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Graphical Abstract

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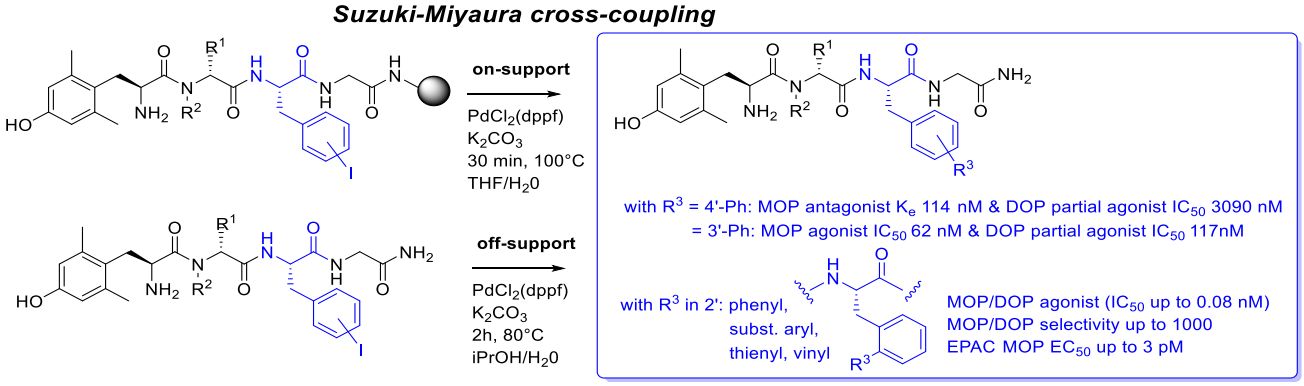

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ABSTRACT

In this study, affinities and activities of derivatized analogues of Dmt-dermorphin[1-4] (i.e. Dmt-D-Ala-Phe-GlyNH$_2$, Dmt = 2',6'-dimethyl-(*S*)-tyrosine) for the μ opioid receptor (MOP) and δ opioid receptor (DOP) were evaluated using radioligand binding studies, functional cell-based assays and isolated organ bath experiments. By means of solid-phase or solution-phase Suzuki-Miyaura cross-couplings, various substituted regioisomers of the phenylalanine moiety in position 3 of the sequence were prepared. An 18-membered library of opioid tetrapeptides was generated *via* screening of the chemical space around the Phe^3 side chain. These substitutions modulated bioactivity, receptor subtype selectivity and highly effective ligands with subnanomolar binding affinities, contributed to higher functional activities and potent analgesic actions. In search of selective peptidic ligands, we show here that the Suzuki-Miyaura reaction is a versatile and robust tool which could also be deployed elsewhere.

Alleviation or treatment of pain remains a significant challenge in pain research. Although opioid therapy is the cornerstone of severe and chronic pain management, serious unwanted effects are associated with their (chronic) administration (e.g., physical dependence, tolerance, nausea).[1] The biological effects of these drugs are exerted via binding to the three opioid receptor subtypes (μ–, δ–, and κ–opioid receptors termed MOP, DOP and KOP, respectively), belonging to the superfamily of G protein-coupled receptors.[2, 3] One possible approach to overcome the limitations of opioids is the use of ligands with mixed activity profile.[4] It has, for example, previously been shown that dual MOP/DOP agonism or mixed agonism/antagonism were advantageous over highly-selective receptor subtype ligands.[5-11]

