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[Dmt[1]]DALDA analogues with enhanced μ opioid agonist potency and with a mixed μ/κ opioid activity profile

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ABSTRACT

Analogues of [Dmt[1]]DALDA (H-Dmt-D-Arg-Phe-Lys-NH_2; Dmt = 2′,6′-dimethyltyrosine), a potent μ opioid agonist peptide with mitochondria-targeted antioxidant activity, were prepared by replacing Phe[3] with various 2′,6′-dialkylated Phe analogues, including 2′,6′-dimethylphenylalanine (Dmp), 2′,4′,6′-trimethylphenylalanine (Tmp), 2′-isopropyl-6′-methylphenylalanine (Imp) and 2′-ethyl-6′-methylphenylalanine (Emp), or with the bulky amino acids 3′-(1-naphthyl)alanine (1-Nal), 3′-(2-naphthyl)alanine (2-Nal) or Trp. Several compounds showed significantly increased μ agonist potency, retained μ receptor selectivity and are of interest as drug candidates for neuropathic pain treatment. Surprisingly, the Dmp[3]-, Imp[3]-, Emp[3]- and 1-Nal[3]-containing analogues showed much increased κ receptor binding affinity and had mixed μ/κ properties. In these cases, molecular dynamics studies indicated conformational preorganization of the unbound peptide ligands due to rotational restriction around the C^{β}—C^{γ} bond of the Xxx[3] residue, in correlation with the observed κ receptor binding enhancement. Compounds with a mixed μ/κ opioid activity profile are known to have therapeutic potential for treatment of cocaine abuse.

1. Introduction

The dermorphin-derived tetrapeptide [Dmt[1]]DALDA (H-Dmt-D-Arg-Phe-Lys-NH_2), containing 2′,6′-dimethyltyrosine (Dmt), is a highly potent μ opioid agonist.[1] It has subnanomolar μ receptor binding affinity (K_i^{μ} = 0.143 nM) and high μ receptor binding selectivity [K_i ratio ($\mu/\delta/\kappa$) = 1:14,700:156]. In the rat tail-flick assay this compound was 3000-fold more potent than morphine with intrathecal (i.th.) administration.[2] The extraordinary potency of [Dmt[1]]-DALDA as a spinal analgesic is due to its triple action as a μ opioid agonist, as a norephinephrine uptake inhibitor and as a releaser of endogenous opioid peptides.[2,3] Surprisingly, [Dmt[1]]DALDA also produced a potent antinociceptive effect in the mouse tail-flick test when given subcutaneously (sc) (40–220 times more potent than morphine), indicating that it is capable of crossing the blood–brain barrier.[4,5] The long-lasting antinociceptive effect of this compound in the acute pain models observed with both i.th. and sc administration is due to its high stability against enzymatic degradation and slow clearance.[2,6] The favorable drug-like properties of [Dmt[1]]-DALDA as a systemically active analgesic have been reviewed.[7]

Confocal laser scanning microscopy carried out with a fluorescent [Dmt[1]]DALDA analogue, H-Dmt-D-Arg-Phe-atn-Dap-NH_2[8] and mitochondrial fractionation studies using a tritiated [Dmt[1]]-DALDA analogue showed that the compound was taken up by cells and was distributed to the inner mitochondrial membrane (IMM).[9] The ability of [Dmt[1]]DALDA to penetrate the cellular membrane and target the IMM is due to its structural motif of alternating aromatic and basic residues. Because the Dmt residue has antioxidant

Abbreviations: Boc, *tert*-butyloxycarbonyl; DAMGO, H-Tyr-D-Ala-Gly-Phe(NMe)-Gly-ol; Dmp, 2′,6′-dimethylphenylalanine; Dmt, 2′,6′-dimethyltyrosine; [Dmt[1]]DALDA, H-Dmt-D-Arg-Phe-Lys-NH_2; DSLET, H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH; EDT, 1,2-ethanediol; Emp, 2′-ethyl-6′-methylphenylalanine; Fmoc, 9-fluorenylmethoxycarbonyl; GPI, guinea pig ileum; HOBt, 1-hydroxybenzotriazole; Imp, 2′-isopropyl-6′-methylphenylalanine; IMM, inner mitochondrial membrane; MVD, mouse vas deferens; 1-Nal, 3′-(1-naphthyl)alanine; 2-Nal, 3′-(2-naphthyl)alanine; Pbf, 2,3-dihydro-2,2,4,6,7-pentamethyl-5-benzofuranyl)sulfonyl; PyBOP, (benzotriazol-1-yl-oxy)tris(pyrrolidino)phosphonium hexafluorophosphate; RP-HPLC, reversed-phase high performance liquid chromatography; TLC, thin layer chromatography; Tmp, 2′,4′,6′-trimethylphenylalanine; U69,593, (5α,7α,8β)-(−)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide.

