Tetrabranched Hetero-Conjugated Peptides as Bifunctional Agonists of the NOP and mu Opioid Receptors

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KEYWORDS: Bifunctional ligands; Multimeric peptides; Dual acting ligands; NOP receptor; MOP receptor; PWT technology.

ABSTRACT: The general aim of the work was the validation of a new synthetic methodology designed for obtaining bifunctional heterotetrabranched peptide ligands. Applying an easy accessible synthetic route, we provided a small series of heteromultimeric peptide conjugates targeting the nociceptin/orphanin FQ (N/OFQ) peptide receptors (NOP) and mu opioid receptors. Among these, H-PWT1-N/OFQ-[Dmt[1]]dermorphin demonstrated a similar and high agonist potency at the NOP and mu receptors. The achieved results confirmed the robustness of the approach that is extremely versatile and virtually applicable to different peptide sequences whose pharmacological activity can be combined for generating dual acting multimeric compounds. These innovative pharmacological tools will be extremely helpful for investigating the consequences of the simultaneous activation and/or blockage of different peptidergic receptors.

INTRODUCTION

Bioactive peptides obtained from natural sources hold great potential for the development of drugs because of their capability to modulate a broad panel of biological functions excelling in potency and selectivity of action with a usually low toxicity profile. However, as largely known, peptides suffer from a short duration of action when administered for therapeutic purposes because of their fast degradation by peptidases. Despite this, the number of peptide drugs being placed on the market has been extraordinarily increasing in the last two decades.[1,2] In most cases, this has been made possible by the identification of chemical strategies useful for improving peptide resistance to peptidases. In the last few years, with the development of the so-called peptide welding technology (PWT), we provided evidence that the multimerization of bioactive peptides can be one of the possible approaches to be exploited for extending their limited half-life.[3] The methodology, that is based on a high efficacy chemical synthesis for the generation of tetra-branched peptide conjugates, is of particular interest since being an elegant way to improve the drug-likeness and pharmacokinetic properties of bioactive peptides in view of their potential therapeutic employment.

All PWT-derivatives reported to date have been efficiently prepared following a convergent synthetic approach based on the conjugation of a clustering core with four linear monomers of the same target peptide after their independent synthesis.[4] Three different cores have been exploited, each of which strategically functionalized with four reactive maleimide groups as depicted in figure 1 (PWT1 with a tris-Lysine motif, PWT2 a cyclam-based scaffold and PWT3 a symmetric lysine-ethylendiamine derivative).[3] The classical procedure consisted in a thiol-Michael reaction between the central reactive scaffold and the peptide of interest in which a key cysteine residue was conveniently introduced.[4]

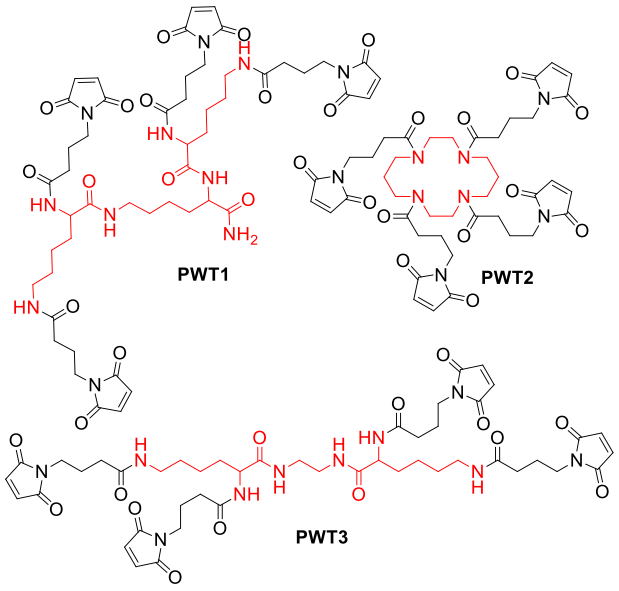


Figure 1. Chemical structures of the clustering cores used for generating PWT peptides.

Thanks to this highly chemo-selective conjugation, different GPCR-targeting peptides have been homotetramerized such as nociceptin/orphanin FQ (N/OFQ),[5] N/OFQ related peptides,[3] tachykinins[6] and neuropeptide S.[7] Of note, the resulting multi branched ligands exhibited an in vitro pharmacological profile similar to that of the native peptides. Even more important, a higher potency and a marked prolongation of action in vivo have been generally observed, possibly due to reduced susceptibility to the action of proteolytic enzymes.[8] Thus, the first generation of PWT derivatives have been considered as valuable

pharmacological tools suitable for investigating in vivo the effects of the potent, selective and prolonged stimulation/blocking of a single target in physiopathological conditions.

