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DEVELOPING CYCLIC OPIOID ANALOGUES: FLUORESCENTLY LABELED BIOCONJUGATES OF BIPHALIN

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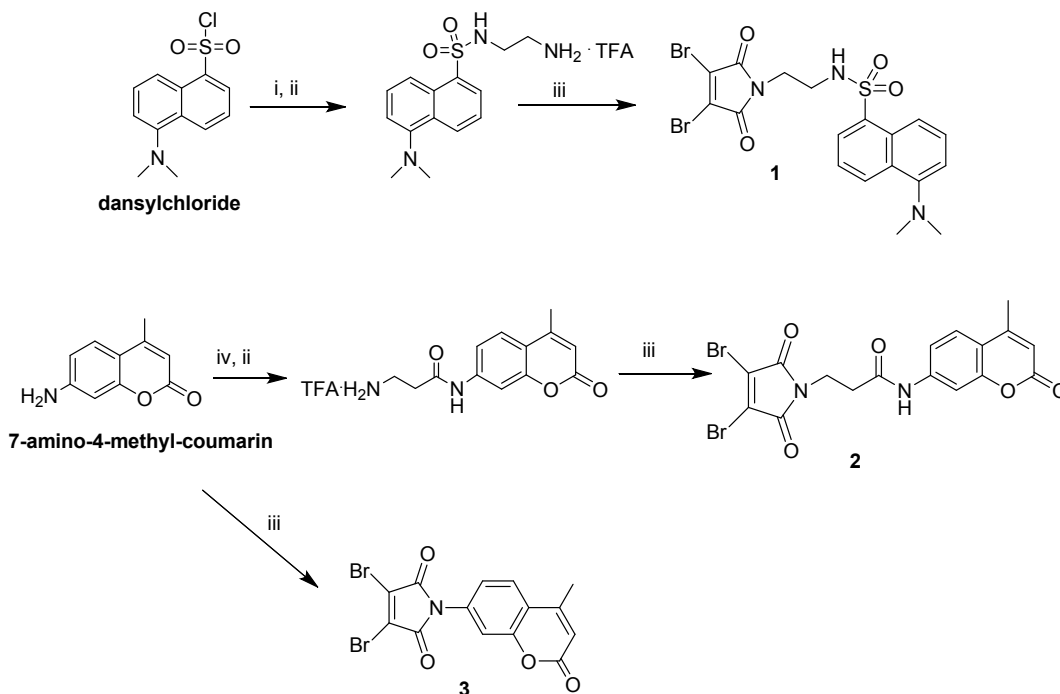
KEYWORDS: biphalin, opioids, fluorescent probes, GPCRs, bioconjugates.

ABSTRACT: The development of bioconjugates is of pivotal importance in medicinal chemistry due to their potential applications as therapeutic agents to improve the targeting of specific diseases, decrease toxicity, or control drug release. In this work we achieved the synthesis and characterization of three novel opioid peptides fluorescently labeled, analogues of cyclic biphalin derivatives, namely **1D**, **1C** and **2C**. Among them, compound **1D**, containing a dansyl-maleimide motif, exhibited an excellent binding affinity and functional potency for the δ -opioid receptor (DOR). **1D** also demonstrated a strong fluorescence emission spectrum ranging from 300 to 700 nm. These features could be highly desirable for medical and biological applications needed for targeting the DOR, including *in vivo* imaging, and as a lead for the design of fluorescent probes.

INTRODUCTION

Bioconjugate peptides occupy an important position in fluorescent probes field, due to their potential application in supramolecular structure design, targeting of biological tissues and diagnostic medicine.¹ Peptides containing a fluorescent dye could be valuable tools for monitoring intracellular reactions and molecular signaling, also fluorescent ligands could be used for pharmacokinetic studies, among other potential applications. Peptide bioconjugates are advantageous, due to their high receptors specificity correlated to the amino acid sequence, low toxicity and low disposal procedures compared to radiolabeled compounds.² Peptide conjugates also could be used in fluorescent microscopy. Studies in living cells require the management of selective probes to investigate the mechanism of internalization of membrane receptors, including the opioid receptors.³ In recent studies opioid peptides such as Dermorphin, deltorphin, TIPP, and endomorphin have been conjugated to the fluorescent dyes BODIPY TR and Alexa Fluor 488.⁴ Most of these peptides were biologically active, showing mixed μ/δ -opioid receptor (MOR/DOR) binding affinity and selectivity. They have been used in internalization study for the MOR and DOR, showing real-time visual tracking of receptor-ligand complexes in living cells, antagonist (naloxone)-sensitive and temperature-dependent.⁵ Leong *et al.* conjugated Endomorphin-1 (EM-1) to tetramethylrhodamine (TAMRA) to analyze the cell biology of the MOR in keratinocytes, revealing a rapid and complete internalization in the endoplasmic reticulum (ER) under resting conditions.⁶ Furthermore, fluorophore-conjugated opioid receptor ligands

