Novel Cyclic Biphalin Analogues by Ruthenium-Catalyzed Ring Closing Metathesis: *in Vivo* and *in Vitro* Biological Profile

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S Supporting Information

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.

KEYWORDS: Biphalin, ring closing metathesis, opioids, olefin, antinociception

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 $\pm 90^\circ$.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-

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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, which 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 EC₅₀ 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, with the analogue containing the *o*-xylene moiety showing 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.

Two cyclic geometric isomers of biphalin *cis*-**5a** and *trans*-**5b** (Figure 2) were separated and purified by RP-HPLC, then

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

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 Supporting Information).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 this group of catalysts suitable for applications in peptide chemistry.^{16,17} We first explored RCM using Zhan-1B and Grubbs I generation catalyst 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, monitored 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 $\geq 95\%$ 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 α position to the double bond (C β , Table 1S see Supporting

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 and (c) fluorescein-maleimide containing analogue via III generation CLIPS technology.¹⁴

Information). 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 nonselective opioid antagonist [³H]-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 $\gg$ KOR, and there is no apparent difference between the two **ABAM** compounds in their binding profile.

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 ³H-Diprenorphine in Membranes from CHO Cells Expressing One of the Human Opioid Receptors^a

ligands	K_i (nM)		
	MOR	DOR	KOR
naloxone	51.2 $\pm$ 2.5	70.0 $\pm$ 2.9	
U50,488			23.9 $\pm$ 4.2
ABAM-A	75.8 $\pm$ 11.9	7.3 $\pm$ 0.7	495 $\pm$ 43
ABAM-B	53.6 $\pm$ 2.7	5.2 $\pm$ 2.7	686 $\pm$ 53

^aData reported as the mean $\pm$ 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 $\pm$ SEM. The naloxone and U50,488 positive controls showed expected values at the receptors, validating the assays.

We then tested the **ABAM** compounds for their functional activity at the opioid receptors using ³⁵S-GTP γ S 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.

Table 2. Opioid Receptor Functional Activity of ABAM-A and ABAM-B^a

ligands	MOR		DOR		KOR	
	EC ₅₀ (nM)	E _{max} (%)	EC ₅₀ (nM)	E _{max} (%)	EC ₅₀ (nM)	E _{max} (%)
DAMGO	19.7 $\pm$ 3.4	100				
SNC80			210 $\pm$ 69	100		
U50,488					7.8 $\pm$ 0.4	100
ABAM-A	19.6 $\pm$ 4.7	91.0 $\pm$ 3.0	NC	NC	1843 $\pm$ 169	69.0 $\pm$ 3.6
ABAM-B	17.7 $\pm$ 2.6	85.7 $\pm$ 2.6	NC	NC	969 $\pm$ 64	73.3 $\pm$ 0.3

^aThe functional agonist activity of the compounds was measured using ³⁵S-GTP γ S coupling in the membranes of CHO cells expressing one of the human opioid receptors. Data reported as the mean $\pm$ 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 (EC₅₀) and efficacy (E_{max}) of each compound from each receptor were calculated separately from each experiment and reported as the mean $\pm$ 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.

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 ³⁵S-GTP γ S coupling (Figure 3).

Figure 3. DOR antagonist activity of ABAM-A and ABAM-B. **ABAM** compounds or naloxone positive control were competed against 100 nM SNC80 at the DOR using ³⁵S-GTP γ S coupling. Data were normalized to the stimulation caused by 100 nM SNC80 (100%) or vehicle (0%) and reported as the mean $\pm$ SEM of $N = 3$ independent experiments. (A) The summary curves combined from each independent experiment are shown. (B) The antagonist potency (IC₅₀) and efficacy (I_{max}) were calculated separately from each experiment and reported as the mean $\pm$ SEM. The I_{max} of each compound was normalized to the inhibition caused by the positive control antagonist naloxone (100%).

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, Supporting Information).