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properties,[9] [Dmt[1]]DALDA acts as a IMM-targeted antioxidant. Since mitochondrial reactive oxygen species (ROS) in spinal cord dorsal horn neurons play a key role in neuropathic pain mechanisms,[10] [Dmt[1]]DALDA was examined for its antinociceptive effectiveness in the rat spinal nerve ligation model and was found to be more effective than morphine in this experimental model of neuropathic pain.[11]

Introduction of conformational constraints into opioid peptides has been shown to have interesting effects on potency and opioid receptor binding selectivity. Global conformational restriction through various types of cyclization resulted in μ or δ receptor-selective opioid agonists.[12–14] Conformational restriction of the side chains of Phe and Tyr (χ constraints, reviewed in Ref. 15) in opioid peptides has also been shown to have significant effects on opioid receptor binding affinities and selectivities. β-Methylation of the Phe residue in cyclic enkephalin analogues produced compounds that retained high δ receptor binding affinity[16] or showed improved δ receptor selectivity.[17] Substitution of Dmt for Tyr[1] in opioid peptides generally enhances opioid receptor binding affinity, as was the case with the δ-selective analogue [Dmt[1]]DPDPE (H-Dmt-c[D-Pen-Gly-Gly-Phe-D-Pen]-OH)[18] and the μ agonist [Dmt[1]]DALDA.[1] Introduction of the 2′,6′-dimethyl groups in Dmt restricts rotation around the C^{β}—C^{γ} bond.

In an effort to obtain [Dmt[1]]DALDA analogues with enhanced μ opioid agonist potency or possibly altered opioid receptor selectivity profiles, we replaced the Phe[3] residue with various alkylated phenylalanine residues, in which rotational mobility around the C^{β}—C^{γ} bond is expected to be restricted. These include 2′,6′-dimethylphenylalanine (Dmp) (**1**), 2′,4′,6′-trimethylphenylalanine (Tmp) (**2**), 2′-isopropyl-6′-methylphenylalanine (Imp) (**3**) and 2′-ethyl-6′-methylphenylalanine (Emp) (**4**) (Fig. 1).[19] Furthermore, analogues containing the bulky aromatic amino acids 3-(1-naphthyl)alanine (1-Nal) (**5**), 3-(2-naphthyl)alanine (2-Nal) (**6**) and Trp (**7**) in place of Phe[3] were also synthesized. The in vitro opioid activity profiles of the compounds were determined in μ-, δ- and κ-opioid receptor binding assays and in the functional guinea pig ileum (GPI) and mouse vas deferens (MVD) assays. The GPI contains both μ and κ opioid receptors, whereas in the MVD δ opioid receptors are predominant with μ and κ receptors also present at lower concentration.

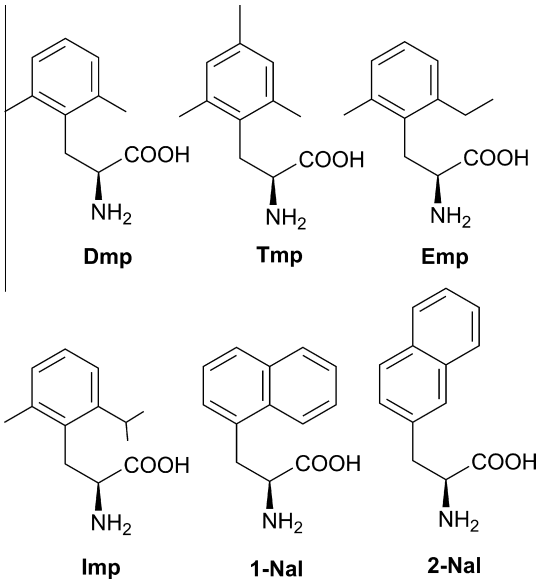

Figure 1. Structural formulas of Dmp, Tmp, Emp, Imp, 1-Nal and 2-Nal.

2. Results and discussion

2.1. Chemistry

Dmp, Tmp, Imp and Emp were synthesized as reported.[19] Peptides were prepared by the solid-phase peptide synthesis method on a Rink amide resin, using 9-fluorenyl-methoxycarbonyl (Fmoc)-protected amino acids and (benzotriazol-1-yl-oxy)tris (pyrr-olidino)phosphonium hexafluorophosphate (PyBOP)/1-hydroxybenzotriazol (HOBt) as coupling agents. The side chains of Lys and Arg were protected with *tert*-butyloxycarbonyl (Boc) and (2,3-dihydro-2,2,4,6,7-pentamethyl-5-benzofuranyl)sulfonyl (Pbf), respectively. Peptides were cleaved from the resin by treatment with TFA/EDT/anisole = 3.8:0.1:0.1(v/v), purified by semi-preparative HPLC, and lyophilized.

Analytical data of the peptides are presented in Table 1.