The innovative purpose of this study is the design, synthesis, and in vitro pharmacological characterization of the first examples of heteromultivalent PWT derivatives of potential interest for the study of multifactorial diseases where the simultaneous modulation of multiple signaling systems can be advantageous compared to the activation/blocking of a single target. To this aim, we focused on the recent evidence that molecules that are able to modulate multiple opioid receptors (currently classified as MOP/μ, DOP/δ, KOP/κ and NOP, the opioid related N/OFQ peptide receptor) may result into novel opioid analgesics possibly with reduced side effects.[9] Most progress has been made in the development of NOP/MOP bifunctional agonists that have been demonstrated to relieve some of the side effects of pure MOP agonists, including tolerance, dependence, constipation and respiratory depression.[9] Of note, NOP receptor is co-localised with the μ opioid receptor in the pain pathways[10] where they seem to be involved in the formation of heterodimers.[11-13] In addition the co-administration of N/OFQ and MOP receptor agonists (i.e. morphine, buprenorphine) in non-human primates was shown to enhance the antinociceptive effects of classical opioid analgesics reducing their remarkable side effects.[14,15]

Overall, these findings inspired the synthesis of peptide and non-peptide bifunctional NOP/MOP ligands. Among these, the small molecule agonists BU08028 e SR16435 have emerged for their antihyperalgesic and antiallodynic action in animal models of inflammatory and neuropathic pain with higher potency if compared to selective NOP or MOP ligands.[16] AT-121 is another relevant example of bifunctional NOP/MOP that displayed analgesic properties without opioid side effects in non-human primates.[17] Furthermore, the mixed opioid/NOP receptors agonist Cebranopadol is a novel first-in-class analgesic currently in clinical development by the company Grünenthal for the treatment of different acute and chronic pain states.[18,19] In addition, a few peptide-based bifunctional NOP/MOP receptor ligands have been obtained through the generation of chimeric peptides containing an opioid and a N/OFQ receptor-binding pharmacophore that have been merged in a linear sequence.[20-23]

A first attempt to combine the PWT technology to the principles of polypharmacology consisted in the homotetramerization of the peptide [Dmt[1]]N/OFQ(1–13)-NH_2 that behaves as a potent agonist for both NOP and classical opioid receptors and elicits robust anti-nociceptive effects after spinal administration in non-human primates.[24] PWT2-[Dmt[1]]N/OFQ(1–13)-NH_2 displayed the same potency of the linear parent peptide in vitro and a prolonged action in vivo.[25] Here we described a new chemical strategy that allowed us to exploit the PWT technology to the obtainment of NOP/MOP bivalent heterotetrameric peptides. In particular, we synthetized a novel PWT core (H-PWT1, depicted in figure 2) featuring two maleimide and two benzaldehyde groups that have been independently reacted with two chains of N/OFQ, as native ligand of the NOP receptor, and two chains of dermorphine or dermorphine-related peptides, as MOP agonists.

RESULTS AND DISCUSSION

Chemistry

For the synthesis of the new heterotetrameric PWT derivatives, we employed the same convergent approach previously applied for the classical homotetrameric compounds. This is generally characterized by high efficiency in terms of feasibility, reaction yields and easy of purification. Thus, we firstly synthesised in liquid phase the central core H-PWT1 starting from a residue of Lys-NH_2 as described in Scheme 1. This was coupled with two molecules of Z-Lys(Boc)-OH in standard conditions to give the trilysine derivative **1**. After the selective removal of Cbz by catalytic hydrogenation, the resulting Boc protected intermediate was coupled with 4-maleimidobutirryc acid leading to the bis-functionalized core **2**. Then, Boc deprotection followed by a second coupling with 4-formylbenzoic acid gave the desired hetero-functionalized H-PWT1 (**3**) as the first building block of the convergent synthetic route.