with peptidic or non-peptidic structures may help to localize receptors at low density, allowing analysis of the receptor ligand-interaction of OR-containing cell populations. Simple labeling methods involve the anchorage of fluorescent dyes through succinimidyl ester or maleimide functional groups with primary amines or thiol groups.^{7,8} Maleimides may quench fluorescence in the conjugated form because the C=C bond undergoes to selective addition reaction for thiols, but when they are linked to chromophores such as pyrene, phthalimide and naphthopyranone, the fluorescence emission results increased. The application of maleimide in peptides and protein bioconjugation is highly desirable due to the prompt *N*-functionalization of maleimide and the possibility to insert the fluorescent molecule by a selective reaction with two cysteine residues present in the peptides. It also allows to establish a precise distance between the fluorescent core and the peptide by the addition of specific linkers.⁹ Biphalin has been used as model scaffold in the design of bioconjugated opioid peptides.¹⁰ Diverse hydrophilic/hydrophobic components and biochemical markers have been attached to biphalin in order to study the biological properties as opioid ligand and to explore its possible application as fluorescent probe.¹¹⁻¹⁶ In 2002 Lipkowski *et al.* demonstrated that one biphalin pharmacophore could be replaced by hydrophobic groups without loss of receptor affinity, with the aim to develop a useful tool for pharmacokinetic and pharmacodynamic studies *in vivo*;¹⁷ the first biphalin analogue so designed contained a dansyl moiety, which expressed high receptor binding affinity for MOR and DOR and antinociceptive activity *in vivo*.¹⁷



Scheme 1. Functionalization of 7-amino-4-methylcoumarin and dansylchloride with Boc- β -Ala-OH and *tert*-Butyl *N*-(2-aminoethyl)carbamate respectively. *Reagents and Conditions:* (i) *tert*-Butyl *N*-(2-aminoethyl)carbamate, EDC·HCl, HOBT, DIPEA in DMF, r.t., overnight; (ii) TFA:DCM = 1:1, r.t. 1h; (iii) 3,4-dibromofuran-2,5-dione, AcOH at r.t. for 6h then reflux for 3h; (iv) Boc- β -Ala-OH, POCl₃, pyridine at -15°C, 1h.

The same fluorescent moiety has been applied to the derivatization of DALDA sequence to give three potent MOR agonists.¹⁸ Further replacement of the dansyl group of one biphalin branch with amino-coumarin resulted in an increased affinity for the MOR and high antinociceptive activity comparable to that of biphalin.¹⁹ These studies establish proof-of-concept for the use of biphalin as a bioconjugate scaffold for targeting the MOR and DOR.

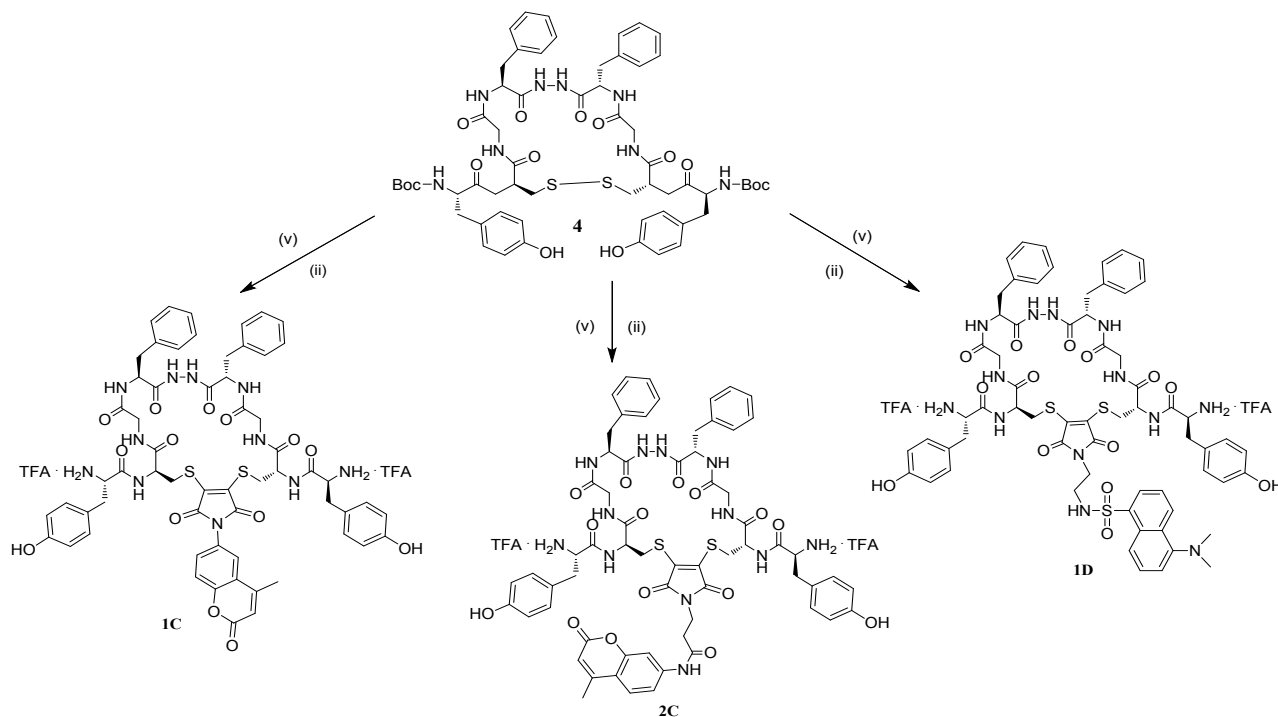
Recently our research group published the first cyclic biphalin analogue incorporating a fluorescein-maleimide moiety with the aim to identify a good fluorescent ligand for *in vivo* and *in vitro* assays, concerning receptor binding and penetration of biological barriers *e.g.* the blood brain barrier (BBB).²⁰ This novel compound exhibited modest affinity for MOR and DOR and was able to partially stimulate their G protein activation. In this study we sought to improve on this initial work. We designed and synthesized three novel cyclic biphalin analogues incorporating different fluorescent-labeled moieties in order to find a good candidate for probe development on opioid receptors for both *in vitro* and *in vivo* studies.