2.2. 2In vitro opioid activity determinations

In the opioid receptor binding assays (Table 2), H-Dmt-D-Arg-Dmp-Lys-NH_2 (**1**) showed 15-fold higher μ receptor binding affinity than the [Dmt[1]]DALDA parent (**8**) with a K_i^{μ} value in the low picomolar range (K_i^{μ} = 9.35 pM). Similar to **8**, analogue **1** displayed high μ receptor binding selectivity (selectivity ratio $K_i^{\mu}/K_i^{\delta}/K_i^{\kappa}$ = 1:11200:350). Despite its increased μ receptor binding affinity, the agonist potency of **1** in the GPI assay was similar to that of **8** (Table 3). A Dmp[3]-analogue of the dermorphin-derived tetrapeptide H-Tyr-D-Arg-Phe-β-Ala-NH_2 had also been reported to have increased μ opioid receptor binding affinity.[20] The enhanced agonist potency of **1** as compared to parent **8** in the MVD assay is due to its 15-fold higher μ receptor binding affinity, resulting in increased activation of μ receptors that are also present in the vas preparation in addition to the predominant δ receptors. In comparison with the [Dmt[1]]DALDA parent (**8**), H-Dmt-D-Arg-Tmp-Lys-NH_2 (**2**) had comparable μ receptor binding affinity, 6-fold higher δ receptor affinity and 2-fold lower κ affinity, and thus also retained high μ receptor preference. In the GPI assay it was a 12-fold less potent agonist than **8**. Compounds **1** and **2** both showed lower potency in the GPI assay than was expected on the basis of their μ receptor binding affinities. This could be explained with their reduced access to receptors in this tissue as a consequence of their increased lipophilic character resulting from the Dmp and Tmp substitutions.. In comparison with the [Dmt[1]]-DALDA parent (**8**), the Imp[3]-analogue (**3**) had similar μ receptor binding affinity, but 100-fold increased κ receptor affinity in the subnanomolar range (K_i^{κ} = 0.236 nM) and higher δ receptor binding affinity (K_i^{δ} = 77.1 nM). Thus, it showed no μ versus κ receptor selectivity, but still quite high preference for μ and κ receptors over δ receptors. The high agonist potency of **3** in the MVD assay (IC_{50} = 1.22 nM) was unexpected, but may be due to its activation of μ and κ receptors in the vas in addition to the agonist effect at

Table 1
Analytical parameters of [Dmt[1]] DALDA analogues

No.	Compound	TLC[a]	HPLC t_R[b] (min)	Mass (M+H)$^+$
1	H-Dmt-D-Arg-Dmp-Lys-NH_2	0.63	7.52	668.1
2	H-Dmt-D-Arg-Tmp-Lys-NH_2	0.62	8.31	682.1
3	H-Dmt-D-Arg-Imp-Lys-NH_2	0.62	9.11	696.2
4	H-Dmt-D-Arg-Emp-Lys-NH_2	0.61	8.40	682.1
5	H-Dmt-D-Arg-1-Nal-Lys-NH_2	0.59	8.44	690.1
6	H-Dmt-D-Arg-2-Nal-Lys-NH_2	0.58	8.32	690.1
7	H-Dmt-D-Arg-Trp-Lys-NH_2	0.56	6.42	679.1

[a] Solvent: *n*-BuOH/Py/HOAc/H_2O = 15:10:3:12.
[b] See Section 4.1 for conditions.

Table 2
Opioid receptor binding affinities of [Dmt[1]]DALDA analogues[a]

Compound		K_i^{μ} (nM)	K_i^{δ} (nM)	K_i^{κ} (nM)	Potency ratio $\mu/\delta/\kappa$
1	H-Dmt-D-Arg-Dmp-Lys-NH_2	0.00935 ± 0.0015	105 ± 13	4.70 ± 0.11	1:11,200:350
2	H-Dmt-D-Arg-Tmp-Lys-NH_2	0.158 ± 0.013	329 ± 9	48.0 ± 0.6	1:2080:304
3	H-Dmt-D-Arg-Imp-Lys-NH_2	0.155 ± 0.007	77.1 ± 3.8	0.236 ± 0.002	1:497:2
4	H-Dmt-D-Arg-Emp-Lys-NH_2	0.0958 ± 0.0101	50.0 ± 6.7	0.961 ± 0.089	1:522:10
5	H-Dmt-D-Arg-1-Nal-Lys-NH_2	0.109 ± 0.012	173 ± 19	1.60 ± 0.09	1:1590:15
6	H-Dmt-D-Arg-2-Nal-Lys-NH_2	0.235 ± 0.057	258 ± 29	18.9 ± 5.6	1:1100:80
7	H-Dmt-D-Arg-Trp-Lys-NH_2	0.0991 ± 0.0019	186 ± 2	16.5 ± 4.6	1:1880:88
8	H-Dmt-D-Arg-Phe-Lys-NH_2[b]	0.143 ± 0.015	2100 ± 310	22.3 ± 4.2	1:14,700:156

[a] Values represent means of 3–4 determinations ± SEM.
[b] Data taken from Ref. 1.