The opioid heptapeptidedermorphin (H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH$_2$), initially derived from the skin of the South American frog *Phyllomedusasauvagei*, was found to be a potent and selective MOP agonist.[12-14] Compared to morphine, dermorphin was shown to possess higher antinociceptive efficacy with decreased adverse effects.[15] SAR studies indicated the *N*-terminal tetrapeptide to be the minimal sequence required for potent opioid responses. In addition, it had been shown that Dmt^1 replacement of tyrosine afforded ligands with enhanced MOP and DOP bioactivity.[16] Here, we attempted to modulate the pharmacological profiles of the peptidic ligands by modifying the second key aromatic pharmacophore group, the Phe^3 side chain. It was hypothesized that the Dmt^1 residue of the opioid peptide would reach deeply into the orthosteric binding pockets, while the Phe^3 side chain would position itself at the outer boundaries of the binding pocket of the receptors. For DOP, the Phe^3 side chain reaches a potential subpocket created by the side chains of H^{301}, K^{108}, Y^{109}, E^{112} and V^{197}. In case of MOP, the side chain may access a pocket defined by the side chains of Q^{124}, H^{319}, W^{318} and I^{322}, and hence in both cases additional hydrophobic, dipole-dipole and cation-π interactions could for instance be made between ligand and receptor by substituting the second aromatic ring in opioid peptides.

In the present work, we report the synthesis and the *in vitro/in vivo* biological evaluation of potent opioid peptide ligands with mixed MOP/DOP activity profile by extension of the Phe^3

Abbreviations: BRET, bioluminescence resonance energy transfer. cAMP, cyclic adenosine monophosphate. Dmt, 2,6-dimethyltyrosine, DOP, δ-opioid receptor. EC_{50}, Half maximal effective concentration. EPAC, exchange protein directly activated by cAMP. GPI, guinea pig ileum. IC_{50}, half maximal inhibitory concentration. K_i, inhibitory constant. KOP: κ-ipioid receptor. MOP, μ-opioid receptor. MVD, mouse vas deferens. P.A., partial agonist. TES, triethylsilane.

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aromatic core. Introduction of a 'halogen derivatization handle' allowed structural modification via Suzuki-Miyaura cross-couplings.[17, 18]

This robust C-C coupling allows the efficient introduction of substituents on arylated amino acid residues in peptide substrates.[19-23] Previously, this bioorthogonal methodology has been applied for the derivatization of peptides,[24-28] leading to altered biological profile and therapeutic effect[27, 29] implementing both solution-phase[21] and solid-phase[30-33] Suzuki-Miyaura reactions. The functional group compatibility of Suzuki-Miyaura reactions with peptidic substrates has recently been reviewed elsewhere.[22, 27]

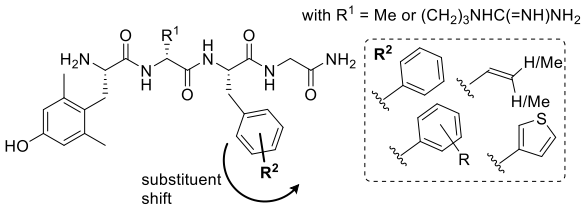


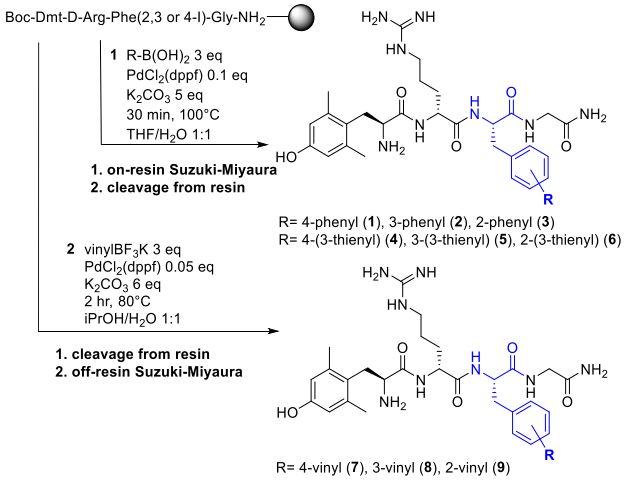
Figure 1. Phe[3] derivatizations within the opioid tetrapeptides H-Dmt[1]-D-Arg/Ala[2]-Phe[3]-Gly[4]-NH_2

The commercial availability of the different regioisomers of iodo-(*S*)-phenylalanine and straightforward incorporation into opioid tetrapeptides provided, after derivatization, a diverse library including (hetero)aromatic or vinylic substituents (Figure1) and imposed profound effects on the biological activity. The dermorphin(1-4) analogues **1-9** were prepared via standard N_α-Fmoc-based solid phase peptide synthesis using Rink Amide resin as solid support. The Dmt[1] residue was inserted as Boc-Dmt-OH (2 eq), and with DIC/HOBt (each 2 eq) to avoid acylation on the unprotected phenol moiety. The Suzuki-Miyaura reactions, making use of (hetero)aromatic boronic acids as coupling partners, were performed on the solid support bearing the fully protected halogenated tetrapeptides (Scheme 1).