RESULTS AND DISCUSSION

Commercially available dansylchloride and 7-amino-4-methylcoumarin were functionalized with *tert*-Butyl *N*-(2-aminoethyl)carbamate and Boc- β -Ala-OH respectively, following the reactions depicted in Scheme 1. Dansyl chloride was functionalized with *tert*-Butyl *N*-(2-aminoethyl)carbamate following the procedure described by Youziel *et al.* so as to give the *N*-Boc protected compound in 93% yield after reaction work-up.²¹ 7-Amino-4-methylcoumarin was also functionalized with Boc- β -Ala-OH following the procedure reported by Heltweg *et al.* to obtain a crude intermediate compound pure on silica gel TLC plate, in 63% yield after reaction work-up.²²

These *N*-Boc protected intermediates were deprotected with a mixture of TFA:DCM = 1:1 at r.t. and promptly reacted with 3,4-dibromofuran-2,5-dione. Maleic anhydride was converted in the organic scaffold 3,4-dibromofuran-2,5-dione, following the procedure reported by Dubernet *et al.*²³ The fluorescent scaffolds were conjugated to 3,4-dibromofuran-2,5-dione following the procedure reported by Stefanucci *et al.*²⁰ so as to give three fluorescent-maleimide moieties in good overall yields (98% yield for **1**, 40% yield for **2**, 71% yield for **3**) (see SI). The cyclic biphalin fluorescent analogs **1C**, **2C**, **1D** were prepared following the well-established procedure reported by us in solution (Scheme 2).²⁰

Compound **4** was prepared following the procedure previously described by our research group,²⁴ and was submitted to *in situ* reduction with the tris(2-carboxyethyl)phosphine hydrochloride solution (TCEP) reagent in presence of the desired fluorescent probe to afford the *N*-Boc protected cyclic compounds.²⁰ They were then treated with a mixture of TFA/DCM in solution at r.t. to give the final products **1D**, **1C** and **2C** as TFA salts in good overall yields (75% yield for **1D**, 61% yield for **1C** and 58% yield for **2C**) and excellent purity after purification by RP-HPLC (see SI). The three novel cyclic peptides were then tested for their ability to bind the MOR, DOR and Kappa OR (KOR) *in vitro* using radioligand displacement binding assays. All 3 compounds showed varying modest to strong binding affinity for MOR and DOR, while all 3 showed very poor binding to the KOR (Figure 1). All compounds also showed a higher affinity for DOR over MOR, with **1D** achieving a strong ~15 nM K_i with all 3 compounds showing ~5-8 fold selectivity for DOR>MOR. Notably, the compounds show incomplete competition at the MOR, which is consistent with an allosteric binding mode.



Scheme 2. Synthesis of novel cyclic peptides **1C**, **2C** and **1D**. *Reagents and conditions.* (ii) TFA:DCM =1:1, r.t. 1h; (v) TCEP, DMF, 1 for compound **1D**, 2 for compound **2C** and 3 for **1C**, r.t. overnight.