Table 3
GPI and MVD assays of [Dmt[1]]DALDA analogues

Compound		GPI IC_{50}[a] (nM)	MVD IC_{50}[a] (nM)
1	H-Dmt-D-Arg-Dmp-Lys-NH_2	2.02 ± 0.02	1.72 ± 0.20
2	H-Dmt-D-Arg-Tmp-Lys-NH_2	17.4 ± 1.7	19.1 ± 1.3
3	H-Dmt-D-Arg-Imp-Lys-NH_2	0.828 ± 0.114	1.22 ± 0.21
4	H-Dmt-D-Arg-Emp-Lys-NH_2	0.474 ± 0.042	0.220 ± 0.010
5	H-Dmt-D-Arg-1-Nal-Lys-NH_2	1.90 ± 0.05	0.755 ± 0.065
6	H-Dmt-D-Arg-2-Nal-Lys-NH_2	71.5 ± 3.9	64.8 ± 16.1
7	H-Dmt-D-Arg-Trp-Lys-NH_2	1.07 ± 0.11	1.67 ± 0.26
8	H-Dmt-D-Arg-Phe-Lys-NH_2[b]	1.41 ± 0.29	23.1 ± 2.0

[a] Values represent means of 3–4 determinations ± SEM.
[b] Data taken from Ref. 1.

the predominant δ receptors. The opioid receptor binding profile of the Emp[3]-analogue, H-Dmt-D-Arg-Emp-Lys-NH_2 (**4**), was similar to that of **3**, but it did show modest preference for μ receptors over κ receptors (K_i^{κ}/K_i^{μ} = 10). It was the most potent agonist of the series both in the GPI assay (IC_{50} = 0.474 nM) and in the MVD assay (IC_{50} = 0.220 nM), in agreement with its very high binding affinities at both μ and κ receptors and its highest δ receptor binding affinity among all compounds.

Substitution of 1-Nal for Phe[3] in [Dmt[1]]DALDA produced a compound (**5**) which retained high μ receptor binding affinity (K_i^{μ} = 0.109 nM) and also showed high κ receptor affinity (K_i^{κ} = 1.60 nM). Similar to peptides **3** and **4**, compound **5** was a potent agonist in both functional assays. The 2-Nal[3]-analogue (**6**) showed lower binding affinity than the 1-Nal[3]-analogue (**5**) at all three opioid receptors, particularly at the κ receptor (K_i^{κ} = 18.9 nM). In agreement with its profile of lower opioid receptor binding affinities, compound **6** displayed significantly reduced agonist potencies both in the GPI assay and in the MVD assay. In comparison with the [Dmt[1]]DALDA (**8**) parent, the Trp[3]-analogue (**7**) had similar μ and κ receptor binding affinities, but 11-fold higher δ receptor affinity. In agreement with the receptor binding data, compounds **7** and **8** were about equipotent in the GPI assay, but **7** was 13-fold more potent than **8** in the MVD assay.

2.3. Molecular modeling studies

To assess the side chain flexibility of the various amino acids substituted for Phe[3] in [Dmt[1]]DALDA, a molecular dynamics study (1 ns duration) was performed at 300 K with some of these amino acids in their N-acetylated and carboxamidated form [Ac—NH—CH(R)—$CONH_2$]. In all amino acids, rotation around χ_1 was not impeded and frequent transitions between the g^+, g^- and t states were observed (data not shown). As expected, conformational flexibility around the C^{β}—C^{γ} bond was observed in the case of Phe with multiple transitions occurring between the two rotational states (χ_2 = +90°, −90°) (Fig. 2a), with a rotational barrier of 3.3 kcal/mol (Table 4). With Dmp, Emp and Imp, the χ_2 (−90°) did not change in the course of the simulation, indicating that rotation around the C^{β}—C^{γ} bond is impeded. In these cases the barrier of rotation was 9–10 kcal/mol for this bond. Rotation around the C^{β}—C^{γ} bond of 1-Nal was also somewhat restricted, as only two transitions between χ_2 = −90° and χ_2 = +90° were observed during the 1 ns simulation with a barrier of rotation of 5.2 kcal/mol. In contrast, four transitions between these two conformational states were seen with 2-Nal, indicating greater rotational flexibility around the C^{β}—C^{γ} bond (barrier of rotation of 3.6 kcal/mol) as compared to 1-Nal. Interestingly, rotational flexibility of the isopropyl substituent on the phenyl ring of Imp was also restricted, with only one conformational state being occupied in the course of the stimulation, whereas the ethyl substituent in Emp enjoyed unhindered rotational freedom (Fig. 2b, τ rotation).

It is well documented that di-*ortho* groups on phenyl rings in ligands of various proteins can promote a favorable conformational preorganization due to increased rotational barriers, resulting in increased binding affinity (for a review, see Ref. 21). Interestingly, the four peptides containing Dmp[3] (**1**), Imp[3] (**3**), Emp[3] (**4**) and 1-Nal[3] (**5**) show markedly increased κ receptor binding affinity as compared to the [Dmt[1]]DALDA parent (**8**). As described above, side chain rotational mobility around the C^{β}—C^{γ} bond of the 3-position residue in these four peptides is restricted and the resulting conformational preorganization in the unbound state may be favorable for their interaction with the κ opioid receptor. The nearly 100-fold κ receptor binding affinity increase seen with the Imp[3]-analogue (**3**) may be due to the combined effect of rotational restriction around the C^{β}—C^{γ} bond and of the isopropyl substituent, with both the phenyl ring and its isopropyl substituent engaging in favorable hydrophobic receptor interactions.

