c.v. administration and at the dose of 1.5 μmol/kg for i.v. administration. * is for $p<0.05$, ** is for $p<0.01$, **** is for $p<0.0001$ vs B. N=10. Data reported as the mean ± SEM of the maximum possible effect (MPE) in the hot plate assay.

EXPERIMENTAL PROCEDURES

General Information.

All reagents, solvents, and starting materials were purchased by Sigma-Aldrich (MI, Italy) and VWR International (Pennsylvania, US). All the intermediate products have been purified by trituration in diethyl ether and then characterized by LRMS and ^{1}H-NMR spectroscopy. The final TFA salts **5a** and **5b** were purified by RP-HPLC on a VP 250/16 Nucleosil 100-7 C18, 5.0 μm, 250 mm × 10 mm column at a flow rate of 7 mL/min with a Waters Binary pump 600, linear gradient of H_2O/ACN-0.1% TFA from 5% to 95% ACN in 30 min. The identity of the N$^{\alpha}$-Boc-protected products was confirmed by ^{1}H-NMR analysis on a Varian Oxford 300 MHz and ESI-LRMS using a LCQ Finnigan-Math mass spectrometer. Peptide structures of the final compounds were determined by NMR spectrometry (Tables 1S,2S see SI) and confirmed by ESI-HRMS mass spectrometry (Table 3S, Figures 1S,2S see SI). Samples were diluted to about 200 fmol/uL in 50:50 ACN-water with 0.1% formic acid. They were infused to an ESI ionization source on a Waters Q-Tof Premier quadrupole-time of flight mass spectrometer operating in positive ion mode. Glu-fibrinopeptide B was used as a lock mass standard. MS data were acquired for 10 minutes with ms scans from 400 to 2000 Da at 5 sec/scan; capillary voltage 3.5 kV, cone voltage 10 V, desolvation temperature 180, desolvation gas flow (N_2) 200 L/h, source temperature 110 °C. The purity of each sample was established by analytical RP-HPLC with a C18-bonded column 4.6 mm × 150 mm at 236 and 268 nm, flow rate of 1 mL/min, gradient of H_2O/ACN-0.1% TFA from 5% to 95% ACN in 30 min, and was found to be ≥95%.

Chemistry. Intermediate compound **1** has been previously described and characterized in literature.[27] The linear intermediate peptides **2** and **3** were synthesized in solution using N$^{\alpha}$-Boc strategy, coupling reactions have been performed using EDC·HCl/anhydrous HOBt /NMM in DMF, and Boc removal with a mixture of TFA/DCM = 1:1 at r.t. for 1 h (Scheme 1S, see SI).[15] For the RCM reaction, a catalytic amount of the second generation Grubbs catalyst (10%) was added to a solution of peptide **3** (100 mg, 0.086 mmoL) in DCE (80 mL) at 80 °C for 78 h. The ring closure was controlled by monitoring with TLC (AcOEt/MeOH = 97:3) until reaction completion. Then the solvent was evaporated by rotavapor and the crude residue was triturated with diethyl ether (3 times). The solid residue was dried in high vacuum to give product **4** as inseparable mixture of *cis/trans* isomers in quantitative yield (see SI). N$^{\alpha}$-Boc protection was removed with a solution of TFA/DCM = 1/1, the two isomeric peptides *cis*-**5a** (**ABAM-A**) and *trans*-**5b** **(ABAM-B)** were isolated by RP-HPLC and obtained in about 1.4:1 ratio, in 52% and 36% overall yields respectively. Assignment of NMR signals was performed using standard set of NMR spectra (ROESY, COSY, TOCSY, and ^{1}H-^{13}C HSQC) (Table 1S, see SI); relevant parameters are given in Table 2S (see SI). The spectra were measured on DDR2 Agilent 600 MHz spectrometer equipped with a Triax probe. The chemical shifts were calibrated on DMSO signal.

c[TFA·NH$_2$-Tyr-D-allyl-Gly-Gly-Phe-NH]$_2$ (*cis*-**5a**). HPLC rt = 16.30 (min); ^{1}H-NMR δ (DMSO-d$_6$): 2.22-2.32 (4H, m, D-allyl-Gly $^{\beta}$CH$_2$), 2.67-2.91 (4H, m, Tyr $^{\beta}$CH$_2$), 2.83-3.07 (4H, m, Phe $^{\beta}$CH$_2$), 3.31-3.94 (4H, dd, Gly $^{\alpha}$CH), 3.84 (2H, q, Tyr $^{\alpha}$CH), 4.32 (2H, q, D-allyl-Gly $^{\alpha}$CH), 4.55 (2H, q, Phe $^{\alpha}$CH), 5.18 (2H, q, D-allyl-Gly $^{\gamma}$CH), 6.68 (4H, dd, Tyr aromatics), 7.02 (4H, dd, Tyr aromatics), 7.20-7.26 (10H, m, Phe aromatics), 7.99 (2H, d, Phe NH), 8.51 (2H, t, Gly NH), 8.57 (2H, d, D-allyl-Gly NH), 9.29 (2H, s, OH Tyr); ^{13}C-NMR δ(DMSO-d$_6$): 29.9, 36.9, 42.3, 51.6, 52.2, 114.9, 126.1, 126.6, 127.8, 128.9, 130.1. ESI-HRMS: Calcd exact mass without TFA for $C_{48}H_{56}N_{10}O_{10}$ m/z = 933.4259 $[M+H]^+$; found m/z 933.4221.