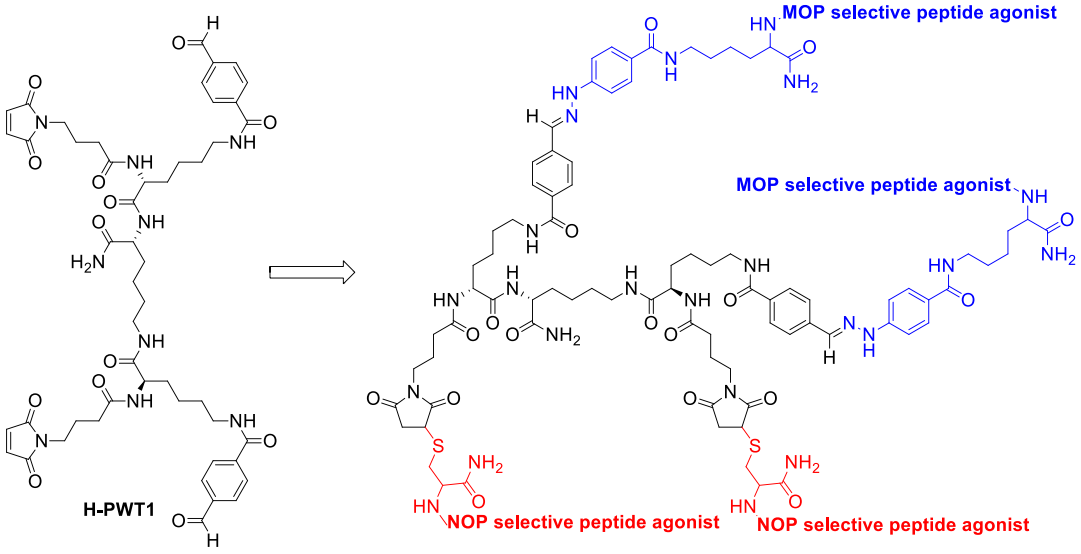
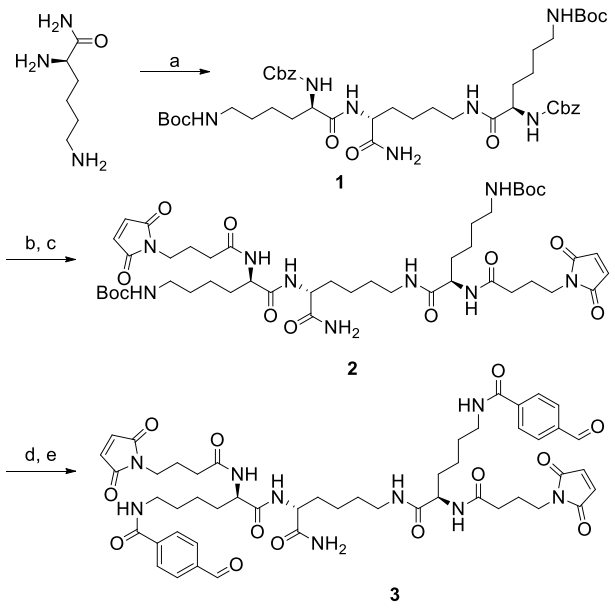


Figure 2. General design for the generation of heterotetrameric H-PWT1 derivatives as NOP/MOP dual acting ligands.

The next stage was the synthesis of the target peptides containing an additional anchoring residue in the linear sequence. In this regard, the native sequence of N/OFQ (FGGFTGARKSARKLANQ) with a supplementary cysteine residue at the C-terminal position was synthesised following a classic solid phase technique as previously described.[4] The thiol residue of [Cys[18]]N/OFQ-NH_2 has been introduced in view of the possible functionalization of H-PWT1 via a thiol-Michael reaction with the two maleimide moieties. The absence of additional cysteine residues in the sequence of N/OFQ ensured the chemoselectivity of the final conjugation step. Previous SAR studies and crystallographic analysis clearly suggested that the C-terminal region of N/OFQ represents an appropriate attachment point that allows peptide modifications without loss of activity and selectivity.[4]

Scheme 1. Synthesis of the orthogonally functionalized H-PWT1 core 3.

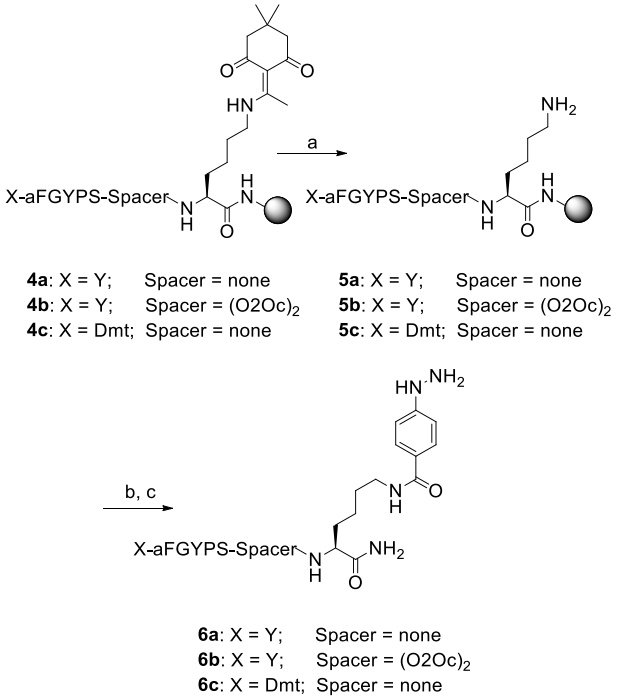


Reagents and conditions: a) Z-Lys(Boc)OH, NMM 30%, Isobutylchloroformate, DMF; b) H_2, Pd/C, MeOH; c) 4-Maleimidobutyric acid, HATU, DIPEA, DMF; d) TFA; e) 4-Carboxybenzaldehyde, HATU, DIPEA, DMF.