3. Conclusions

The performed amino acid substitutions at the 3-position residue of [Dmt[1]]DALDA resulted in several analogues with enhanced μ opioid agonist potency with one of them, H-Dmt-D-Arg-Dmp-Lys-NH_2 (**1**), showing low picomolar μ receptor binding affinity. These peptides retain the alternating aromatic-basic structural motif required for mitochondria-targeted antioxidant activity and may have improved therapeutic potential for the treatment of neuropathic pain states. In general, the new analogues had somewhat increased, but still modest δ receptor binding affinity (K_i^{δ} = 50–300 nM). Previously, it had been demonstrated that endomorphin-2 analogues containing Dmp, Tmp, Emp or Imp in place of Phe[3], or Dmp, Tmp, 1-Nal or 2-Nal in place of Phe[4] also showed enhanced μ and δ receptor binding affinities.[22,23] Unexpectedly, most of the peptides containing 2′,6′-disubstituted phenylalanine analogues (**1**, **3**, **4**) or 1-Nal (**5**) displayed much enhanced κ

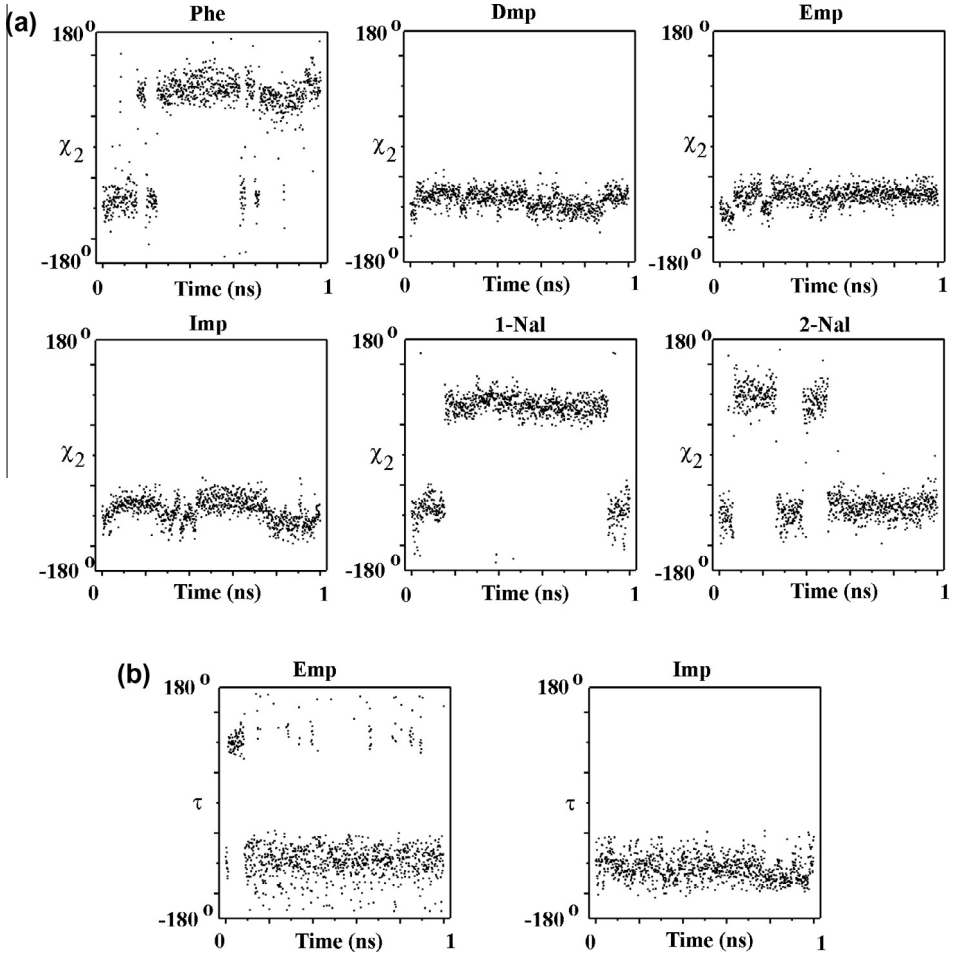

Figure 2. Molecular dynamics simulation of NAc-Xxx^3-CONH$_2$. (a) χ_2 of Xxx^3 = Phe, Dmp, Emp, Imp, 1-Nal and 2-Nal. (b) τ of ethyl and isopropyl substituents of Emp and Imp.

