

Opioid receptor activity and analgesic potency of DPDPE peptide analogues containing a xylene bridge

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ABSTRACT: D-Pen²,D-Pen⁵ enkephalin (DPDPE) is one of the most selective synthetic peptide agonist targeting the δ -opioid receptor. Three cyclic analogues of DPDPE containing a xylene bridge in place of disulphide bond have been synthesized and fully characterized as opioid receptors agonists. The *in vitro* activity was investigated showing a good affinity of **7a-c** for μ and δ -receptors. *In vivo* biological assays revealed that **7b** is the most potent analogue with the ability to maintain high level of analgesia from 15 to 60 minutes following intracerebroventricular (i.c.v.) administration, whereas DPDPE was slightly active until 45 min. **7b** induced long lasting analgesia also after subcutaneous administration, whereas DPDPE was inactive.

The cloning of opioid receptors has provided direct structural evidence of the “multiple opioid receptors” concept as powerful tools for physiological and pharmacological evaluation of their roles in both normal and acute pain states.¹⁻²

Delta-opioid receptors (DOP) are appealing drug targets for pain relief, due to the lack of unwanted side effects and the strong antinociceptive activity showed by their selective agonists. Ligands possessing dual agonist activities at the δ - and μ -receptors may allow for the effective treatment of pain with lessened μ -receptor-mediated side effects.³ Thus, it is highly desirable the design of “unbalanced” dual-acting opioid drugs as full δ -opioid receptor agonist and only as partial agonist of the μ -opioid receptor, which is strongly related to the development of tolerance and physical dependence.⁴ Met-Enkephalin and Leu-Enkephalin are linear endogenous pentapeptides with high affinity for DOP regulating human nociception; numerous structural modifications have been explored during the last years to investigate the structure activity relationships (SAR) and to improve their selectivity;^{5,6} H-Tyr-c[D-Pen-Gly-Phe-D-Pen]-OH (DPDPE) is the first synthetic prototype of highly selective constrained cyclic peptide for this receptor (Figure 1).⁷

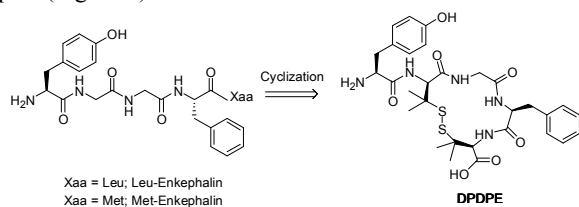
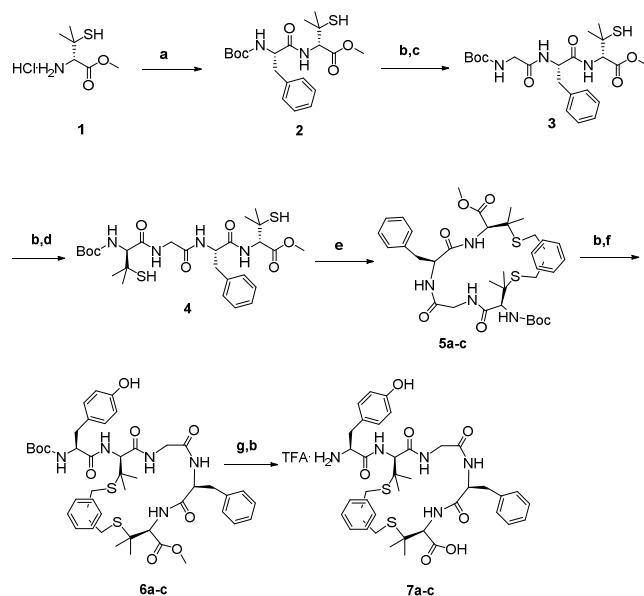


Figure 1. DPDPE cyclization strategy from Leu/Met-Enkephalins.

