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A novel cyclic enkephalin analogue with potent opioid antagonist activity

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Abstract—2′,6′-Dimethyl substitution of the Tyr[1] residue in opioid agonist peptides and deletion of the N-terminal amino group, as achieved by replacement of Tyr[1] with 3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid (Dhp), have been shown to produce opioid antagonists. To examine the effect of β-methylation of Dhp[1] in opioid peptides on the activity profile, stereoselective syntheses of (3*S*)- and (3*R*)-3-methyl-3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid [(3*S*)- and (3*R*)-Mdp] were carried out. In comparison with the cyclic parent antagonist peptide Dhp-c[D-Cys-Gly-Phe(*p*NO_2)-D-Cys]NH_2, the methylated analogue (3*S*)-Mdp-c[D-Cys-Gly-Phe(*p*NO_2)-D-Cys]NH_2 showed higher μ, δ and κ antagonist potencies in functional assays and higher binding affinities for μ, δ and κ opioid receptors (K_i^{μ} = 2.03 nM; K_i^{δ} = 2.34 nM; K_i^{κ} = 49.5 nM), whereas the corresponding (3*R*)-Mdp[1]-analogue was less potent by 1–2 orders of magnitude.

2′,6′-Dimethyl substitution of the Tyr[1] residue of opioid agonist peptides and deletion of the positively charged N-terminal amino group have recently been shown to represent a general structural modification to convert opioid peptide agonists into antagonists.[1–3] This conversion requires the synthesis of opioid peptide analogues containing 3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid (Dhp) in place of Tyr[1].[1] The cyclic enkephalin analogue Dhp-c[D-Cys-Gly-Phe(*p*NO_2)-D-Cys]NH_2 (**8**) turned out to be a quite potent μ and δ opioid receptor antagonist and a somewhat less potent κ antagonist[2] (Tables 1 and 2). In this study, we examine the effect of β-methylation of Dhp[1] in Dhp-c[D-Cys-Gly-Phe(*p*-NO_2)-D-Cys]NH_2 (**8**) on the in vitro opioid activity profile. This requires replacement of Dhp in the cyclic peptide with (3*S*)- or (3*R*)-3-methyl-3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid [(3*S*)- or (3*R*)-Mdp (**6a** or **6b**)]. The presence of a β-methyl group might either enhance or decrease opioid receptor binding affinity and it will be of interest to determine the stereochemical requirements for receptor binding. Therefore, both (3*S*)- and (3*R*)-Mdp-c[D-Cys-Gly-Phe(*p*NO_2)-D-Cys]NH_2 (**7a** and **7b**) were prepared. These compounds will also allow for an interesting comparison with β-methylated Tyr(2′,6′-Me_2)[1]-containing opioid peptide agonists[4] in terms of potency changes and stereochemical requirements.

The stereoselective synthesis of (3*S*)-Mdp (**6a**) is outlined in Scheme 1, which is based on a published approach to the synthesis of chiral β-branched carboxylic acids.[5] Basic hydrolysis of methyl-3-(4-*tert*-butoxycarbonyloxy-2,6-dimethylphenyl)propenoate **1**[6] afforded acid **2**. Incorporation of the chiral auxiliary (*S*)-(+)-4-phenyl-2-oxazolidinone[5] was carried out in the standard manner[7] to yield **3a**.[8] Asymmetric Michael addition was performed using the organocuprate prepared from methylmagnesium bromide in THF/$(CH_3)_2S$ to furnish **4a**[8] with a diastereomeric excess of 48%. It should be noted that the diastereoselectivity of the Michael addition to **3a** was less than that reported in the literature,[5] even though the reaction was carried out in an analogous manner. The diastereomers were easily separated by flash column chromatography (silica gel) to yield chirally pure **4a** in 55% yield. Removal of the chiral auxiliary[9] and Boc group then gave **6a**.[8] Compound **6b**[8] was

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Table 1. Receptor binding affinities of opioid peptide analogues