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 $\sim$ 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 buphalin, 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]NH₂ exerted agonism at MOR and KOR, while displaying antagonism at DOR in the GTP γ S binding assay;²¹ our novel compounds have lost the high potency KOR agonism exhibiting a biological profile that closely resembles 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

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 μ mol/kg for i.v. administration. * p < 0.05, ** p < 0.01, *** p < 0.0001 vs B. N = 10. Data reported as the mean $\pm$ SEM of the maximum possible effect (MPE) in the hot plate assay.

compound, with vehicle administration as a negative control. ABAM-B produced an MPE% $\geq$ 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 time points. 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 DIPPVNH₂ described by Schiller et al.²³ and KSK-103 reported by Purington et al.²⁴ showed poor BBB penetration and have scarce 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 biphalin²⁷ with an olefin bridge leads to geometrical isomer analogues with less affinity at MOR and DOR with 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.

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 ¹H 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 μ m, 250 mm $\times$ 10 mm column at a flow rate of 7 mL/min with a Waters Binary pump 600, linear gradient of H₂O/ACN–0.1% TFA from 5% to 95% ACN in 30 min. The identity of the N ^{α} -Boc-protected products was confirmed by ¹H 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 spectroscopy (Tables 1S and 2S, see [Supporting Information](#)) and confirmed by ESI-HRMS mass spectrometry (Table 3S, Figures 1S and 2S, see [Supporting Information](#)). Samples were diluted to about 200 fmol/ μ L 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 min with ms scans from 400 to 2000 Da at 5 s/scan; capillary voltage 3.5 kV, cone voltage 10 V, desolvation temperature 180, desolvation gas flow (N₂) 200 L/h, source temperature 110 $^{\circ}$ C. The purity of each sample was established by analytical RP-HPLC with a C18-bonded column 4.6 mm $\times$ 150 mm at 236 and 268 nm, flow rate of 1 mL/min, gradient of H₂O/ACN–0.1% TFA from 5% to 95% ACN in 30 min, and was found to be $\geq$ 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 ^{α} -Boc strategy, coupling reactions have been performed using EDC-HCl/anhydrous HOBt/MM in DMF, and Boc was removed with a mixture of TFA/DCM = 1:1 at r.t. for 1 h (Scheme 1S, see [Supporting Information](#)).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 [Supporting Information](#)). N^α-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 ¹H–¹³C HSQC) (Table 1S, see [Supporting Information](#)); relevant parameters are given in Table 2S. 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-NH₂-Tyr-D-allyl-Gly-Gly-Phe-NH]₂ (*cis*-5a). HPLC *rt* = 16.30 (min); ¹H NMR δ (DMSO-*d*₆): 2.22–2.32 (4H, m, D-allyl-Gly ^βCH₂), 2.67–2.91 (4H, m, Tyr ^βCH₂), 2.83–3.07 (4H, m, Phe ^βCH₂), 3.31–3.94 (4H, dd, Gly ^αCH), 3.84 (2H, q, Tyr ^αCH), 4.32 (2H, q, D-allyl-Gly ^αCH), 4.55 (2H, q, Phe ^αCH), 5.18 (2H, q, D-allyl-Gly ^γCH), 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); ¹³C 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 C₄₈H₅₆N₁₀O₁₀ *m/z* = 933.4259 [M + H]⁺; found *m/z* 933.4221.

c[TFA-NH₂-Tyr-D-allyl-Gly-Gly-Phe-NH]₂ (*trans*-5b). HPLC *rt* = 17.04 (min); ¹H NMR δ (DMSO-*d*₆): 2.14–2.19 (4H, m, D-allyl-Gly ^βCH₂), 2.68–2.89 (4H, m, Tyr ^βCH₂), 2.80–3.09 (4H, m, Phe ^βCH₂), 3.25–3.99 (4H, dd, Gly ^αCH), 3.89 (2H, q, Tyr ^αCH), 4.33 (2H, q, D-allyl-Gly ^αCH), 4.53 (2H, q, Phe ^αCH), 5.22 (2H, q, D-allyl-Gly ^γCH), 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); ¹³C 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 C₄₈H₅₆N₁₀O₁₀ *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 ³H-diprenorphine binding, can be found in Stefanucci et al.¹⁴ The cells were maintained in 1:1 DMEM/F12 culture media (Gibco), with 1× penicillin/streptomycin, and 10% heat-inactivated fetal bovine serum (Gibco) in a 5% CO₂ 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 ³H-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 ³H-diprenorphine alone (100%) or nonspecific binding measured by the inclusion of 10 μM naloxone (0%). IC₅₀ values for each curve and the calculated *K_i* using the established *K_D* of ³H-diprenorphine in each cell line was calculated using GraphPad Prism 7.0, and reported as the mean ± SEM.