DPDPE is employed as radio-labeled δ receptor full agonist in the opioid binding assays due to its resistance to proteolytic degradation and permeability to the blood-brain barrier (BBB).⁸

The X-ray crystal structure of DPDPE published in 1994,⁹ has been assumed as starting point in a recent computational study based on MD simulations,¹⁰ from which a series of putative bioactive conformations has been provided: Gem-dimethyl groups of the D-Penicillamine residues are responsible for the rigidified structure and their adverse steric interactions for the low μ -opioid (MOP) receptor affinity. Disulphide bond is prone to reduction following ring opening, so incorporation of different types of linkers could provide conformational diversity involving different cyclization strategies, global and local constrictions;⁷ the replacement of disulphide bond with more stable bridges can increase metabolic stability and change the overall conformation, in order to improve the receptor binding affinity of the novel compounds.^{11,12} A systematic and synergistic multi-disciplinary approach has been used, over the last decades, for the design of novel peptide ligands with unique biological activity profiles.¹³ In this paper we propose the design, synthesis and biological evaluation of new cyclic DPDPE analogues **7a-c** containing *o*-, *m*-, *p*-xylene regioisomers respectively as μ/δ mixed opioid receptor agonists, using *in vitro* and *in vivo* models to examine the antinocicep-

tion activity of the new entities. The C-terminal free carboxyl group in DPDPE improves the δ/μ selectivity,¹⁴ then it has been maintained in our cyclic peptides. We envisaged that the introduction of different disulphide bridge in DPDPE would lead to different μ/δ affinity, increased stability and activity of the newly designed peptides; according to this working hypothesis, a *side-chain-to-side-chain* cyclization involving the two thiols groups and three dibromo-xylene regioisomers has been performed. The xylene-type bridging reaction utilizes the exquisite reactivity of dibromo-xylene scaffolds toward free thiols groups of penicillamine residues of compound **4**,¹⁵ providing cyclic peptides containing two robust thioether bonds (**5a-c**). The novel cyclic peptides **7a-c** have been also investigated by molecular docking study, to discern the structural influences of different xylene regioisomers on the molecular interactions of the cyclic peptides at the δ -opioid receptors. Other similar organic scaffolds may offer new insights for the preparation of further constrained DPDPE analogues (e.g. *N*-substituted bromomaleimide motif),¹⁶ and could be selected for future development.



Scheme 1. Synthesis of final compounds 7a-c. Reagents and Conditions. (a) 1.1 equiv BocPhe-OH, 1.1 equiv EDC HCl, 1.1 equiv HOBt anhydrous, 3.3 equiv DIPEA, DMF under N₂ atmosphere, r.t., overnight; (b) TFA/DCM = 1:1 under N₂ atmosphere, r.t., 1h; (c) 1.1 equiv BocGly-OH, 1.1 equiv EDC HCl, 1.1 equiv HOBt anhydrous, 3.3 equiv DIPEA, DMF under N₂ atmosphere, r.t., overnight; (d) 1.1 equiv Boc(D)Pen-OH, 1.1 equiv EDC HCl, 1.1 equiv HOBt anhydrous, 3.3 equiv DIPEA, DMF under N₂ atmosphere, r.t., overnight; (e) 2.1 equiv of *o*-dibromo-xylene, 6 days for **5a**, 1.3 equiv of *m*-dibromo-xylene, 4 days for **5b**, 2.1 equiv of *p*-dibromo-xylene, 6 days for **5c**, 2.6 equiv DIPEA in DMF under N₂ atmosphere, r.t.; (f) 1.1 equiv BocTyr-OH, 1.1 equiv EDC HCl, 1.1 equiv HOBt anhydrous, 3.3 equiv DIPEA, DMF under N₂ atmosphere, r.t., overnight; (g) 4 equiv NaOH 1M in THF, 5h for **7a**, 2 equiv NaOH 1M in THF, 2h for **7b**, 3.5 equiv NaOH 1M in THF, 3h for **7c**, r.t.

Solution phase peptide synthesis has been performed following the standard method of EDC HCl/HOBt anhydrous/DIPEA in DMF,¹⁷ in accordance with Boc protection

strategy to afford the three cyclic peptides **7a-c** as TFA salts (for the general procedures see SI). The commercially available D-Penicillamine has been converted in its methyl ester derivative **1** following the well-established literature procedure,¹⁸ and then it was submitted to coupling reaction with BocPhe-OH. Coupling reactions have been repeated together with Boc-deprotection cocktail treatment, until to reach the key intermediate compound Boc-(D)Pen-Gly-Phe-(D)Pen-OMe (**4**), which was used for the subsequent cyclization reaction (Scheme 1).