Dermorphin has been selected for the development of the MOP agonist component of the designed heterotetrameric NOP/MOP dual acting ligands. In one of the investigated examples the native sequence of the peptide (YaFGYPS-NH_2) was synthetized in solid phase and elongated at the C-terminal position with a Lysine(Dde) residue till the obtainment of the resin supported peptide **4a** as depicted in Scheme 2. Then, the selective deprotection of Dde with 20% hydrazine solution in DMF[26] led to a free amine group on the lysine side chain (**5a**) that was coupled with 4-(2'-*N*-Boc-hydrazino)benzoic. The final cleavage with a cocktail of TFA/H_2O/triisopropylsilane gave the desired Dermorphin derivative **6a** functionalized with a reactive hydrazine group suitable for the linking to the aldehyde moieties of **3**. As in the case of N/OFQ, the c-terminal derivatization of dermorphine has been shown to be well tolerated for the maintainance of the peptide activity.[20]

In the second Dermorphin analog **6b**, two polyoxyethylene units have been introduced as spacer between the MOP pharmacophore and the C-terminal anchoring residue. This has been made with the aim to improve the water solubility of the resulting tetrabranched derivative and to evaluate the effect of the lengthening of the distance between the message domain and the central core. Finally, we synthesised **6c**, a novel Dermorphine derivative in which the Tyr[1] residue has been replaced by the non-natural aminoacid 2,6-dimethyl tyrosine (Dmt). Dmt has been extensively exploited for its contribution in increasing the potency and metabolic stability of opioid peptides when introduced instead of Tyr at the first position.[27-30] Nevertheless, to the best of our knowledge this kind of modification has never been performed before on the message domain of dermorphine. Thus, we decided to adopt this strategy speculating that it could be effective in improving opioid agonist activities in our bivalent tetramers.

Scheme 2. Synthesis of the hydrazinobenzoic dermorphin derivatives 6a-c.



4a: X = Y; Spacer = none
4b: X = Y; Spacer = $(O2Oc)_2$
4c: X = Dmt; Spacer = none

5a: X = Y; Spacer = none
5b: X = Y; Spacer = $(O2Oc)_2$
5c: X = Dmt; Spacer = none

6a: X = Y; Spacer = none
6b: X = Y; Spacer = $(O2Oc)_2$
6c: X = Dmt; Spacer = none

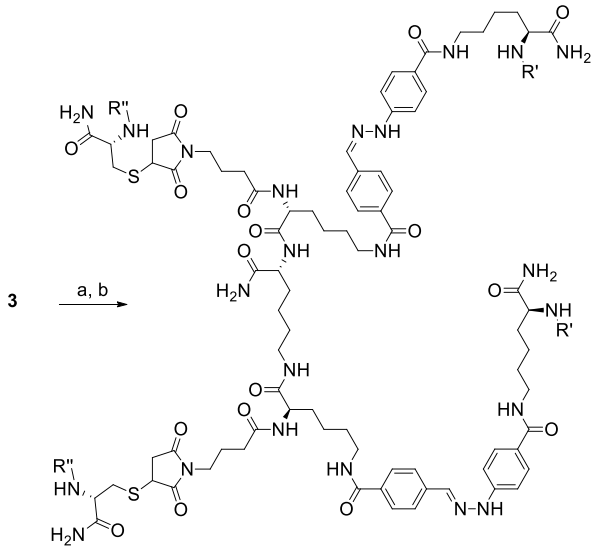
Reagents and conditions: a) 20% Hydrazine, DMF; b) 4-(2'-N-Boc-hydrazino)benzoic acid, HATU, DIPEA, DMF; c) TFA/H_2O/Triisopropylsilane.