³⁵S-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 ³⁵S-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 (EC₅₀) 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 min 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 h. Cells serum starved for 4 h, followed by 500 μM IBMX for 20 min (IBMX in all treatment solutions). ABAM compounds or naltrindole positive control incubated on cells for 15 min, followed by agonist (16.7 nM SNC80) combined with forskolin (100 μM) for 10 min. The cells were then processed as reported above, and cAMP levels determined by competition with [³H]-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 25 × 25 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 cutoff 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)/(cutoff time – baseline latency) × 100.³⁰

Data Analysis and Statistics. Experimental data were expressed as mean ± SEM. Significant differences among the groups were evaluated with an analysis of variance followed by Tukey's posthoc comparisons using the GraphPad Prism 6.03 software. Statistical significance was set at *P* < 0.05.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.8b00495.

Details of compounds synthesis, characterization, and *in vitro* assays (PDF)

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Author Contributions

A.S.: synthesis, characterization, and writing of the text. W.L. and J.S.: *in vitro* binding and function experiments. S.P.: *in vivo* assays. M.P.D.: purification of the novel compounds. G.L. and E.N.: writing and revision of the text. M.N. and W.K.: NMR analysis. S.M. and G.Z.: design of the molecules. J.S.: cowrote the manuscript. AM: designed, cowrote, and coordinated all the research units.³¹

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

DOR, δ -opioid receptor; MOR, μ -opioid receptor; KOR, κ -opioid receptor; RCM, ring closing metathesis; PK, pharmacokinetic; DCE, dichloroethane; EDC, 1-ethyl-(3-(dimethylamino)propyl)-carbodiimide; DAMGO, [DAla(2), N-Me-Phe-(4), Gly-ol(5)] enkephalin; SNC80, (+)-4-[(α R)- 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, dimethyl sulfoxide; CHO, Chinese hamster ovary; DMEM/F12, Dulbecco's modified eagle medium/nutrient mixture F-12; EDTA, ethylenediamine-tetraacetic acid; PBS, phosphate buffered saline

REFERENCES

- (1) Zhang, R. Y.; Thapa, P.; Espiritu, M. J.; Menon, V.; Bingham, J. P. From nature to creation: Going around in circles, the art of peptide cyclization. *Bioorg. Med. Chem.* **2018**, *26*, 1135–1150.
- (2) Qvit, N.; Rubin, S. J. S.; Urban, T. J.; Mochly-Rosen, D.; Gross, E. R. Peptidomimetic therapeutics: scientific approaches and opportunities. *Drug Discovery Today* **2017**, *22*, 454–462.
- (3) Wang, M.; Jiang, X. Sulfur-Sulfur bond construction. *Top. Curr. Chem. (Cham)*. **2018**, *376*, 14.
- (4) Keller, O.; Rudinger, J. Synthesis of (1,6- α , α' -diaminosuberic acid)oxytocin (dicarba-oxytocin). *Helv. Chim. Acta* **1974**, *57*, 1253–1259.