Based on the work of Benito and Meldal on bicyclic organopeptides¹⁹ we applied the same chemoselective cyclization reaction relying on the chemical linkage of peptides onto scaffolds (CLIPS) technology,²⁰ to afford our desired cyclic intermediates **5a-c** in 51%, 63% and 39% yields respectively, after silica gel chromatography. The reaction between linear intermediate **4** and 1.3 equiv. of *m*-dibromo-xylene is completed after 4 days, while the cyclization reaction to form **5a,c** requires 1 week to reach the completeness with 2.1 equiv. of alkylation reagent; this is possibly due to the steric hindrance of halogen atoms in *o*-dibromo-xylene, which makes difficult the attack of the two thiols groups; in the opposite case, the distance between the two bromine atoms in the *p*-substituted regioisomer, hampers the ring closure, thus prolonging further the reaction time and requiring more equivalents of reagent. Then the Boc protecting group has been removed from **5a-c** and Boc-Tyr-OH has been attached to obtain the full cyclic sequences **6a-c**. Finally, saponification under basic condition occurred following the same behavior of the *side chain-to-side chain* cyclization in terms of time and reagents converting the free acids compounds into the corresponding TFA salts. The purification by RP-HPLC chromatography allowed to obtain the desired cyclic peptides **7a-c** with a grade of purity $\geq 95\%$ in 41%, 55% and 40% overall yields respectively. Analysis of cyclic penta peptides incorporating thioether bridge is tricky and complex in order to achieve a rigorous proof of structure; according to a recent paper by Johnson *et al.*,²¹ full characterization of the final products **7a-c** has been performed through MS-UPLC (Figures S1-S3), analytical RP-HPLC and ¹H-NMR.

Cpds.	(K _i)		Ratio μ/δ
	[³ H]IleDelt II (δ)	[³ H]DAMGO (μ)	
DPDPE	(4.5 nM)	(438.1 nM)	97.3
7a	(16.9 nM)	(115 nM)	6.7
7b	(34.9 nM)	(292.2 nM)	8.3
7c	(546 nM)	(249 nM)	0.5

Table 1. The affinity values ($\log IC_{50} \pm$ S.E.M. and K_i) and μ/δ selectivity ratios of **7a-c** compounds and DPDPE in [³H]IleDelt II and [³H]DAMGO competition binding assays in rat brain membrane homogenates. The $\log IC_{50}$ and K_i values were calculated based on the competition binding curves seen in Figure S4.

The method applied resulted to be efficient and straightforward to furnish exhaustive qualitative and structural information on our novel molecular entities, which were used for

the competition binding assays versus μ and δ -opioid receptors;^{22,23} all the cyclic compounds **7a-c** showed good binding profile for δ -opioid receptor, although they displayed a lower affinity than DPDPE (Figure S4, Table 1). However, they showed a considerable higher affinity for μ -opioid receptor compared to DPDPE, indicating that the structural modifications on the parent compound significantly reduced its δ -opioid receptor selectivity (Table 1). This behavior closely resembles that of small cycle enkephalin analogues containing a thiourea bridge.⁶ In particular, cyclic peptide **7a** had the best K_i value for DOR and MOR (Table 1).

Antinociception assays have been also carried out to investigate the *in vivo* potential activity of the newly designed compounds.^{24,25} From the tail flick (TF) and hot plate (HP) tests we observed that compound **7b** exerted a potent analgesic effect over 60% MPE, ranging from 15 to 60 minutes, after i.c.v. administration; this result has been also confirmed by formalin test (Figure 2). The compounds **7a-c** and DPDPE were docked to the δ -opioid receptor (PDB:4RWD) to explore the impact of xylene bridge incorporation on the biological activity profile. This has been carried out by using Glide tool embedded in maestro 9.2 and employing the generated grid around 10Å from the crystallographic ligand H-Dmt-Tic-Phe-Phe-NH₂ (DIPP-NH₂).²⁶

The best docking pose for each ligand was selected basing on XP scoring function. The interaction between residue Asp128 and the amino terminus of each ligand is the most important and was preserved in all 5 models; the orientation of Tyr amino acid is overall very similar (Figure 3).