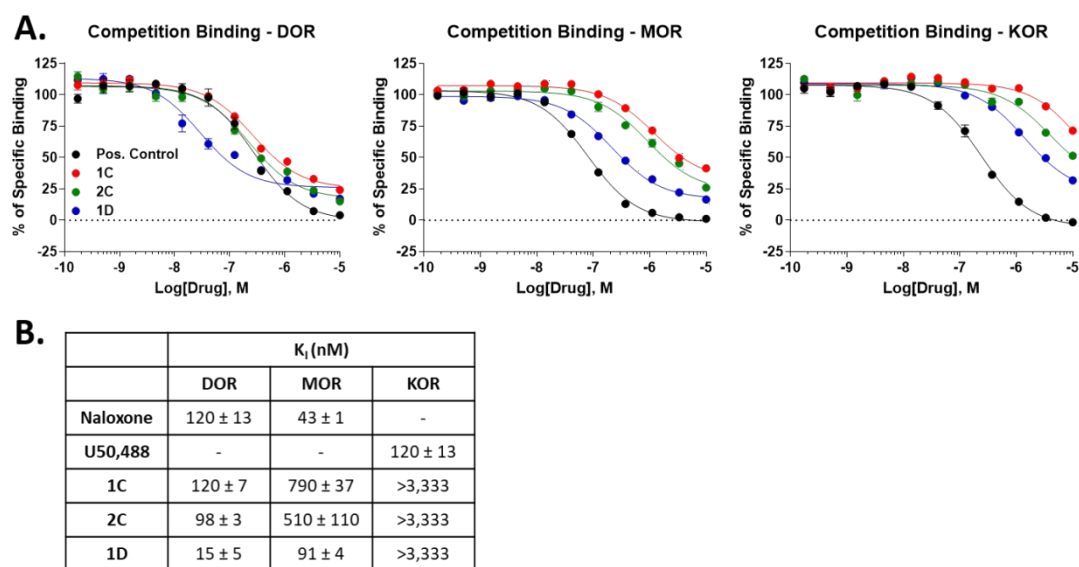


Figure 1. Opioid Receptor Binding Affinity of Novel Compounds. All compounds were competed against ^3H -diprenorphine using membranes from CHO cells expressing the human opioid receptors (DOR, MOR, or KOR). Data reported as the mean \pm SEM of $N = 3$ independent experiments. A) Concentration-response curves shown for all 3 compounds and positive control (naloxone for MOR and DOR; U50,488 for KOR) at each receptor. All compounds show competition at each receptor, with affinity $\text{DOR} > \text{MOR} > \text{KOR}$. The compounds show incomplete competition at the MOR, which is consistent with an allosteric binding mode. B) Affinity values (K_i) shown for each compound at each receptor derived from the curves in A.

These profiles of MOR/DOR binding are somewhat similar to the biphalin parent scaffold, and also suggest that these ligands could be effective labeling agents for the DOR and/or MOR. We next measured the functional activity of all compounds at the opioid receptors using the same CHO cell lines used above for the binding. We used ^{35}S -GTP γ S coupling,

which we've used extensively to characterize the functional activity of our compounds.^{20,25}

We first found that all 3 compounds were partial agonists at the DOR (E_{MAX} 47-62%), with modest potencies (EC_{50} 40-250 nM, **Figure 2**). These potencies were all about 2 fold worse than the binding affinities measured in **Figure 1**, suggesting that these ligands have somewhat poor intrinsic efficacy at the DOR. The reverse was true for the MOR, where all 3 compounds had

improved potency vs. binding affinity, suggesting good intrinsic efficacy, while all 3 were near-full to full agonists (E_{MAX} 79-120%). Lastly, all 3 compounds were weak potency partial agonists at KOR, as suggested by the poor affinities above, however they did show complete curves with improved potency vs. affinity, suggesting good intrinsic efficacy at KOR. These results altered the binding selectivity profile at the different opioid receptors found in **Figure 1**, with all compounds now nearly equi-potent at MOR/DOR with little to

no selectivity between them, at least at the *functional* level. These results should be kept in mind for evaluating these compounds *in vivo*. For the purposes of binding and labeling, for which the fluorescent moieties would be most useful, these compounds will likely be DOR-preferring. However, for any functional studies, they will likely be non-selective between MOR and DOR, similar to the parent scaffold biphalin. Taken together, these results show that the fluorescent moieties do not prevent effective binding and activation of the opioid receptors.