- (5) Mollica, A.; Feliciani, F.; Stefanucci, A.; Fadeev, E.; Pinnen, F. (Acyloxy) alkoxy moiety as amino acids protecting group for the synthesis of (R,R)-2,7 diaminosuberic acid via RCM. *Protein Pept. Lett.* **2012**, *19*, 1245–1249.
- (6) Stymiest, J. L.; Mitchell, B. F.; Wong, S.; Vederas, J. C. Synthesis of biologically active dicarba analogues of the peptide hormone oxytocin using ring-closing metathesis. *Org. Lett.* **2003**, *5*, 47–49.
- (7) Schiller, P. W. Opioid peptide analog design: from cyclic enkephalins to orally active analgesics. *Biopolymers* **2005**, *80*, 492.
- (8) Berezowska, I.; Nga, N. C.; Lemieux, C.; Wilkes, B. C.; Schiller, P. W. Dicarba analogues of the cyclic enkephalin peptides H-Tyr-c[D-Cys-Gly-Phe-D (or L)-Cys]NH₂ retain high opioid activity. *J. Med. Chem.* **2007**, *50*, 1414–1417.
- (9) Han, J. C.; Li, C. C. Ruthenium-catalyzed metathesis cascade reactions in natural products synthesis. *Chem. Rec.* **2017**, *17*, 499–517.
- (10) Remesic, M.; Lee, Y. S.; Hruby, V. J. Cyclic opioid peptides. *Curr. Med. Chem.* **2016**, *23*, 1288–1303.
- (11) Cowell, S. M.; Lee, Y. S. Biphallin: The foundation of bivalent ligands. *Curr. Med. Chem.* **2016**, *23*, 3267–3284.
- (12) Mollica, A.; Carotenuto, A.; Novellino, E.; Limatola, A.; Costante, R.; Pinnen, F.; Stefanucci, A.; Pieretti, S.; Borsodi, A.; Samavati, R.; Zador, F.; Benyhe, S.; Davis, P.; Porreca, F.; Hruby, V. J. Novel cyclic biphallin analogue with improved antinociceptive properties. *ACS Med. Chem. Lett.* **2014**, *5*, 1032–1036.
- (13) Stefanucci, A.; Carotenuto, A.; Macedonio, G.; Novellino, E.; Pieretti, S.; Marzoli, F.; Szucs, E.; Erdei, A. I.; Zador, F.; Benyhe, S.; Mollica, A. Cyclic biphallin analogues incorporating a xylene bridge: synthesis, characterization, and biological profile. *ACS Med. Chem. Lett.* **2017**, *8*, 858–863.
- (14) Stefanucci, A.; Lei, W.; Hruby, V. J.; Macedonio, G.; Luisi, G.; Carradori, S.; Streicher, J. M.; Mollica, A. Fluorescent-labeled bioconjugates of the opioid peptides biphallin and DPDPE incorporating fluorescein-maleimide linkers. *Future Med. Chem.* **2017**, *9*, 859–869.
- (15) Mollica, A.; Pinnen, F.; Feliciani, F.; Stefanucci, A. New potent biphallin analogues containing *p*-fluoro-L-phenylalanine at the 4,4' positions and non-hydrazine linker. *Amino Acids* **2010**, *40*, 503–511.
- (16) Arisawa, M. Development of environmentally benign organometallic catalysis for drug discovery and its application. *Chem. Pharm. Bull.* **2007**, *55*, 1099–1118.
- (17) Binder, J. B.; Raines, R. T. Olefin metathesis for chemical biology. *Curr. Opin. Chem. Biol.* **2008**, *12*, 767–773.
- (18) Montgomery, T. P.; Ahmed, T. S.; Grubbs, R. H. Stereoretentive olefin metathesis: An avenue to kinetic selectivity. *Angew. Chem., Int. Ed.* **2017**, *56*, 11024–11036.
- (19) Dounis, P.; Feast, W. J.; Kenwright, A. M. Ring-opening metathesis polymerization of monocyclic alkenes using molybdenum and tungsten alkylidene (Schrock-type) initiators and ¹³C nuclear magnetic resonances studies of the resulting polyalkenamers. *Polymer* **1995**, *36*, 2787–2796.
- (20) Beierbeck, H.; Saunders, J. K. A reinterpretation of beta, gamma, and delta substituent effects on ¹³C chemical shifts. *Can. J. Chem.* **1976**, *54*, 2985–2995.
- (21) Purington, L. C.; Pogozheva, I. D.; Traynor, J. R.; Mosberg, H. I. Pentapeptides displaying μ opioid receptor agonist and δ opioid receptor partial agonist/antagonist properties. *J. Med. Chem.* **2009**, *52*, 7724–7731.
- (22) Przydzial, M. J.; Pogozheva, I. D.; Ho, J. C.; Bosse, K. E.; Sawyer, E.; Traynor, J. R.; Mosberg, H. I. Design of high affinity cyclic pentapeptide ligands for kappa-opioid receptors. *J. Pept. Res.* **2005**, *66*, 255–262.
- (23) Schiller, P. W.; Fundytus, M. E.; Merovitz, L.; Weltrowska, G.; Nguyen, T. M.; Lemieux, C.; Chung, N. N.; Coderre, T. J. The opioid mu agonist/delta antagonist DIPP-NH(2)[Psi] produces a potent analgesic effect, no physical dependence, and less tolerance than morphine in rats. *J. Med. Chem.* **1999**, *42*, 3520–3526.
- (24) Purington, L. C.; Sobczyk-Kojiro, K. S.; Pogozheva, I. D.; Traynor, J. R.; Mosberg, H. I. Development and *in vitro* characterization