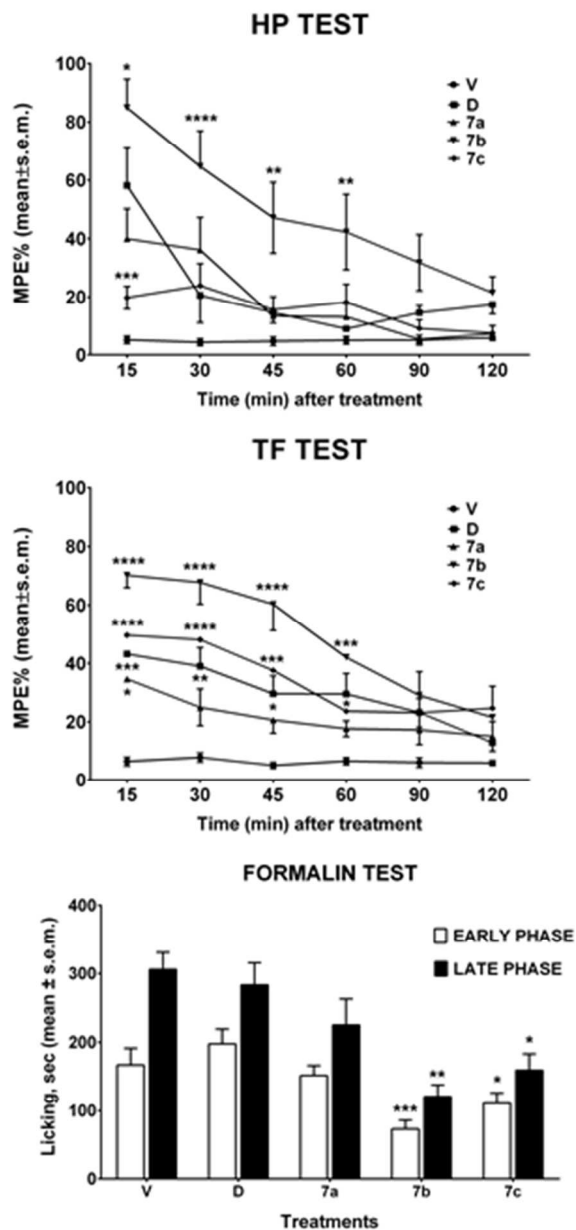


Figure 2. Tail Flick, Hot plate and Formalin Test assays on cyclic peptides **7a-c**. V is for vehicle, D is for DPDPE. In the HP and TF test, drugs were injected i.c.v. at the dose of 23 nmol/mouse. In the formalin test, drugs were administered s.c. at the dose of 150 nmol/mouse, 15 minutes before formalin. **** is for $P < 0.0001$, *** is for $p < 0.001$, ** is for $p < 0.01$, * is for $p < 0.05$ vs D. N=8-10.

The interaction with residue Arg192 has been also maintained in **7a** and **7c**, but **7b** gained the best docking score; its docking pose is stabilized by hydrogen bonds with residues Asp128, Lys214 and a saline bridge with Lys108 (Figure S5). It is worth noting that the xylene bridge enclosed in **7a-c** is typically very similar to the Tic moiety present in DIPP-NH₂.²⁶

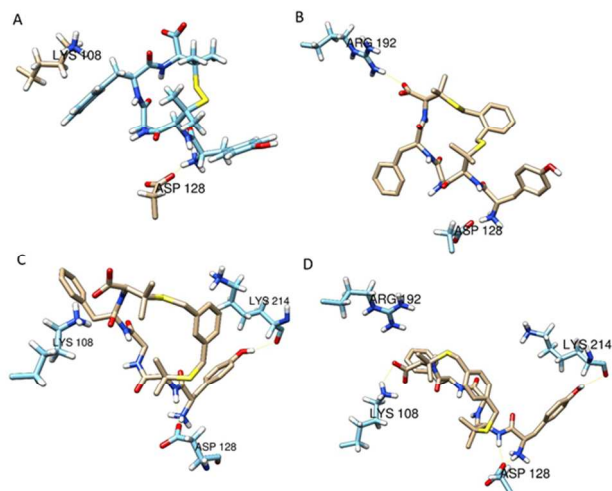


Figure 3. Best docking poses of DPDPE (A), compound **7a** (B), compound **7b** (C) and compound **7c** (D) at the DOR (only the involved residues are depicted).