In the last step of the synthetic method, the desired heteromultimeric peptide derivatives were obtained via conjugation of **3** with two molecules of [Cys[18]]N/OFQ-NH_2 and two molecules of **6a**, **6b** or **6c** following the one-pot, two-steps approach depicted in scheme 3. First, two equivalents of [Cys[18]]N/OFQ-NH_2 were reacted with **3**, in mild condition, using $NaHCO_3$ as catalysts for the thiol-Michael addition between the C-terminal cysteine residue and the maleimide functions. After 5 minutes, UPLC-MS analysis indicated the complete consumption of the starting reagents and the quantitative formation of the corresponding semi-functionalised thiol-ether intermediate. In the second step, the hydrazine group of the dermorphin-related peptides were reacted with the remaining aldehyde functions of the core. The formation of the hydrazone adduct was performed in

the same reactor by adding CH_3COOH until reaching pH 5 before the addition of **6a-c**. UPLC-MS analysis indicated the quantitative formation of **7a** (H-PWT1-N/OFQ-dermorphin), **7b** (H-PWT1-N/OFQ-dermorphin$(O2Oc)_2$) and **7c** (H-PWT1-N/OFQ-[Dmt^1]dermorphin) within 30 minutes. The target compounds were then easily purified via preparative HPLC.

In the resulting tetrabranched derivatives, the four peptide chains were stably linked to the central core through thioether or hydrazone bonds in analogy to the strategies currently in use for the generation of chemoimmunoconjugates.[31] In order to confirm the actual stability of these linkers in the conditions required for the biological assay discussed below, H-PWT1-N/OFQ-dermorphin was dissolved in HBSS supplemented with 20 mM HEPES (pH 7.5) and incubated at 40 °C for a maximum of 4h. Samples of the solution were taken up after 1 and 4 hours and analysed via RP-HPLC. The resulting chromatograms confirmed that no appreciable degradation of the molecule occurred within the considered times (see Figure S1 of the supporting information).

Scheme 3. One-pot, Two-step strategy for the hetero bioconjugation of peptides.



7a: R' = YaFGYPS; R'' = FGGFTGARKSARKLANQ
7b: R' = YaFGYPS$(O2Oc)_2$; R'' = FGGFTGARKSARKLANQ
7c: R' = Dmt-aFGYPS; R'' = FGGFTGARKSARKLANQ

Reagents and conditions: a) [Cys^{18}]N/OFQ-NH_2, 5% $NaHCO_3$, DMF; b) CH_3COOH, **6a-c**, DMF.

The following linear monomers have been synthetized as internal references in standard solid phase conditions: N/OFQ, YaFGYPS-NH_2 (dermorphin), YaFGYPS$(O2Oc)_2$-NH_2 (dermorphin$(O2Oc)_2$-NH_2), Dmt-aFGYPS-NH_2, ([Dmt^1]dermorphin). In addition, for comparative reasons, the homo tetrabranched derivatives PWT1-N/OFQ,[5] PWT1-dermorphin, PWT1-dermorphin$(O2Oc)_2$ and PWT1-[Dmt^1]dermorphin have been prepared in analogy to the previously described approach (HPLC chromatograms and ESI mass spectra have been reported in the supporting material).[4]

Pharmacology

The described ligands were assayed in calcium mobilization studies performed in CHO cells coexpressing either the human recombinant NOP or MOP receptors and the chimeric protein $G\alpha_{qi5}$.[32,33] The NOP and MOP agonist potencies of the heterotetramers **7a-c** were expressed as pEC_{50}s and compared to those of the parent peptide monomers and the reference homotetrabranched derivatives investigated under the same experimental conditions (see Table 1). This calcium mobilization assay has been previously validated with a large panel of NOP and opioid ligands[32,33] and the standard agonists used in the present work (N/OFQ and dermorphin) displayed potencies and selectivity profiles consistent with these studies. In particular, N/OFQ evoked a concentration dependent stimulation of calcium release in cells expressing the human NOP receptor with high potency (pEC_{50} = 9.52) and maximal effects (308 ± 30% over basal values), while dermorphin was inactive up to micromolar concentrations. Conversely, dermorphin behaved as a potent full agonist of the MOP receptor (pEC_{50} = 8.09; E_{max} 324 ± 18% over basal values) toward which N/OFQ resulted inactive.

In the first instance, the standard opioid agonists N/OFQ and dermorphin have been tetramerized in compound H-PWT1-N/OFQ-dermorphin (**7a**) that elicited a potent stimulatory action both in cells expressing the NOP receptor and in those transfected with the MOP receptor with maximal effects similar to those of the corresponding linear monomers.

These encouraging results demonstrated that the new heteromultimerization strategy did not compromise the pharmacological activity of the starting bioactive peptides, in line with what previously observed in the same assay for most of PWT-based homotetramers.[3,5-7] Even though H-PWT1-N/OFQ-dermorphin displayed an interesting profile of mixed NOP/MOP full agonist, the potency of the compound in activating the NOP receptor (pEC_{50} = 8.09) was almost 30-fold lower if compared to that of unconjugated N/OFQ. Likewise, **7a** resulted 10-fold less potent than dermorphin in stimulating the MOP receptor (pEC_{50} = 7.13). This would indicate that the H-PWT1-based assembling of the analysed bioactive sequences disfavours to some degree their interaction with the respective biological targets. This effect was also evident from the in vitro pharmacological profile of the homotetrabranched derivatives PWT1-N/OFQ (Table 1)[5] and PWT1-dermorphin that behaved, respectively, as a NOP-selective and a MOP-selective agonist 3-10 fold less potent than the native peptide precursors.