Table 4
Barriers of χ_2 rotation of Phe, Dmp, Emp, Imp, 1-Nal and 2-Nal

Amino acid	Energy (χ_2 = +90°) (kcal/mol)	Energy (χ_2 = +180°) (kcal/mol)	Δ energy (kcal/mol)
Phe	2.30	5.60	3.30
Dmp	4.01	13.41	9.40
Emp	4.38	14.30	9.92
Imp	5.67	16.41	10.74
1-Nal	3.88	9.03	5.15
2-Nal	2.15	5.76	3.61

receptor binding affinities and had a mixed μ/κ opioid profile. A molecular dynamics study revealed rotational restriction around the C^{β}—C^{γ} bond of the 3-position residue in these peptides. The resulting conformational preorganization of the unbound ligands selectively strengthens κ receptor binding. Compounds with a mixed μ/κ opioid activity profile have therapeutic potential for the treatment of cocaine abuse.[24]

4. Experimental section

4.1. General methods

Precoated plates (silica gel F254, Qiangdao, China) were used for ascending TLC in the following solvent systems: *n*-BuOH/Py/HOAc/H_2O (15:10:3:12). RP-HPLC was performed on a Waters Delta 600 liquid chromatograph using the following solvent system: solvent A, 0.05% TFA in water; solvent B, 0.05% TFA in CH_3CN. Semi-preparative RP-HPLC was performed on a SunFire™ Prep C18 column (20 × 150 mm) with a linear gradient of 90% A to 50% A over 15 min at a flow rate of 10 ml/min. Analytical RP-HPLC was performed on a SunFire™ Prep C18 column (4.6 × 150 mm) with a linear gradient of 90% A to 10% A over 30 min at a flow rate of 1.2 ml/min and the retention time, t_R (min), was determined. ^{1}H and ^{13}C NMR spectra were recorded on an AVANCE AV-500 or AV-300 spectrometer at 25 °C. Chemical shifts are indicated as δ values (ppm) relative to tetramethylsilane (TMS). Molecular masses of peptides were determined by electron spray mass spectrometry on a Agilent 1100 mass spectrometer.

4.2. Peptide synthesis

Peptide synthesis was performed by the manual solid phase technique using a Rink Amide resin (1% cross-linked, 100–200 mesh, 0.24 m equiv/g) obtained from GL Biochem (Shanghai) Ltd. Peptides were assembled using Fmoc-protected amino acids and PyBOP and HOBt as coupling agents. The side chain protection was Boc for Lys and Pbf for Arg. The phenolic hydroxyl group of Dmt was unprotected. The following steps were performed in each cycle: (1) addition of Fmoc-amino acid (2.5 equiv) in DMF; (2) addition of HOBt (2.5 equiv); (3) addition of PyBOP (2.5 equiv); (4) addition of DIEA (2.5 equiv) and mixing for 2.5 h at 26 °C; (5) washing with DMF (5 × 5 ml); (6) monitoring

completion of the reaction with the ninhydrin test; (7) Fmoc deprotection with 20% (v/v) piperidine in DMF (5 min); (8) Fmoc deprotection with 20% (v/v) piperidine in DMF (30 min). After complete peptide assembly, Fmoc protection was removed by treatment with piperidine in DMF. The resin was washed with DMF (3 × 5 ml), CH_2Cl_2 (3 × 5 ml), MeOH (3 × 5 ml), CH_2Cl_2 (3 × 5 ml), MeOH (3 × 5 ml) and was dried in a desiccator. Peptides were cleaved from the resin by treatment with TFA/EDT/anisole (3.8:0.1:0.1) for 2 h at 26 °C (8 ml of the mixture/g of resin), and the cleavage procedure was repeated twice. The filtrates were combined and precipitated with ice-cold ether/hexane (1:1). The crude peptides were purified by semi-preparative HPLC. Each peptide was at least 98% pure as assessed by analytical reversed-phase HPLC. Molecular weights were confirmed by MS.

4.2.1. H-Dmt-D-Arg-Dmp-Lys-NH_2 (1)

73.1 mg. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 0.82 (s, 2H), 1.00–1.12 (m, 2H), 1.29 (s, 2H), 1.51–1.55 (t, J = 6.66 Hz, 3H), 1.63–1.65 (m, 1H), 2.16 (s, 6H), 2.28 (s, 6H), 2.77–2.85 (m, 6H), 2.96–3.11 (m, 2H), 3.89 (s, 1H), 4.12–4.24 (m, 2H), 4.57–4.65 (q, J = 7.65 Hz, 1H), 6.40 (s, 2H), 6.86–6.94 (m, 3H), 7.07 (s, 2H), 7.17 (s, 2H), 7.44 (s, 1H), 7.92–7.95 (d, J = 8.04 Hz, 1H), 8.19–8.22 (d, J = 7.68 Hz, 1H), 8.33–8.36 (d, J = 7.98 Hz, 3H), 9.07 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 19.87, 19.96, 22.26, 23.82, 26.65, 28.75, 30.61, 31.36, 32.28, 51.29, 51.90, 52.22, 52.35, 114.86, 121.86, 125.99, 127.79, 134.05, 136.88, 138.31, 155.60, 156.75, 158.06, 158.47, 168.24, 170.00, 170.73, 173.04.