c[TFA·NH$_2$-Tyr-D-allyl-Gly-Gly-Phe-NH]$_2$ (*trans*-**5b**). HPLC rt = 17.04 (min); ^{1}H-NMR δ (DMSO-d$_6$): 2.14-2.19 (4H, m, D-allyl-Gly $^{\beta}$CH$_2$), 2.68-2.89 (4H, m, Tyr $^{\beta}$CH$_2$), 2.80-3.09 (4H, m, Phe $^{\beta}$CH$_2$), 3.25-3.99 (4H, dd, Gly $^{\alpha}$CH), 3.89 (2H, q, Tyr $^{\alpha}$CH), 4.33 (2H, q, D-allyl-Gly $^{\alpha}$CH), 4.53 (2H, q, Phe $^{\alpha}$CH), 5.22 (2H, q, D-allyl-Gly $^{\gamma}$CH), 6.68 (4H, dd, Tyr aromatics), 7.02 (4H, dd, Tyr aromatics), 7.20-7.27 (10H, m, Phe aromatics), 8.15 (2H, d, Phe NH), 8.46 (2H, t, Gly NH), 8.52 (2H, d, D-allyl-Gly NH), 9.30 (2H, s, OH Tyr); ^{13}C-NMR δ(DMSO-d$_6$): 35.2, 36.8, 42.0, 51.6, 52.3, 114.9, 126.1, 127.8, 127.9, 128.8, 130.1. ESI-HRMS: Calcd exact mass without TFA for $C_{48}H_{56}N_{10}O_{10}$ m/z = 933.4259 $[M+H]^+$; found m/z 933.4241.

In vitro studies

Cell lines and cell culture

Chinese hamster ovary (CHO) cells expressing the human MOR, DOR, or KOR were used for all experiments. The

details for these cell lines, including their K_D values for ^{3}H-diprenorphine binding, can be found in Stefanucci *et al.*[14] The cells were maintained in 1:1 DMEM/F12 culture media (Gibco), with 1X penicillin/streptomycin, and 10% heat-inactivated fetal bovine serum (Gibco) in a 5% CO_2 atmosphere 37°C incubator. For experiments, cells were harvested using 5 mM EDTA in PBS, collected, centrifuged, and stored at -80°C. Membrane preparations for binding or GTPγS coupling were created using the same protocol as reported.[14]

Competition radioligand binding

Competition radioligand binding was performed exactly as reported in Stefanucci *et al.*[14] Membrane preparations of receptor-containing CHO cells were combined with concentration curves of **ABAM** compounds or positive control, and a fixed concentration (4.33 – 5.25 nM) of ^{3}H-diprenorphine (PerkinElmer). Vehicle concentrations were equalized between each reaction. Reactions were incubated for 1 h at r.t. The resulting plates were read using a PerkinElmer MicroBeta2 6-detector 96-well format scintillation counter. The data was normalized to ^{3}H-diprenorphine alone (100%) or non-specific binding measured by the inclusion of 10 μM naloxone (0%). IC_{50} values for each curve and the calculated K_i using the established K_D of ^{3}H-diprenorphine in each cell line was calculated using GraphPad Prism 7.0, and reported as the mean ± SEM.

^{35}S-GTPγS coupling assay

The GTPγS assay was again performed exactly as reported in Stefanucci *et al*.[14] Membrane preparations of receptor-containing CHO cells were combined with concentration curves of **ABAM** compound or positive control along with 0.1 nM ^{35}S-GTPγS (PerkinElmer), and incubated at room temperature for 1 h. Vehicle concentrations were normalized between each reaction. The resulting plates were read as above, and the data normalized to the stimulation caused by positive control compound (100%) or vehicle (0%). The potency (EC_{50}) and efficacy (E_{Max}) values were calculated for each curve using GraphPad Prism 7.0 and reported as the mean ± SEM. The efficacy was defined for each compound in relation to the maximum efficacy of the positive control compound, defined as 100%. The antagonist experiments with DOR cells were performed as above, except the membranes were incubated with concentration curves of antagonist for 5 minutes before the addition of 100 nM SNC80. The data was normalized to 100 nM SNC80 (100%) or vehicle (0%), and analyzed as above, using naloxone as a positive control antagonist.