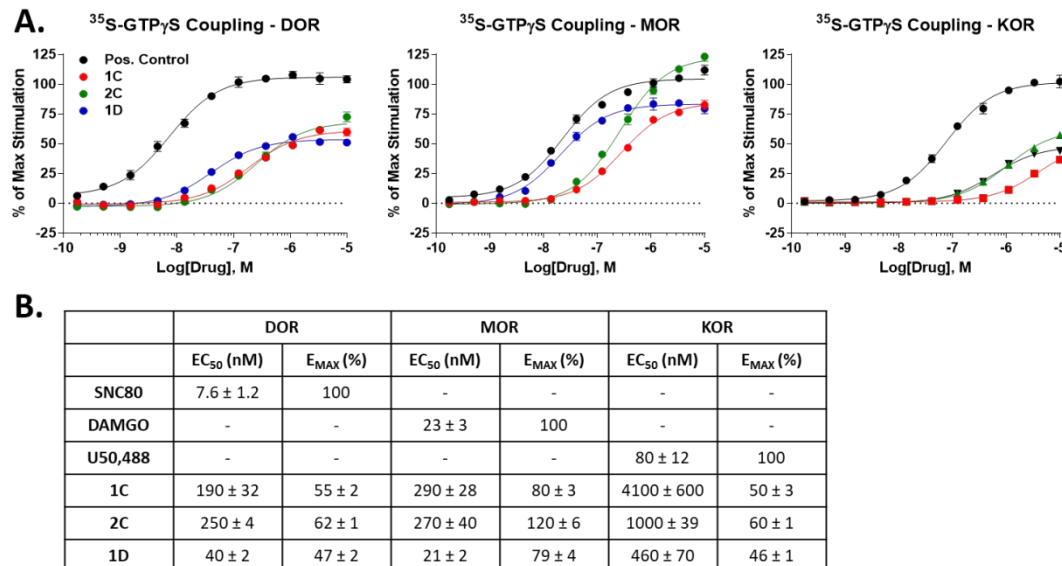


Figure 2. Opioid Receptor Functional Activity of Novel Compounds. All compounds were used to stimulate ^{35}S -GTP γ S accumulation using membranes from CHO cells expressing the human opioid receptors (DOR, MOR, or KOR). Data reported as the mean \pm SEM of N = 3 independent experiments. A) Concentration-response curves shown for all 3 compounds and positive control (DAMGO for MOR, SNC80 for DOR, U50,488 for KOR) at each receptor. All compounds show partial to full agonist activity at each receptor, with potency DOR=MOR>KOR. B) Potency (EC₅₀) and Efficacy (E_{MAX}) values shown for each compound at each receptor derived from the curves in A.

After establishing the molecular pharmacology of our compounds, we finally sought to evaluate their potential as fluorescent labeling tools. We thus measured the excitation and emission spectra of our highest affinity cyclic peptide **1D**, using an Edinburg Instruments FLS-920 fluorimeter in 100% methanol matrix at r.t., with a sample concentration of 10^{-2} μM (**Figure 3**). The fluorescence of the fluorescent-conjugated molecule is strongly dependent on the polarity of the solvent and chemical surroundings. The fluorescence emission spectrum of our novel compound **1D** shows an emission band ranging from 300 to 700 nm, with a maximum at 450 nm in methanol. In agreement with the findings of Guy *et al.* a maximum at 605 nm is present in the spectra, which represents the fluorescence emission of *N*-substituted dansylamide.²⁶ Two strong emission bands are present around 300 and 450 nm associable to tyrosine residues and conjugated hydrazine into the cyclic peptide.^{27,28} These results establish that **1D** is indeed a fluorescent ligand, with potential for use as a labeling and imaging agent. The good separation between excitation and emission spectra means that the compound would show little bleed through or crosstalk if the excitation and emission gates were correctly chosen. In conclusion three novel chemical entities have been synthesized and characterized with the aim to find a useful fluorescent bioconjugate of cyclic biphalin able to bind opioid receptors. Among them, compound **1D** exhibits the best affinity profile, being able to bind MOR/DOR and to stimulate the G protein coupled receptors with high affinity and potency. This compound represents an ideal candidate for

further future work to define the pharmacokinetic properties of cyclic biphalin compounds, and a valuable tool for binding studies, cellular uptake, and tissue distribution measurement of opioid receptors.

EXPERIMENTAL PROCEDURES

General information

Amino acids and reagents were acquired from Sigma-Aldrich (Milano, Italy), and HPLC grade solvents from VWR (Milano, Italy). The three novel cyclic peptides as TFA salts were purified by RP-HPLC on a Waters XBridgeTM Prep BEH130 C18, 5.0 μm , 250 mm \times 10 mm column; flow rate of 7 mL/min; Waters Binary pump 1525 (Waters, MA, USA); eluent: linear gradient of H₂O/ACN 0.1% TFA from 5 to 90% ACN in 35 min. *N*⁴-Boc-protected products were identified by NMR analysis on a Varian Mercury 300 MHz instrument and mass spectrometry ESI-LRMS (Thermo Finnigan, NJ, USA). The purity of the final compounds as TFA salts was calculated using analytical reverse phase high performance liquid chromatography (RP-HPLC; C18-bonded 4.6 \times 150 mm) at a flow rate of 1 mL/min; gradient eluent of H₂O/ACN 0.1% TFA from 5 to 95% ACN in 26 min, and was found to be $\geq 90\%$ pure. The identity of all final compounds was determined by ¹H NMR and ESI-LRMS. Fluorescence measurement was performed using an Edinburg Instruments FLS-920 fluorometer. The fluorescence measure was done in 100% methanol matrix at r.t., sample concentration was 10^{-2} μM ; wavelength ranging from 200 to 900 nm in 10 minutes.