Moreover, the bifunctional profile of **7a** was considered suboptimal in view of the unbalanced NOP/MOP potency i.e. 10-fold higher potency for the NOP receptor. This prompted us to investigate suitable strategies for the structural modification of the dermorphin sequence with the aim to increase the MOP potency of the resulting heterotetrabranched derivatives. Firstly, we designed compound **7b** (H-PWT1-N/OFQ-dermorphin$(O2Oc)_2$) in which the MOP pharmacophore was spaced from the central PWT core through two polyoxyethylene units. The hydrophilic linker was selected because of the low water solubility experienced with the homotetrabranched derivative PWT2-dermorphin previously described.[3] In addition, we speculated about the possibility that a flexible linker of the proper length could facilitate the interaction of the message sequence of dermorphin with the binding pocket of the MOP receptor. Compared to **7a**, H-PWT1-N/OFQ-dermorphin$(O2Oc)_2$ displayed a substantial maintenance of NOP potency (pEC_{50} = 8.21) but only a marginal increase in MOP potency (pEC_{50} = 7.42). However, it has to be considered that the modification of the dermorphin sequence in the derivative dermorphin$(O2Oc)_2$-NH_2 resulted per se slightly detrimental for MOP potency.

Table 1. In vitro effects of the synthetized compounds in calcium mobilization studies performed on CHO cells coexpressing either the NOP or MOP receptor and the $G\alpha_{qi5}$ chimeric protein.

	NOP		MOP		NOP/MOP
	pEC_{50} ($CL_{95\%}$)	**α ± sem**	**pEC_{50} ($CL_{95\%}$)**	**α ± sem**	
Linear peptide monomers					
N/OFQ	9.52 (8.97-10.06)	1.00	< 6	-	> 1000
Dermorphin	< 6	-	8.09 (7.44-8.74)	1.00	< 0.01
Dermorphin(O2Oc)$_2$NH$_2$	< 6	-	7.74 (7.21-8.27)	0.94 ± 0.06	< 0.1
[Dmt^1]dermorphin	< 6	-	8.76 (8.42-9.10)	0.91 0.07	< 0.01
Homotetrameric PWT1 derivatives					
PWT1-N/OFQ	8.44 (8.13-8.75)	1.08 ± 1.07	< 6	-	> 100
PWT1- dermorphin	< 6	-	7.53 (7.23-7.84)	0.88 ± 0.05	< 0.1
PWT1-dermorphin(O2Oc)$_2$	< 6	-	7.34 (6.81-7.88)	0.80 ± 0.08	< 0.1
PWT1-[Dmt^1]dermorphin	< 6	-	9.64 (9.20-10.08)	1.04 ± 0.06	< 0.001
Heterotetrameric H-PWT1 derivatives					
H-PWT1-N/OFQ-dermorphin (7a)	8.09 (7.98-8.20)	0.99 ± 0.04	7.13 (6.90-7.37)	0.93 ± 0.03	0.11
H-PWT1-N/OFQ-dermorphin(O2Oc)$_2$ (7b)	8.21 (8.03-8.40)	1.09 ± 0.09	7.42 (7.25-7.59)	0.91 ± 0.03	0.16
H-PWT1-N/OFQ-[Dmt^1]dermorphin (7c)	8.35 (7.71-8.99)	0.88 ± 0.03	8.75 (8.32-9.19)	1.00± 0.04	2.5

Thus, in the case of **7b** the H-PWT1-derivatization of the dermorphin component did not significantly affect the MOP potency of the linear precursor. This was also confirmed in the homotetrameric derivative PWT1-dermorphin(O2Oc)$_2$ displaying similar potency as PWT1-dermorphin.