Catalyst screening showed that the $PdCl_2$(dppf) catalyst, which was shown to be compatible to peptide diversification in solution phase,[22] allowed to prepare the cross-coupled products with excellent conversions on support. Hence, the cross-couplings on solid-phase were realized with $PdCl_2$(dppf) (10 mol%) as the precatalyst system in combination with K_2CO_3 (5 eq) and (hetero)aromatic boronic acids (3 eq). Near complete conversions were attained after 30 min in a mixture of THF/H_2O (1/1) at 100°C, while gently stirring the resin beads in MW vials. After successful cross-coupling, the peptide analogues were cleaved from the resin by treatment with a mixture of TFA/TES/H_2O (95:2.5:2.5 v/v/v). The crude peptides were obtained after evaporation of the cleavage mixture and purified by preparative HPLC to yield the target peptides (>95% purity).

Due to the occurrence of side product formation during cleavage, a different strategy was pursued to access the vinylated analogues. The vinyl group was chosen as a substituent of limited size which additionally offers a possibility for further diversification or peptide cyclization. Here, the peptides were first assembled on solid support and cleaved to obtain the iodinated precursor peptides (Scheme 1). As mentioned above, this (still convergent) strategy was followed due to the limited stability of vinylated products toward highly acidic (95% TFA) conditions. After optimization of the reaction conditions, $PdCl_2$(dppf) (5mol%) was again found suitable for these transformations in combination with K_2CO_3 (6 eq) as the base and potassium vinyltrifluoroborate (3 eq) as the boron coupling partner. For these couplings trifluoroborates, bench-stable analogues of boronic acid, were used.[34, 35] In this case, optimal conversion was reached in a 1:1 mixture of H_2O/*i*PrOH at 80°C for 2 hours. The peptides were obtained after purified by preparative HPLC with a yield ranging from 13-39% (>95% purity).

Scheme 1. Preparation of the first peptide set using on-resin and off-resin Suzuki-Miyaura cross-couplings



The obtained set of peptide analogues (**1-9**) was evaluated for biological activity (see Table 1). In addition to MOP and DOP binding and functional signaling bioassay (EPAC cAMP BRET-based biosensor test), the guinea pig ileum (GPI, functional test representative of MOP agonist activity) and mouse vas deferens (MVD, functional test representative of DOP agonist activity) assays were carried out. From this first data set, it could be concluded that the peptides with highest MOP and DOP agonist activity contained a substituent at the *ortho*-position (**3, 6, 9**). Especially the vinyl group (**9**) attracted interest due to its subnanomolar binding affinity for MOP and its potent activity in the functional bioassays. Indeed, compound **9** exhibited respectively 20-fold and 70-fold increase in potency at inhibiting cAMP production and GPI contraction, compared to the reference MOP agonist DAMGO (Tables 1 and 2).

Interestingly, two peptides with *para*-substitution (**1, 4**) demonstrated*in vitro* μ-opioid antagonism in the functional GPI assay and analogues **1, 2, 4** and **5** behaved as δ partial agonists in the MVD assay. This antagonism or partial agonism of the 4'-substituted ligands could be shifted to improved agonist activity by either *meta*- or *ortho*-substitution, with the latter giving way to the most potent compounds (i.e., for **3/6/9**). Such a tendency was noticed for both MOP and DOP. Activities in both assays were highest for the *ortho*-substituted tetrapeptides, which clearly shows the benefit of these focused derivatizations realized by the Suzuki-Miyaura reaction. Based on these findings, we focused our efforts on more subtle structural changes at the *ortho*-position of the Phe[3] side chain, providing ligands **10-18**.