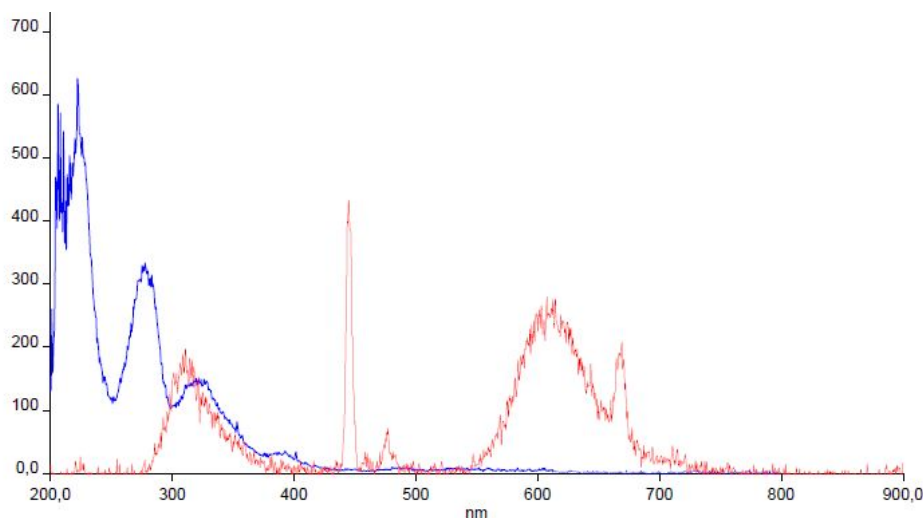


Figure 3. Adsorption (blue)/emission (red) spectrum of compound **1D** in 100% methanol. Y axis: Absorbance in A.U.; X axis: wavelength in nm unit.

Chemistry

The organic scaffold 3,4-dibromofuran-2,5-dione was prepared starting from the commercially available maleic anhydride following the procedure reported by Dubernet *et al.*²³ The fluorescent intermediates reported in **Scheme 1** were synthesized in solution as described by Youziel *et al.*,²¹ and Heltweg *et al.*²² respectively. Compounds **1-3** were prepared starting from the treatment of dibromomaleic anhydride with the appropriate fluorescent intermediates as TFA salts or 7-amino-4-methylcoumarin at room temperature (r.t.), followed by reflux in AcOH to give the desired fluorescent maleimides in good yields. All linear intermediate peptides were obtained by solution phase peptide synthesis using the EDC·HCl/HOBt hydrate/*N,N*-Diisopropylethylamine (DIPEA) in the dimethylformamide (DMF) coupling method. Deprotection of the *N*^α terminus of peptides from Boc-protecting group was performed with a mixture of TFA in DCM 1:1 at r.t. The intermediate TFA salts were used for subsequent reactions without further purification. *N*^βBoc-protected intermediates were isolated by silica gel column chromatography when required. The cyclic biphalin intermediate **4** was synthesized in solution, following the well-established procedure reported by us.²⁴ Then this intermediate was treated with tris(2-carboxyethyl)phosphine hydrochloride solution (TCEP) reagent in DMF at r.t. and the fluorescent-probes **1-3** were added *in situ* to promptly react with the thiol groups, so as to form the cyclic compounds **1C**, **1D** and **2C** after *N*-Boc deprotection.²⁰ The fluorescent cyclic intermediates were obtained in good yields after an easy work-up, and used as such for the following reaction without further purification. All final biphalin fluorescent analogs were deprotected with a mixture of TFA:DCM = 1:1, purified by RP-HPLC and characterized as TFA salts. ¹H NMR, ESI-LRMS and analytical RP-HPLC were performed for all final compounds, details of the experimental procedures are reported in the SI.

Competition-binding assays

Opioid receptor-expressing CHO cells were used as previously reported.¹⁹ The cells were grown using 50:50 DMEM/F12 culture media with 10% heat-inactivated fetal bovine serum, 1X penicillin/streptomycin, with propagation cultures maintained with 500 µg/mL G418 (all culture reagents

from ThermoFisher, Gibco brand). The cells were grown in a 37°C humidified incubator with 5% CO₂. Cells were grown and pellets harvested for both binding and G protein coupling assays as reported.²⁰ The competition binding assay was also performed as reported.²⁰ Briefly, 20-25 µg of cell membrane protein was combined with concentration curves of experimental compound or positive control and a fixed concentration (1.2-4.6 nM) of ³H-diprenorphine (PerkinElmer) in a 200 µL reaction volume. The reactions were incubated for 1 hr at r.t., and terminated by rapid filtration onto GF/B filter plates (PerkinElmer) using a Brandel cell harvester. The plates were dried, 40 µL of Microscint PS (PerkinElmer) was added, and the data was collected using a MicroBeta2 96 well format scintillation counter (PerkinElmer). The data was analyzed using GraphPad Prism 8.2 with a 1-site competition binding model using the previously measured K_D of the ³H-diprenorphine in each cell line. The data was further normalized to radioligand alone (100%) or in the presence of 10 µM naloxone (non-specific binding, 0%). The resulting affinity (K_i) values were reported as the mean ± SEM of N=3 independent experiments.

³⁵S-GTPγS coupling assay

The cells were grown and harvested as above; the assay protocol used was also reported previously in Stefanucci *et al.*^{20,25} 15 µg of cell membrane protein was combined with concentration curves of experimental or positive control agonist and 0.1 nM ³⁵S-GTPγS (PerkinElmer) in a 200 µL volume. Reactions were incubated at 30°C for 1 hr, then harvested and data collected as above. The data was normalized to the stimulation caused by positive control agonist (100%) or vehicle treatment (0%). GraphPad Prism 8.2 was used to analyze the data using a 3-variable non-linear regression model (Hill Slope defined as 1). The resulting potency (EC₅₀) and efficacy (E_{MAX}) values were reported as the mean ± SEM of N=3 independent experiments.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Details of compound synthesis and characterization included (PDF).