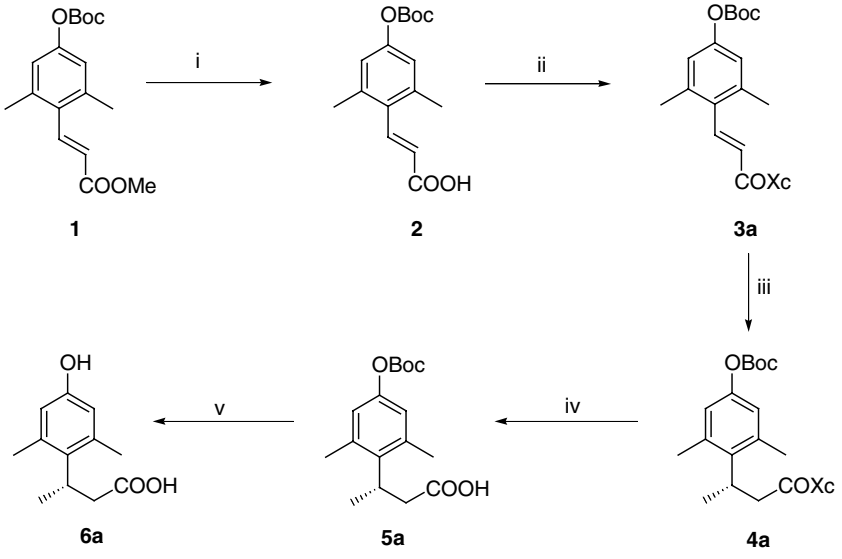
Compound	K_i^{μ} [nM][a]	K_i^{δ} [nM][a]	K_i^{κ} [nM][a]
7a (3*S*)-Mdp-c[D-Cys-Gly-Phe(pNO_2)-D-Cys]NH_2	2.10 ± 0.21	2.03 ± 0.09	49.5 ± 2.1
7b (3*R*)-Mdp-c[D-Cys-Gly-Phe(pNO_2)-D-Cys]NH_2	94.4 ± 13.6	497 ± 112	6970 ± 820
8 Dhp-c[D-Cys-Gly-Phe(pNO_2)-D-Cys]NH_2	4.79 ± 0.39	11.6 ± 1.1	299 ± 57
9 H-Dmt-c[D-Cys-Gly-Phe(pNO_2)-D-Cys]NH_2	0.247 ± 0.026	0.704 ± 0.042	3.77 ± 0.72

[a] Mean of 3–4 determinations ± SEM.

Table 2. GPI and MVD assay of opioid peptide analogues

Compound	GPI		MVD
	K_e^{μ} [nM][a,b]	K_e^{κ} [nM][a,c]	K_e^{δ} [nM][a,d]
7a (3*S*)-Mdp-c[D-Cys-Gly-Phe(pNO_2)-D-Cys]NH_2	1.40 ± 0.25	5.81 ± 1.20	55.0 ± 5.4
7b (3*R*)-Mdp-c[D-Cys-Gly-Phe(pNO_2)-D-Cys]NH_2	845 ± 135	1630 ± 290	3280 ± 430
8 Dhp-c[D-Cys-Gly-Phe(pNO_2)-D-Cys]NH_2	3.68 ± 0.45	22.6 ± 3.0	63.3 ± 10.5
9 H-Dmt-c[D-Cys-Gly-Phe(pNO_2)-D-Cys]NH_2[e]	IC_{50} = 0.541 ± 0.125 nM		IC_{50} = 0.182 ± 0.030 nM

[a] Mean of 3–5 determinations ± SEM.
[b] Determined with TAPP (H-Tyr-D-Ala-Phe-Phe-NH_2) as agonist.
[c] Determined with U50,488 as agonist.
[d] Determined with DPDPE as agonist.
[e] Agonist.



Scheme 1. Reagents and conditions: (i) 1 N aq NaOH/THF, 81%; (ii) Et_3N, PvCl, THF, −78 to 0 °C, then treated with *n*-BuLi, Xc, THF, −78 to 0 °C, 70%; (iii) CuBr–Me_2S complex, THF, CH_3MgBr, ether, 0 °C, 55%; (iv) LiOH, H_2O_2, THF/H_2O, 90%; (v) 95% TFA/H_2O, 0 °C, 98%. X_c=(*S*)-(+)-4-phenyl-2-oxazolidinone.

prepared in an analogous manner, using (*R*)-(−)-4-phenyl-2-oxazolidinone as chiral auxiliary and following the same sequence of reactions.