of a novel bifunctional mu-agonist/delta antagonist opioid tetrapeptide. *ACS Chem. Biol.* **2011**, *6*, 1375–1381.

(25) Polt, R.; Dhanasekaran, M.; Keyari, C. M. Glycosylated neuropeptides: a new vista for neuropsychopharmacology? *Med. Res. Rev.* **2005**, *25*, 557–585.

(26) Mosberg, H. I.; Yeomans, L.; Anand, J. P.; Porter, V.; Sobczyk-Kojiro, K.; Traynor, J. R.; Jutkiewicz, E. M. Development of a bioavailable μ opioid receptor (MOPr) agonist, δ opioid receptor (DOPr) antagonist peptide that evokes antinociception without development of acute tolerance. *J. Med. Chem.* **2014**, *57*, 3148–3153.

(27) Mollica, A.; Davis, P.; Ma, S.-W.; Porreca, F.; Lai, J.; Hruby, V. J. Synthesis and biological activity of the first cyclic biphalin analogues. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 367–372.

(28) Stevenson, G. W.; Luginbuhl, A.; Dunbar, C.; LaVigne, J.; Dutra, J.; Atherton, P.; Bell, B.; Cone, K.; Giuvelis, D.; Polt, R.; Streicher, J. M.; Bilsky, E. J. *Pharmacol., Biochem. Behav.* **2015**, *132*, 49–55 The mixed-action delta/mu opioid agonist MMP-2200 does not produce conditioned place preference but does maintain drug self-administration in rats and induces in vitro markers of tolerance and dependence. .

(29) McGrath, J. C.; Lilley, E. Implementing guidelines on reporting research using animals (ARRIVE etc.): new requirements for publication in *BJP. Br. J. Pharmacol.* **2015**, *172*, 3189–3193.

(30) Pieknielna-Ciesielska, J.; Mollica, A.; Pieretti, S.; Fichna, J.; Szymaszkiewicz, A.; Zielińska, M.; Kordek, R.; Janecka, A. Antinociceptive potency of a fluorinated cyclopeptide Dmt-c[D-Lys-Phe-p-CF₃-Phe-Asp]NH₂. *J. Enzyme Inhib. Med. Chem.* **2018**, *33*, 560–566.

(31) Curtis, M. J.; Bond, R. A.; Spina, D.; Ahluwalia, A.; Alexander, S. P.; Giembycz, M. A.; Gilchrist, A.; Hoyer, D.; Insel, P. A.; Izzo, A. A.; Lawrence, A. J.; MacEwan, D. J.; Moon, L. D.; Wonnacott, S.; Weston, A. H.; McGrath, J. C. Experimental design and analysis and their reporting: new guidance for publication in *BJP. Br. J. Pharmacol.* **2015**, *172*, 3461–3471.