4.2.2. H-Dmt-D-Arg-Tmp-Lys-NH_2 (2)

22.4 mg. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 0.77 (s, 2H), 1.02–1.08 (m, 2H), 1.26–1.34 (m, 5H), 1.50 (s, 1H), 2.16–2.23 (m, 13H), 2.59–2.65 (t, J = 7.80 Hz, 2H), 2.73–2.89 (m, 2H), 2.94–3.05 (m, 4H), 3.89–3.92 (m, 2H), 4.23–4.26 (m, 1H), 4.35 (s, 1H), 6.42 (s, 2H), 6.74 (s, 2H), 7.04 (s, 2H), 7.17 (s, 2H), 7.27 (s, 1H), 7.44 (s, 1H), 7.78–7.81 (d, J = 7.89 Hz, 4H), 8.24–8.34 (m, 4H), 9.10 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 19.70, 19.87, 20.40, 21.73, 24.11, 26.43, 28.98, 30.38, 30.77, 31.64, 51.55, 52.31, 52.37, 52.82, 114.91, 122.04, 128.53, 130.56, 134.70, 136.73, 138.30, 155.58, 156.75, 170.48, 170.71, 173.63.

4.2.3. H-Dmt-D-Arg-Imp-Lys-NH_2 (3)

151.8 mg. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 0.79–0.82 (m, 2H), 1.02–1.05 (m, 1H), 1.12–1.14 (dd, J_1 = 6.55 Hz, J_2 = 4.60 Hz, 7H), 1.27–1.33 (m, 2H), 1.51–1.57 (m, 3H), 1.65–1.70 (m, 1H), 2.16 (s, 6H), 2.29 (s, 3H), 2.76–2.85 (m, 7H), 2.98–3.03 (m, 1H), 3.13–3.17 (dd, J_1 = 14.10 Hz, J_2 = 6.50 Hz, 1H), 3.88 (s, 1H), 4.18–4.23 (m, 2H), 4.51–4.56 (q, J = 7.00 Hz, 1H), 6.40 (s, 2H), 6.87–6.89 (d, J = 6.45 Hz, 1H), 6.99–7.04 (m, 3H), 7.08 (s, 1H), 7.18 (s, 2H), 7.40–7.43 (t, J = 5.30 Hz, 1H), 7.80–7.82 (m, 4H), 8.22–8.24 (d, J = 7.80 Hz, 1H), 8.31–8.35 (m, 4H), 9.05 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 19.84, 20.50, 22.19, 23.74, 23.86, 24.37, 26.67, 27.90, 28.69, 30.63, 30.99, 31.53, 51.29, 52.15, 52.39, 53.21, 114.86, 121.84, 122.77, 126.39, 127.44, 132.57, 136.77, 138.31, 155.59, 156.72, 157.85, 158.09, 158.34, 158.59, 168.31, 170.05, 170.49, 173.07.

4.2.4. H-Dmt-D-Arg-Emp-Lys-NH_2 (4)

96.5 mg. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 0.84 (s, 2H), 1.02–1.10 (m, 4H), 1.15–1.18 (m, 1H), 1.25–1.30 (m, 2H), 1.50–1.54 (m, 3H), 1.64–1.68 (m, 1H), 2.16 (s, 6H), 2.29 (s, 3H), 2.61–2.69 (m, 2H), 2.74–2.77 (t, J = 7.10 Hz, 2H), 2.79–2.87 (m, 4H), 2.97–3.02 (m, 1H), 3.07–3.11 (q, J = 6.65 Hz, 1H), 3.87–3.88 (d, J = 5.60 Hz, 1H), 4.15–4.25 (m, 2H), 4.54–4.59 (q, J = 7.65 Hz, 1H), 6.39 (s, 2H), 6.89–6.91 (m, 2H), 6.93–6.98 (m, 1H), 7.06–7.07 (d, J = 6.20 Hz, 3H), 7.42 (s, 1H), 7.77 (s, 2H), 7.85–7.87 (d, J = 8.15 Hz, 2H), 8.19–8.20 (d, J = 7.65 Hz, 2H), 8.33–8.34 (d, J = 8.80 Hz, 3H), 9.04 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ (ppm): 15.45, 19.85, 20.04, 22.22, 23.88, 25.22, 26.66, 28.77, 30.70, 31.40, 31.49, 51.35, 52.27, 52.56, 114.85, 115.99, 118.38, 121.92, 125.91, 126.26, 127.73, 133.27, 136.92, 138.30, 142.83, 155.58, 156.71, 157.83, 158.07, 158.32, 170.00, 170.61, 172.99.