The target peptides (3*S*)- and (3*R*)-Mdp-c[D-Cys-Gly-Phe(pNO_2)-D-Cys]NH_2 (**7a**,**b**) were prepared by a combination of manual solid-phase and solution techniques. The linear precursor peptide of H-c[D-Cys-Gly-Phe(p-NO_2)-D-Cys]NH_2 was assembled on a *p*-methylbenzhydrylamine resin using Boc protection of the α-amino function and acetamidomethyl (Acm) protection of the Cys side chain. After cleavage from the resin by HF/anisole treatment, disulfide bond formation was carried out in MeOH/H_2O using iodine as oxidation agent and the resulting cyclic peptide was purified by preparative reversed-phase HPLC. (3*S*)-Mdp or (3*R*)-Mdp were attached to the N-terminal amino group of H-c[D-Cys-Gly-Phe(pNO_2)-D-Cys]NH_2 using 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) as coupling agent. The final peptide products **7a** and **7b** were purified by preparative reversed-phase HPLC and their purity and structural identity were established by TLC, analytical HPLC and FAB-MS.[10]

In comparison with the Dhp-c[D-Cys-Gly-Phe(pNO_2)-D-Cys]NH_2 parent peptide (**8**), the (3*S*)-Mdp[1]-analogue (**7a**) showed significantly higher μ-, δ- and κ-receptor binding affinities (Table 1). This result is in contrast to the observation made with the (2*S*,3*R*)-Tmt[1]-analogue of the δ agonist DPDPE (H-(2*S*,3*R*)-Tmt-c[D-Pen-Gly-

Phe(pNO_2)-D-Pen]OH; Tmt = 2′,6′-dimethyl-β-methyltyrosine),[4] which has the same stereochemistry at the methylated β-carbon of the N-terminal residue as **7a**. [(2*S*,3*R*)-Tmt^1]DPDPE showed significantly lower μ and δ receptor binding affinities and lower δ agonist potency in the mouse vas deferens (MVD) assay as compared to the parent peptide H-Dmt-c[D-Pen-Gly-Phe(pNO_2)-D-Pen]OH; Dmt = 2′,6′-dimethyltyrosine).[11] Obviously, the β-methyl group of (3*S*)-Mdp in **7a** is able to effectively interact with a lipophilic binding site at all three opioid receptors to strengthen binding, whereas the β-methyl group of (2*S*,3*R*)-Tmt in [2*S*,3*R*)-Tmt^1] DPDPE decreases binding affinity, most likely due to some steric interference. The ability of the β-methyl group of (3*S*)-Mdp to enhance receptor binding affinity may be due to the greater conformational flexibility of the (3*S*)-Mdp residue as compared to the (2*S*,3*R*)-Tmt residue. It is also possible that the conformational requirements of the active and inactive receptor conformations differ from one another with regard to the interaction of the N-terminal residue of the agonist peptide and the antagonist peptide.

In agreement with the receptor binding data, the (3*S*)-Mdp^1-analogue **7a** also showed higher μ-, δ- and κ-antagonist potencies than the Dhp^1-parent **8** in the functional guinea pig ileum (GPI) and MVD assays (Table 2). In comparison with the (3*S*)-Mdp^1-analogue **7a**, the diastereomeric (3*R*)-Mdp^1-analogue **7b** displayed drastically lower binding affinities and antagonist potencies at all three receptors. This result is in agreement with observations that (2*S*,3*R*)-Tmt^1-analogues of opioid agonist peptides generally showed higher opioid receptor binding affinities and higher agonist potencies in functional assays than their corresponding (2*S*,3*S*)-Tmt^1-analogues.[4] It thus appears that the stereochemical requirements at the β-carbon of the 1-position side chain of β-methylated Dhp^1-antagonist peptides and β-methylated Dmt^1-agonist peptides for opioid receptor binding are the same. Furthermore, these results indicate that the overall mode of opioid receptor binding of these agonists and antagonists is similar but not identical.