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Author Contributions

AS designed and he novel compounds synthesized the novel compounds, co-wrote and organized the manuscript. MPD helped in the purification and characterization of the novel compounds. GL and EN revised the entire manuscript for English language; GZ collaborated in the interpretation and rationalization of the data. JMS participated with and supervised GM in the performance of the *in vitro* experiments and co-wrote the manuscript. AM, co-wrote and organized the manuscript, and coordinated all the research units.

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ABBREVIATIONS

DOR, δ -opioid receptor; MOR, μ -opioid receptor; KOR, κ -opioid receptor; 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-methoxybenzyl]-N,N-diethylbenzamide; TAMRA, tetramethylrhodamine; ER, endoplasmic reticulum; SEM, Standard error of measurement; HOBt, 1-hydroxybenzotriazole; DIPEA, *N,N*-Diisopropylethylamine; DMF, dimethylformamide; EtOAc, ethyl acetate; RP-HPLC, reversed phase high performance liquid chromatography; ACN, acetonitrile; NMR, nuclear magnetic resonance; ESI-LRMS, electrospray ionization low resolution mass spectrometry; HRMS, high resolution mass spectrometry; TFA, trifluoroacetic acid; DCM, dichloromethane, TLC, thin layer chromatography; BBB, blood brain barrier; CNS, central nervous system; GTP, guanosine triphosphate; DMSO, dimethylsulfoxide; CHO, chinese hamster ovary; DMEM/F12, Dulbecco's modified eagle medium/nutrient mixture F-12; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate buffered saline.

REFERENCES

- Benizri, S.; Gissot, A.; Martin, A.; Vialet, B.; Grinstaff, M. W.; Barthél my, P. Bioconjugated oligonucleotides: Recent developments and therapeutic applications. *Bioconjug. Chem.* **2019**, *30*, 366-383.
- Chang, A.-C.; Chao, C. C.; Takemori, A. E.; Gekker, G.; Hu, S.; Peterson, P. K.; Portoghese, P. S. Arylacetamide-derived fluorescent probes: Synthesis, biological evaluation, and direct fluorescent labeling of κ opioid receptors in mouse microglial cells. *J. Med. Chem.* **1996**, *39*, 1729-1735.
- Huynh, A. S.; Estrella, V.; Stark, V. E.; Cohen, A. S.; Chen, T.; Casagni, T. J.; Josan, J. S.; Lloyd, M. C.; Johnson, J.; Kim, J.; Hruby, V. J.; Vagner, J.; Morse, D. L. Tumor targeting and pharmacokinetics of a near-Infrared fluorescent-labeled δ -opioid receptor antagonist agent, Dmt-Tic-Cy5. *Mol. Pharm.* **2016**, *13*, 534-544.
- Arttamangkul, S.; Alvarez-Maubecin, V.; Thomas, G.; Williams, J. T.; Grandy, D. K. Binding and internalization of fluorescent opioid peptide conjugates in living cells. *Mol. Pharmacol.* **2000**, *58*, 1570-1580.
- Dado, R. J.; Law, P. Y.; Loh, H. H.; Elde, R. Immunofluorescent identification of a δ -opioid receptor on primary afferent nerve terminals. *Neuroreport* **1993**, *5*, 341-344.
- Leong, C.; Neumann, C.; Ramasamy, S.; Rout, B.; Yi, W. L.; Bigliardi-Qi, M. Investigating endogenous μ -opioid receptors in human keratinocytes as pharmacological targets using novel fluorescent ligand. *Plos One* **2017**, *12*(12): e0188607. <https://doi.org/10.1371/journal.pone.0188607>
- Smith, M. E. B.; Felix, F.; Schumacher, C. P.; Lauren, R.; Tedaldi, M.; Papaioannou, D.; Waksman, G.; Caddick, S.; Baker, J. R. Protein modification, bioconjugation, and disulfide bridging using bromomaleimides. *J. Am. Chem. Soc.* **2010**, *132*, 1960-1965.
- Robin, M. P.; Wilson, P.; Mabire, A. B.; Kiviahio, J. K.; Raymond, J. E.; Haddleton, D. M.; O'Reilly, R. K. Conjugation-induced fluorescent labeling of proteins and polymers using dithiomaleimides. *J. Am. Chem. Soc.* **2013**, *135*, 2875-2878.
- Marculescu, C.; Kossen, H.; Morgan, R. E.; Mayer, P.; Fletcher, S. A.; Tolner, B.; Chester, K. A.; Jones, L. H.; Baker, J. R. Aryloxymaleimides for cysteine modification, disulfide bridging and the dual functionalization of disulfide bonds. *Chem. Commun.* **2014**, *50*, 7139-7142.
- Dvoracko, S.; Stefanucci, A.; Novellino, E.; Mollica, A. The design of multitarget ligands for chronic and neuropathic pain. *Future Med. Chem.* **2015**, *7*, 2469-2483.
- Stefanucci, A.; Carotenuto, A.; Macedonio, G.; Novellino, E.; Pieretti, S.; Marzoli, F.; Sz cs, E.; Erdei, A. I.; Z dor, F.; Benyhe, S.; Mollica, A. Cyclic biphalin analogues incorporating a xylene bridge: synthesis, characterization, and biological profile. *ACS Med. Chem. Lett.* **2017**, *8*, 858-863.
- 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 biphalin analogue with improved antinociceptive properties. *ACS Med. Chem. Lett.* **2014**, *5*, 1032-1036.
- Mollica, A.; Costante, R.; Stefanucci, A.; Pinnen, F.; Lucente, G.; Fidanza, S.; Pieretti, S. Antinociceptive profile of potent opioid peptide AM94, a fluorinated analogue of biphalin with non-hydrazine linker. *J. Pept. Sci.* **2012**, *19*, 233-239.
- Costante, R.; Pinnen, F.; Stefanucci, A.; Mollica, A. Potent biphalin analogs with μ/δ mixed opioid activity: *in vivo* and *in vitro* biological evaluation. *Archiv der Pharmazie* **2014**, *347*, 305-312.
- Mollica, A.; Pinnen, F.; Costante, R.; Locatelli, M.; Stefanucci, A.; Pieretti, S.; Davis, P.; Lai, J.; Rankin, D.; Porreca, F.; Hruby, V. J. "Biological active analogues of the opioid peptide biphalin: Mixed α/β -peptides" *J. Med. Chem.* **2013**, *56*, 3419-3423.
- Mollica, A.; Pinnen, F.; Feliciani, F.; Stefanucci, A.; Lucente, G.; Davis, P.; Porreca, F.; Ma, S. W.; Lai, J.; Hruby, V. J. New potent biphalin analogues containing p-fluoro-L-phenylalanine at the 4,4' positions and non-hydrazine linkers. *Amino Acids* **2011**, *40*, 1503-1511.
- Lipkowski, A. W.; Misicka, A.; Kosson, D.; Kosson, P.; Lachwa-From, M.; Brodzik-Bienkowska, A.; Hruby, V. J. Biological properties of a new fluorescent biphalin fragment analogue. *Life Sci.* **2002**, *70*, 893-897.
- Berezowska, I.; Lemieux, C.; Chung, N. N.; Zelent, B.; Schiller, P. W. Damsylated analogues of the opioid peptide [Dmt1] DALDA: *in vitro* activity profiles and fluorescence parameters. *Acta Biochim Pol.* **2004**, *51*, 107-113.
- Lukowiak, M.; Kosson, P.; Hennink, W.; Lipkowski, A. W. Synthesis and pharmacological properties of a new fluorescent opioid peptide analog. *Pharmacological Rev.* **2009**, *61*, 727-731.
- 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 biphalin and DPDPE incorporating fluorescein-maleimide linkers. *Future Med. Chem.* **2017**, *9*, 859-869.