Forskolin induced [3H]cAMP assay

The cAMP assay was performed as reported in Stevenson *et al.*[28] The same DOR-expressing CHO cells were used as above, plated live in 96 well plates (20,000 cells/well) and recovered for 24 hrs. Cells serum starved for 4 hours, followed by 500 μM IBMX for 20 min (IBMX in all treatment solutions). **ABAM** compounds or naltrindole positive control incubated on cells for 15 minutes, followed by agonist (16.7 nM SNC80) combined with forskolin (100 μM) for 10 minutes. The cells were then processed as reported above, and cAMP levels determined by competition with [^{3}H]-cAMP for binding to recombinant PKA. The resulting signal was normalized to percent of forskolin stimulation (100%) or vehicle alone (0%), and analyzed using Prism 7.0.

In vivo studies

Animals. Male CD-1 mice (Harlan, Italy) weighing 25-30 g were used in all the experiments. Before the experimental sessions, the mice were housed in colony cages (7 mice per cage) under standard light (from 7:00 AM to 7:00 PM), temperature (21±1°C) and relative humidity (60±10%) for at least 1 week. Food and water were available ad libitum. The research protocol was approved by the Service for Biotechnology and Animal Welfare of the Istituto Superiore di Sanità and authorized by the Italian Ministry of Health, according to Legislative Decree 26/14, which implemented the European Directive 2010/63/UE on laboratory animal protection in Italy. Animal welfare was routinely checked by veterinarians from the Service for Biotechnology and Animal Welfare. Animal studies are reported in compliance with the ARRIVE guidelines.[29]

Drugs and treatment procedure. DMSO was purchased from Merck (Italy). On each test day, peptides solutions were freshly prepared using saline containing 0.9% NaCl and DMSO in the ratio DMSO: saline 1:5 v/v. These solutions were injected at a volume of 10 μL/mouse for intracerebroventricular (i.c.v.) administrations, or at a volume of 10 ml/kg for intravenous (i.v.) administrations.

Surgery for i.c.v. injections. For i.c.v. injections, mice were lightly anesthetized with isoflurane, and an incision was made in the scalp. Injections were performed using a 10 μL Hamilton microsyringe at a point 2-mm caudal and 2-mm lateral from the bregma.

Hot plate test. Thermal nociception was assessed as previously reported, using a commercially available apparatus consisting in a metal plate 25x25 cm (Ugo Basile, Italy) heated to a constant temperature of 55.0 ± 0.1° C, on which a plastic cylinder (20 cm diameter, 18 cm high) was placed. The time of latency (s) was recorded from the moment the animal was inserted inside the cylinder up to when it licked its paws or jumped off the hot plate; the latency exceeded the cut-off time of 60 s. The baseline was calculated as mean of three readings recorded before testing at intervals of 15 min. The time course of latency was then determined at 15, 30, 45, 60, 90 and 120 min after treatment. Data from the hot plate test, were reported as time course of the percentage of maximum effect (%MPE) = (post drug latency – baseline latency) / (cut-off time – baseline latency) x 100.[30]

Data analysis and statistics. Experimental data were expressed as mean ± s.e.m. Significant differences among the groups were evaluated with an analysis of variance followed by Tukey's post-hoc comparisons using the GraphPad Prism 6.03 software. Statistical significance was set at $P<0.05$.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Details of compounds synthesis, characterization, in vitro assays (PDF).

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Author Contributions

AS: synthesis, characterization and writing of the text. WL and JS: *in vitro* binding and function experiments. SP: *in vivo* assays; MPD: purification of the novel compounds. GL and EN: writing and revision of the text; MN and WK: NMR analysis. SM and GZ: design of the molecules. JS: co-wrote the manuscript. AM: design, co-wrote and coordinated all the research units.[31]

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ABBREVIATIONS

DOR, δ-opioid receptor; MOR, μ-opioid receptor; KOR, k-opioid receptor; RCM, Ring Closing Metathesis; PK, pharmacokinetic properties; DCE, dichloroethane, EDC, 1-ethyl-(3-(dimethylamino)propyl)-carbodiimide; DAMGO, [DAla(2), N-Me-Phe-(4), Gly-ol(5)] enkephalin; SNC80, (+)-4-[(αR)-α-((2S,5R)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-methoxy benzyl]-N,N-diethylbenzamide; HOBt, 1-hydroxybenzotriazole; i.c.v., intracerebroventricular; i.v., intravenous; NMM, N-methylmorpholine; DMF, dimethylformamide;; RP-HPLC, reversed phase high performance liquid chromatography; ACN, acetonitrile; NMR, nuclear magnetic resonance; ROESY, rotating-frame nuclear Overhauser effect correlation spectroscopy; COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; HSQC, heteronuclear single quantum correlation; LRMS, low resolution mass spectrometry; HRMS, high resolution mass spectrometry; TFA, trifluoroacetic acid; DCM, dichloromethane; BBB, blood brain barrier; CNS, central nervous system; GTP, guanosine triphosphate; MPE, maximum possible effect; DMSO, dimethylsulfoxide; CHO, chinese hamster ovary; DMEM/F12, Dulbecco's modified eagle medium/nutrient mixture F-12; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate buffered saline.

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SYNOPSIS TOC