In the last derivative H-PWT1-N/OFQ-[Dmt^1]dermorphin (**7c**), the dermorphin component was directly linked to the core but modified at the N-terminal portion where the Tyr^1 residue was replaced with a 2,6-dimethyl tyrosine. The corresponding linear precursor [Dmt^1]dermorphin was shown to be 5-fold more potent than the native peptide analogue (pEC_{50} = 8.76) confirming the important effect of this chemical modification in promoting the interaction of opioid peptide ligands with the MOP receptor.[27-30] Surprisingly, and unlike most of the previously reported PWT derivatives, the newly described homotetrabranched PWT1-[Dmt^1]dermorphin exhibited a significantly improved MOP-agonist potency compared to the parent peptide monomer (pEC_{50} = 9.64). This would suggest that, in the case of compound PWT1-[Dmt^1]dermorphin, the beneficial effect of the introduction of Dmt^1 was amplified by the presence of multiple modified message domains that would cooperate synergistically in MOP receptor binding and activation. It can be speculated that the molecular flexibility and the relatively high distance between the four pharmacophores of the investigated multivalent ligand, make it possible the concurrent targeting of functional dimers or oligomers of the mu-opioid receptor.[34]

Of relevance for the aim of this work, the introduction of a Dmt^1 residue had also a positive impact on the pharmacological profile of compound **7c**. Indeed, the MOP potency of H-PWT1-N/OFQ-[Dmt^1]dermorphin was significantly incremented in comparison with **7a** and **7b** to such an extent that the NOP/MOP potency ratio was reversed (NOP pEC_{50} = 8.35, MOP pEC_{50} = 8.75). Even in this case, the maximal effects of the compound at both the investigated receptors were comparable to those of the unconjugated peptide precursors with no changes in ligand efficacy. Thus, H-PWT1-N/OFQ-[Dmt^1]dermorphin exhibited a profile of dual acting full agonist with high and balanced potencies for NOP and MOP receptors.

In order to confirm this result with a different assay, H-PWT1-N/OFQ-[Dmt^1]dermorphin has been investigated in DMR studies performed in CHO cells expressing the human MOP or NOP receptors (figure 3). DMR is a label free assay that gives the

possibility to measure, in a noninvasive manner, receptor-dependent holistic cellular responses.[35] DMR studies have been performed in recent years investigating the pharmacological profile of several GPCR including classical opioid[36] and NOP[37] receptors. As shown in figure 3, N/OFQ elicited a concentration dependent DMR response in cells expressing the NOP receptor (pEC_{50} 9.48, Emax 236 ± 33 pm) being inactive in MOP cells. Opposite results were obtained with dermorphin that evoked DMR responses in cells expressing the MOP receptor (pEC_{50} 8.98, Emax 152 ± 28 pm) being inactive in NOP cells. Similar results have been previously obtained with both peptides in our laboratories.[37] Importantly N/OFQ and dermorphin up to 1 µM were inactive in wild type CHO cells (data not shown). H-PWT1-N/OFQ-[Dmt^1]dermorphin produced similar DMR responses in cells expressing the NOP or the MOP receptors. However, the potency and maximal effects of H-PWT1-N/OFQ-[Dmt^1]dermorphin could not be estimated in these experiments since at 1 µM the tetrameric peptide elicited statistically significant DMR responses in wild type CHO cells (data not shown). This result was not completely unexpected since similar findings has been previously reported in DMR studies investigating the homomeric PWT derivatives of N/OFQ[37] and neuropeptide S.[38] Corroborating these findings, bioassay studies performed with tissues taken from wild type and NOP receptor knockout mice demonstrated that PWT-N/OFQ displays reduced selectivity than the parent peptide. The implications of these findings are twofold: i) a certain loss of selectivity compared with the parent peptide(s) is probably a common feature of both homo and heteromeric PWT derivatives and ii) the label free DMR assay is superior than single end point assays for revealing this aspect. Despite the above mentioned limitations, DMR experiments confirmed that H-PWT1-N/OFQ-[Dmt^1]dermorphin behaves as mixed NOP/MOP agonist able to stimulate the two receptors in the same range of concentrations.