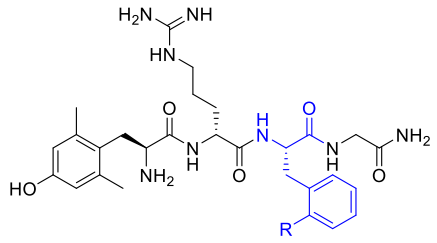
Table 1. Affinity and *in vitro* functional activity data for the first set of diversified opioid tetrapeptides

Peptides	MOP IC_{50} (nM)	DOP IC_{50} (nM)	Selectivity $IC_{50}^{\delta}/IC_{50}^{\mu}$	IC_{50} (GPI) (nM)	IC_{50} (MVD) (nM)
MOP-selective DAMGO	1.39±0.3	1533±172	/	28.3	950
DOP-selective DeltII	627±100	1.00±0.14	/	854	0.147
Dmt-D-Arg-Phe(4-phenyl)-Gly-NH_2 **1**	7.08±3.34	42.3±35	6	K_e= 114 ±21[1]	3090±340 (P.A. IC_{20})
Dmt-D-Arg-Phe(3-phenyl)-Gly-NH_2 **2**	0.85±0.15	268.8±237	316	61.7±3.9	117 ±15 (P.A. IC_{40})
Dmt-D-Arg-Phe(2-phenyl)-Gly-NH_2 3	**0.54±0.16**	**20.25±2.31**	**38**	**3.47±0.06**	**6.99±0.69**
Dmt-D-Arg-Phe(4-(3-thienyl))-Gly-NH_2 **4**	5.04±4.1	309±19.2	61	K_e= 138 ±21[1]	3960±190 (P.A. IC_{20})
Dmt-D-Arg-Phe(3-(3-thienyl))-Gly-NH_2 **5**	1.03±0.05	601±536	583	30.4±3.8	52.8±4.6 (P.A. IC_{35})
Dmt-D-Arg-Phe(2-(3-thienyl))-Gly-NH_2 6	**1.45±0.5**	**625±18**	**431**	**1.54±0.25**	**2.75±0.37**
Dmt-D-Arg-Phe(4-vinyl)-Gly-NH_2 **7**	1.82±0.14	1415±282	777	24.1±2.6	430±13
Dmt-D-Arg-Phe(3-vinyl)-Gly-NH_2 **8**	0.46±0.17	165±19.8	359	1.43±0.12	9.74±1.17
Dmt-D-Arg-Phe(2-vinyl)-Gly-NH_2 9	**0.47±0.23**	**18±4.46**	**38**	**0.389±0.062**	**0.406±0.038**
Dmt-D-Arg-Phe(2-(4-MeO-phenyl))-Gly-NH_2 **10**	0.47±0.24	70.2±27.2	149	0.901±0.224	5.23±0.28
Dmt-D-Arg-Phe(2-(4-CF_3-phenyl))-Gly-NH_2 11	**0.07±0.09**	**39.2±17**	**560**	**1.26±0.17**	**1.78±0.32**
Dmt-D-Arg-Phe(2-(4-F-phenyl))-Gly-NH_2 **12**	0.47±0.06	435±26	925	0.801±0.086	2.95±0.26
Dmt-D-Arg-Phe(2-(4-Cl-phenyl))-Gly-NH_2 **13**	0.35±0.06	346±10	989	2.39±0.09	1.63±0.22
Dmt-D-Arg-Phe(2-(2-methyl-1-propenyl)-Gly-NH_2 14	**0.57±0.15**	**585±10**	**1026**	**0.701±0.114**	**0.630±0.113**
Dmt-D-Ala-Phe(2-phenyl)-Gly-NH_2 15	**0.35±0.2**	**19.8±4.8**	**57**	**1.60±0.12**	**2.60±0.41**
Dmt-D-Ala-Phe(2-(3-thienyl))-Gly-NH_2 **16**	240±73	687±188	4	1.27±0.16	2.16±0.45
Dmt-D-Ala-Phe(2-vinyl)-Gly-NH_2 17	**0.25±0.05**	**8±1.9**	**32**	**1.18±0.17**	**2.81±037**
Dmt-*N*-Me-D-Ala-Phe(2-vinyl)-Gly-NH_2 **18**	0.36±0.09	59.4±0.8	165	0.641±0.045	0.689±0.060
Dmt-D-Arg-Phe-Gly-NH_2 **19**	0.1±0.06	14±2.7	140	2.26±0.37	0.316±0.059
Dmt-D-Ala-Phe-Gly-NH_2 **20**	0.14±0.05	22.6±4.6	161	1.50±0.12	1.15±0.15