Acknowledgements

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8. All new compounds were fully characterized by optical rotation measurements, ^{1}H and ^{13}C NMR spectra and HRMS. Data of selected compounds:
3a. $[\alpha]_D^{20}$ +30.5 (*c* 0.98, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.92–7.88 (d, 1H, *J* = 16.1 Hz), 7.56–7.52 (d, 1H, *J* = 16.1 Hz), 7.44–7.36 (m, 5H), 6.89 (s, 2H), 5.56–5.53 (dd, 1H, *J* = 3.84, 8.8 Hz), 4.76–4.72 (m, 1H), 4.34–4.31 (dd, 1H, *J* = 3.84, 8.8 Hz), 2.37 (s, 6H), 1.59 (s, 9H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 164.9, 154.0, 152.0, 150.8, 143.9, 139.4, 139.3, 131.7, 129.4, 129.0, 126.3, 122.6, 121.3, 83.8, 70.2, 58.1, 27.9, 21.7; HRMS (FAB) *m/e* calcd for $C_{25}H_{27}NO_6$ $[M+K]^+$ 476.1475, found: 476.1477.
4a. $[\alpha]_D^{20}$ +80.5 (*c* 1.03, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40–7.25 (m, 5H), 6.76 (s, 2H), 5.37–5.34 (dd, 1H, *J* = 3.48, 8.8 Hz), 4.56–4.61 (m, 1H), 4.26–4.22 (dd, 1H, *J* = 3.48, 8.8 Hz), 3.94–3.84 (m, 1H), 3.50–3.44 (dd, 1H, *J* = 6.7, 17.1 Hz), 3.38–3.32 (dd, 1H, *J* = 7.8, 17.1 Hz), 2.45–2.34 (d, 6H, *J* = 44.6 Hz), 1.55 (s, 9H), 1.25–1.23 (d, 3H, *J* = 7.3 Hz); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 171.9, 152.5, 148.7, 139.3, 129.4, 128.8, 126.0, 122.7, 120.9, 83.5, 70.1, 57.9, 40.6, 30.1, 27.9, 21.8, 19.0; HRMS (FAB) *m/e*, calcd for $C_{26}H_{31}NO_6$ $[M+K]^+$ 492.1788, found: 492.1790.
6a. $[\alpha]_D^{20}$ +35.3 (*c* 1, MeOH); ^{1}H NMR (400 MHz, CD_3COCD_3) δ 10.46 (s, 1H), 7.83 (s, 1H), 6.47 (s, 2H), 3.81–3.72 (m, 1H), 2.7–2.6 (m, 2H), 2.33 (s, 6H), 1.32–1.30 (d, 3H, *J* = 7.32 Hz); ^{13}C NMR (100.6 MHz, CD_3COCD_3) δ 206.1, 173.9, 155.0, 137.5, 132.7, 117.0, 115.3, 39.8, 30.94, 30.90, 21.0, 18.9; HRMS (EI) *m/e* calcd for $C_{12}H_{16}O_3$ $[M^+]$ 208.1099, found: 208.1104.
6b. $[\alpha]_D^{20}$ −35.2 (*c* 1, MeOH); ^{1}H NMR (400 MHz, CD_3COCD_3) δ 10.46 (s, 1H), 7.84 (s, 1H), 6.46 (s, 2H), 3.80–3.72 (m, 1H), 2.75–2.64 (m, 2H), 2.33 (s, 6H), 1.31–1.29 (d, 3H, *J* = 7.1 Hz); ^{13}C NMR (100.6 MHz, CD_3COCD_3) δ 206.1, 173.9, 155.0, 137.5, 132.7, 117.0, 115.3, 39.8, 30.9, 30.8, 21.1, 18.9; HRMS (EI) *m/e* calcd for $C_{12}H_{16}O_3$ $[M]^+$ 208.1099, found: 208.1110.
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10. Analytical data of peptides **7a** and **7b**:
7a. TLC R_f 0.90 (*n*-BuOH/AcOH/H_2O, 4:1:1), R_f 0.22 ($CHCl_3$/MeOH/AcOH, 85:10:5); FAB-MS $[M+H]^+$ 661.
7b. TLC R_f 0.92 (*n*-BuOH/AcOH/H_2O, 4:1:1), R_f 0.22 ($CHCl_3$/MeOH/AcOH, 85:10:5); FAB-MS $[M+H]^+$ 661.
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