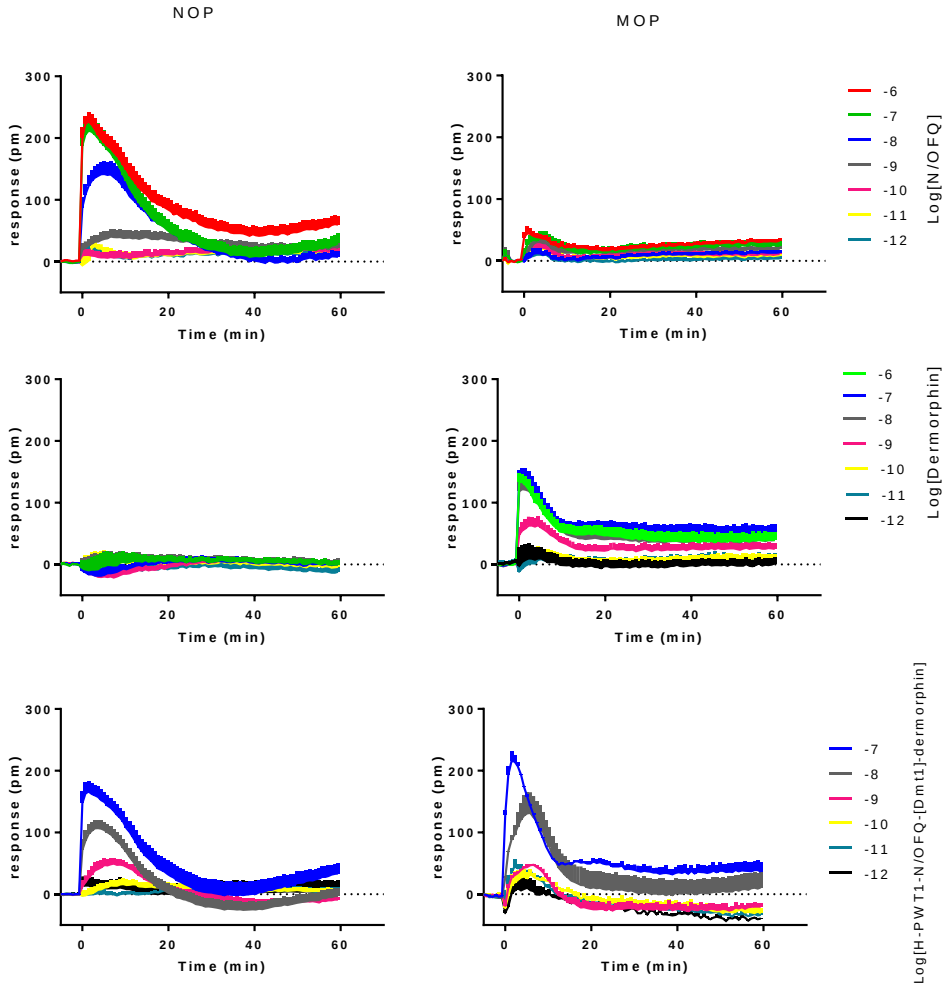


Figure 3. DMR traces of N/OFQ (top panels), dermorphin (middle panels) and H-PWT1-N/OFQ-[Dmt^1]dermorphin (bottom panels) in CHO cells expressing the NOP (left panels) and the MOP (right panels) receptors. Data are the mean ± sem of 4 experiments performed in duplicate.

CONCLUSION

In conclusion, with this work we validated an easy accessible synthetic methodology that allows obtaining heterotetrameric peptide ligands with a bifunctional pharmacological profile. Overall, the results of this study will let the scientific community to increase the knowledge regarding the design and synthesis of bivalent ligands for peptidergic receptors. As above detailed, the approach has been successfully applied to the identification of the NOP/MOP mixed full agonist H-PWT1-N/OFQ-[Dmt[1]]dermorphin which exhibited a balanced potency at the investigated targets with EC_{50} values in the low nanomolar range. The available evidence suggests that dual acting NOP/MOP agonists may have therapeutic potential in the treatment of pain as non-addictive analgesics and possibly as medications to treat drug abuse.[23,17,39]

The newly disclosed compound H-PWT1-N/OFQ-[Dmt[1]]dermorphin can be considered a valuable tool for future in vivo studies aimed at investigating the effects of the simultaneous stimulation of the NOP and MOP receptors in disease models. This will allow the understanding and, possibly, the expansion of the therapeutic potential of opioid bivalent ligands for treating any disease that can likely benefit by multi-target drug treatments. In vivo studies will be useful to establish if the newly described methodology can be exploited to enhance the in vivo potency of the investigated peptides and particularly their duration of action, as previously demonstrated with different examples of homotetrameric PWT derivatives.[3] Of note, the strategy herein described is extremely versatile and virtually applicable to any peptide sequence, thus, this approach could be translated in the future to the development of bivalent compounds targeting other receptor systems. As a consequence, the achieved results could be extremely helpful to explore new therapeutic perspectives and pave the way to innovative pharmacological treatments for diseases in which the concurrent activation or blockade of two different receptors is more effective / safer than the selective modulation of a single target.

ASSOCIATED CONTENT

The Supporting Information is available free of charge on the ACS Publications website.

Detailed synthetic procedures, HPLC chromatograms and ESI mass spectra of compounds **1-3**, **6a-c**, **7a-c**, **PWT1-N/OFQ, PWT1-dermorphin**, **PWT1-dermorphin(O2Oc)$_2$ PWT1-[Dmt[1]]dermorphin**, procedures for biological experiments (PDF).

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Author Contributions

S.P., V.A., D.I., A.F., E.M. performed the chemical synthesis; F.F., C.S., J. A. N., C.R. performed the pharmacological studies; G.C., R.G., D.P. designed the experiments, analysed the data and wrote the manuscript.

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Notes

The authors declare no competing financial interest.

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