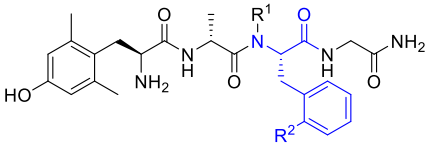
[1]antagonist. P.A.: partial agonist. Binding affinities of compounds for MOP and DOP receptors were determined by competition binding studies of [^{125}I]-DAMGO (MOP-selective) and [^{125}I]-Deltorphin II (DOP-selective) using membrane preparations of HEK293 cells expressing MOP or DOP. The GPI functional assay is representative of MOP activation, whereas the MVD is a DOP-representative assay.

Additionally, we envisaged the substitution of the D-Arg^2 residue by D-Ala^2 (**15-17**) could influence these profiles and potentially enhanced hydrophobicity could be reached by insertion of *N*Me-D-Ala (**18**). The biological results of this second subset of compounds are summarized in Table 2. Moreover, both parent peptides (with either D-Arg (**19**) or D-Ala (**20**)), were included in Table 1 for comparison. Remarkably, all ligands with the exception of compound **16** were highly selective for the MOP and showed subnanomolar affinities. Of note, up to a 7-fold increase in affinity could be observed through minor changes (such as CF_3 group *versus* F). Tetrapeptide **11** possessed the highest affinity, showing an IC_{50} of 70 pM.

Figure 2. Structures of the second set [Dmt^1,D-Arg^2]- and [Dmt^1,D-Ala^2]dermorphin(1-4) analogues



with R
4-MeO-phenyl **10**
4-CF_3-phenyl **11**
4-F-phenyl **12**
4-Cl-phenyl **13**
2-methyl-1-propenyl **14**



with
R^1= H, R^2= phenyl **15**
R^1= H, R^2= thienyl **16**
R^1= H, R^2= vinyl **17**
R^1= Me, R^2= vinyl **18**

Replacement of D-Arg by D-Ala (**15/16/17** vs. **3/6/9**, resp.) had a marginally positive effect, except for **16** which, unexpectedly, showed poor affinity for MOP. Very high MOP over DOP selectivity was obtained for ligand **14**, indicating that very subtle changes, such as methyl group insertions, can induce a profound effect on the opioid receptor selectivity. Likewise, compounds **11-13** carrying an *ortho*-substitution on the aromatic side chain, exhibited improved selectivity for MOP over DOP.

Table 2. EPAC cAMP BRET-based biosensor assay used to determine the potency (EC_{50}) of selected ligands at activating DOP and MOP.

Peptide	EPAC MOP EC_{50} (nM)	EPAC DOP EC_{50} (nM)
DAMGO	0.26±0.07	49±32.9
DeltII	16±12.3	0.1±0.04
6 with R= 2-(3-thienyl)	0.04 ± 0.03	49 ± 33
9 with R = 2-vinyl	0.012 ± 0.001	7 ± 2.896
11 with R = 2-(4-CF_3-phenyl)	0.12 ± 0.05	26.8 ± 6
14 with R = 2-methyl-1-propenyl	0.1 ± 0.4	19.4 ± 12.8
15 with R^2 = 2-phenyl	0.12 ± 0.01	4.9 ± 1
16 with R^2 = 2-(3-thienyl)	34.8 ± 6.3	56 ± 127 [a]
17 with R^2 = 2-vinyl	0.006 ± 0.001	5.8 ± 1
18 with R^2 = 2-vinyl	0.003 ± 0.004	4.4 ± 2
Dmt-D-Arg-Phe-Gly-NH_2 **19**	0.008 ± 0.002	1.13 ± 0.2
Dmt-D-Ala-Phe-Gly-NH_2 **20**	0.012 ± 0.0007	2.4 ± 1