21. Youziel, J.; Akhbar, A. R.; Aziz, Q.; Smith, M. E.; Caddick, S.; Tinker, A.; Baker, J. R. Bromo- and thiomaleimides as a new class of thiol-mediated fluorescence 'turn-on' reagents. *Org. Biomol. Chem.* **2014**, *12*, 557-560.
22. Heltweg, B.; Dequiedt, F.; Marshall, B. L.; Brauch, C.; Yoshida, M.; Nishino, N.; Verdin, E.; Jung, M. Subtype selective substrates for histone deacetylases. *J. Med. Chem.* **2004**, *47*, 5235-5243.
23. Dubernet, M.; Caubert, V.; Guillard, J.; Viaud-Massuard, M. C. Synthesis of substituted bis(heteroaryl)maleimides. *Tetrahedron* **2005**, *61*, 4585-4593.
24. 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.
25. Stefanucci, A.; Lei, W.; Pieretti, S.; Dimmito, M. P.; Luisi, G.; Novellino, E.; Nowakowski, M.; Koźmiński, W.; Mirzaie, S.; Zengin, G.; Streicher, J. M.; Mollica, A. Novel cyclic biphalin analogues by ruthenium-catalyzed ring closing metathesis: *in vivo* and *in vitro* biological profile. *ACS Med. Chem. Lett.* **2019**, *10*, 450-456.
26. Guy, J.; Caron, K.; Dufresne, S.; Michnick, S. W.; Jeffrey, S.; Keillor, W. Convergent preparation and photophysical characterization of dimaleimide dansyl fluorogens: Elucidation of the maleimide fluorescence quenching mechanism. *J. Am. Chem. Soc.* **2007**, *129*, 11969-11977.
27. Tang, J.; Yin, H.; Qiu, J.; Tucker, M. J.; DeGrado, W. F.; Gai, F. Using two fluorescent probes to dissect the binding, insertion, and dimerization kinetics of a model membrane peptide. *J. Am. Chem. Soc.* **2009**, *131*, 3816-3817.
28. Grigoryan, K. R.; Shilajyan, H. A.; Fluorescence 2D and 3D spectra analysis of tryptophan, tyrosine and phenylalanine. *Proc. of the Yerevan State Univ. Chemistry and Biology* **2017**, *51*, 3-7.