However, an important difference is the orientation of the fourth aromatic ring, which may discriminate the agonist vs antagonist activity of the DPDPE cyclic analogues vs the crystallographic ligand DIPP-NH₂ (Figure S6).^{26,27}

Simple variation of xylene bridge regioisomer in a small cycle greatly impacts the biological activity, while maintaining the same peptide sequence. Considering that xylene substitution could influence the bridge orientation rendering the cycle less flexible and leading to a less favorable conformation, compound **7b** incorporating *m*-xylene bridge represent an exquisite example of well-balanced equilibrium between δ -opioid receptor affinity and analgesic potency. Compound **7b** was tested *in vivo* displaying antinociceptive activity in TF, HP and Formalin test, after i.c.v. and subcutaneous injections. Our data validate the hypothesis that the design of novel chemical entities specifically targeted at known receptors will provide advantages for pain-relief in many pathological situations. Local peripheral administration of opioid endeavored antinociceptive effects in chronic inflammatory conditions,^{28,29} although this effect was reached at relatively high doses. Previous report showed that DPDPE induced antinociceptive effects after subcutaneous administration in the formalin test, but in the second phase only.³⁰

Our data demonstrate that the novel compounds induced robust and long-lasting antinociceptive effects both after central and local peripheral administration.

EXPERIMENTAL SECTION

Methods and Materials. Boc-protected amino acids, EDC·HCl, HOBt anhydrous, DIPEA, solvents and other reagents were purchased from Sigma-Aldrich (Milano) and Iris-Biotech (Germany) and were used without further purification. Solvents for RP-HPLC and ESI-MS were of HPLC grade. All the reactions were conducted under nitrogen atmosphere. Final products **7a-c** were purified by RP-HPLC using a Waters XBridge Prep BEH130 C18, 5.0 μ m, 250 mm \times 10 mm column at a flow rate of 4 mL/min on a Waters Binary pump 1525, using as eluent a linear gradient of H₂O/acetonitrile 0.1% TFA ranging from 5% acetonitrile to 90% acetonitrile in

45 min. The purity of the N^t-Boc-protected products was confirmed by NMR analysis on a Varian Inova 300 MHz and mass spectrometry ESI-LRMS. The purity of all final TFA salts was confirmed by NMR analysis, ESI-LRMS, and analytical RP-HPLC (C18-bonded 4.6 mm \times 150 mm) at a flow rate of 1 mL/min, using as eluent a gradient of H₂O/acetonitrile 0.1% TFA ranging from 5% acetonitrile to 95% acetonitrile in 50 min and was found to be \geq 95%. ¹H NMR spectra were performed in DMSO-*d*₆ solution on a Varian Inova operating at the ¹H frequency of 300 MHz. Chemical shifts were referred to the residual proton signal of DMSO at 2.5 ppm. Peptide structures were also confirmed by UPLC-MS (see SI).

ASSOCIATED CONTENT

Supporting Information

Details of compounds characterization, synthetic procedures, molecular modeling and biological assays. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

Boc, *tert*-butyloxycarbonyl; [3H]DAMGO, [3H]-[DAla(2),N-Me-Phe-(4),Gly-ol(5)]enkephalin; [3H]-U69593, [3H]-(+)-(5 α ,7 α ,8 β)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide; DCM, dichloromethane; DIPEA, diisopropylethylamine; DPDPE, [2-D-penicillamine,5-D-penicillamine]enkephalin; [³H]IleDelt II, ile^{5,6} deltorphin II; DMF, N,N-dimethylformamide; DMSO, dimethyl sulfoxide; DOR, δ opioid receptor; EDC, 1-ethyl-(3-dimethylaminopropyl)carbodiimide; HOBt, 1-hydroxybenzotriazole; MOR, μ opioid receptor; MVD, mouse vas deferens; DIPEA, N,N-diisopropylethylamine; RP-HPLC, reversed phase high performance liquid chromatography; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TMS, tetramethylsilane; MS-UPLC, ultra-performance liquid-chromatography tandem mass spectrometry.

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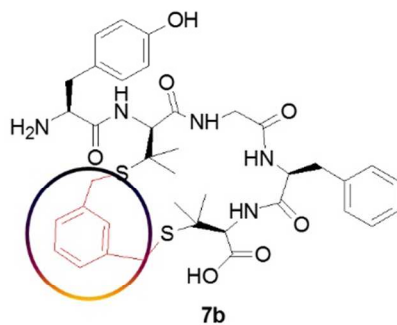
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$K_i^{\delta} = 34.9 \text{ nM}$