[a]Partial agonist (E_{max}: 70%)

Similarly, parent ligands **19** and **20** also resulted in higher affinity for the MOP receptor (10-fold increase compared to DAMGO), a result which is to be expected since they originate from the MOP-selective dermorphin. In the EPAC cAMP BRET-based biosensor assay, selected examples of the second tetrapeptide set were very potent in reducing cAMP production (Table 2). Overall, these results revealed strong correlation between binding and functional data.

The analgesic effectiveness of selected compounds was then evaluated using the tail-flick test on rats after intrathecal administration. Our results revealed that compounds **14** and **15**, as well as their respective parent peptides **19** and **20**, showing either high-affinity for both receptors or selectivity at MOP, exerted potent analgesic action on thermal nociception for times exceeding 60 min (Figure 3). Importantly, these analgesic effects were 10 times more effective in reversing the nociceptive behavior than DAMGO, a selective and potent MOP agonist, used as the reference compound.

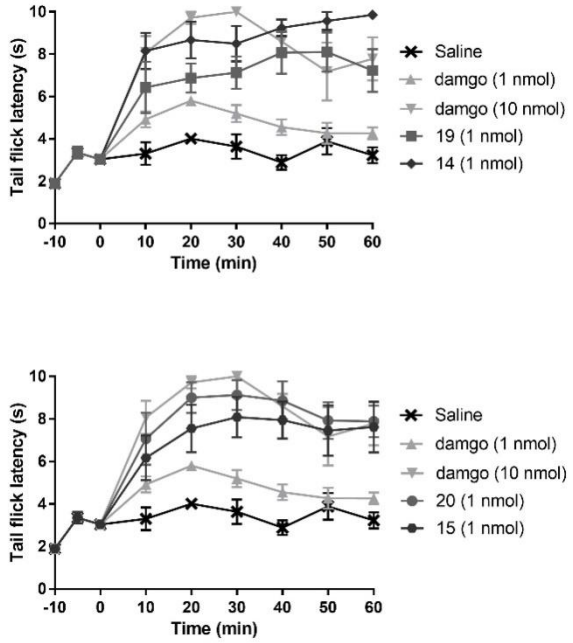


Figure 3. Analgesic effects of selected *ortho*-substituted Phe[3] opioid tetrapeptides**14** and **15.**

In conclusion, a series of truncated and derivatized dermorphintetrapeptide analogues was prepared by means of the Suzuki-Miyaura reaction. Straightforward introduction of (hetero)aromatic and vinylic substituents was realized, allowing a spatial screening of the chemical space around the Phe[3] side chain. Depending on the position and size of the newly incorporated substituent, different pharmacological profiles were accessed, with some of the ligands even possessing picomolar binding affinities for MOP. After intrathecal administration, some selected analogues (compounds **14** and **15**) were also very effective in reducing the withdrawal nociceptive responses to thermal stimuli in rats. A potential binding pose of derivatized ligand **15** is provided in the supporting information. Given the increased hydrophobicity of the derivatized ligands (see supporting information for a comparison of cLogP values and HPLC retention times), the described ligands have potentially improved pharmacokinetic properties, and increased absorption after systemic administration may result. In due time, the antinociceptive effect of the derivatized ligands after peripheral administration will be reported and compared to the parent structures. Moreover, the described ligands could possibly profit from an increased metabolic stability, after derivatization by the Suzuki-Miyaura reaction, since they encompass unnatural amino acid derivatives. Hence, the described derivatization approach could also be applied elsewhere in view of the above advantages.

Acknowledgments

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A. Supplementary data

The Supporting data associated with this article can be found, in the online version.

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