4.2.5. H-Dmt-D-Arg-1-Nal-Lys-NH_2 (5)

170.4 mg. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 0.76 (s, 2H), 0.94–1.07 (m, 2H), 1.34 (s, 2H), 1.53–1.69 (m, 4H), 2.15 (s, 6H), 2.78–2.84 (m, 5H), 2.95–3.03 (t, J = 11.49 Hz, 1H), 3.08–3.18 (t, J = 10.20 Hz, 1H), 3.63–3.66 (d, J = 10.53 Hz, 1H), 3.88 (s, 1H), 4.15–4.24 (m, 2H), 4.63 (s, 1H), 6.38 (s, 2H), 7.15 (s, 4H), 7.33–7.41 (m, 4H), 7.48–7.58 (m, 2H), 7.75–7.77 (d, J = 7.02 Hz, 2H), 7.87–7.90 (d, J = 7.62 Hz, 2H), 7.97–8.00 (d, J = 8.10 Hz, 1H), 8.19–8.24 (t, J = 6.18 Hz, 4H), 8.55–8.58 (d, J = 8.4 Hz, 1H), 9.05 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 19.83, 22.30, 24.02, 26.67, 28.54, 30.67, 31.39, 34.73, 51.36, 52.24, 52.49, 53.41, 114.84, 121.89, 123.62, 125.11, 125.48, 125.99, 127.05, 127.55, 128.53, 131.39, 133.39, 133.44, 138.29, 155.57, 156.68, 158.44, 168.45, 170.29, 170.66, 173.35.

4.2.6. H-Dmt-D-Arg-2-Nal-Lys-NH_2 (6)

58 mg. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 0.65 (s, 2H), 0.93–1.06 (m, 2H), 1.30–1.33 (d, J = 7.53 Hz, 2H), 1.52–1.67 (m, 4H), 2.14 (s, 6H), 2.74–2.18 (t, J = 7.71 Hz, 3H), 2.86–3.02 (m, 2H), 3.32 (s, 3H), 3.87 (s, 1H), 4.17–4.21 (t, J = 6.06 Hz, 2H), 4.66 (s, 1H), 6.36 (s, 2H), 7.10 (s, 4H), 7.27 (s, 1H), 7.41–7.49 (m, 4H), 7.71–7.84 (m, 6H), 8.13–8.15 (t, J = 3.57 Hz, 4H), 8.37–8.40 (d, J = 8.73 Hz, 1H), 9.00 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm): 20.33, 22.79, 24.18, 27.18, 29.40, 31.21, 31.21, 31.92, 51.86, 52.83, 54.31, 115.32, 122.44, 125.80, 126.35, 127.66, 127.81, 128.09, 128.18, 132.20, 132.27, 135.84, 138.73, 156.03, 157.13, 158.44, 158.83, 170.57, 171.20, 173.80.

4.2.7. H-Dmt-D-Arg-Trp-Lys-NH_2 (7)

99.9 mg. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 0.73–0.82 (m, 2H), 1.05–1.15 (m, 2H), 1.26–1.31 (m, 2H), 1.49–1.54 (m, 3H), 1.62–1.68 (m, 1H), 2.16 (s, 6H), 2.76–2.79 (m, 4H), 2.81–2.85 (dd, J_1 = 14.05 Hz, J_2 = 4.85 Hz, 1H), 2.88–2.93 (dd, J_1 = 14.65 Hz, J_2 = 9.45 Hz, 1H), 2.97–3.02 (t, J = 10.90 Hz, 1H), 3.15–3.18 (dd, J_1 = 14.50 Hz, J_2 = 4.45 Hz, 1H), 3.90 (s, 1H), 4.15–4.24 (m, 2H), 4.51–4.55 (m, 1H), 6.39 (s, 2H), 6.94–6.97 (t, J = 7.50 Hz, 2H), 7.02–7.07 (dd, J_1 = 14.15 Hz, J_2 = 7.15 Hz, 3H), 7.11–7.12 (d, J = 2.15 Hz, 2H), 7.30–7.32 (d, J = 8.10 Hz, 1H), 7.34–7.36 (m, 1H), 7.60–7.61 (d, J = 7.85 Hz, 1H), 7.78 (s, 3H), 7.91–7.93 (d, J = 8.00 Hz, 1H), 8.19–8.21 (d, J = 7.75 Hz, 1H), 8.27–8.31 (m, 4H), 9.04 (s, 1H), 10.70 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 19.85, 22.26, 23.87, 26.70, 27.83, 28.80, 30.61, 31.40, 51.29, 52.21, 52.32, 53.45, 109.80, 111.17, 114.86, 118.09, 118.39, 120.78, 121.84, 123.81, 127.81, 135.99, 138.30, 155.59, 156.69, 158.07, 158.32, 168.24, 170.11, 171.07, 173.37.

4.3. Molecular dynamics studies

All calculations were performed using SYBYL version 7.0 (Tripos Associates, St. Louis, MO). The Tripos force field was used for energy calculations with a dielectric constant of 78. Phenylalanine was taken from a fragment library and modified as needed to generate the desired amino acid derivatives. In all cases the N-terminal amino group was acetylated and the C-terminal carboxylic acid group was amidated. Molecular dynamics simulations were carried out at 300 K for 1 ns. The barrier of rotation around the C^{β}—C^{γ} bond of the amino acids Phe, Dmp, Emp, Imp, 1-Nal and 2-Nal was determined using the torsion driver subroutine of SYBYL, with χ_1 set at −60°. The χ_2 bond was rotated by 5° increments and each structure was minimized. Calculations using the standard χ_1 values of

+60° and 180° were also performed with select amino acids and produced very similar results (data not shown).

4.4. In vitro bioassays and receptor binding assays

The GPI[25] and MVD[26] bioassays were carried out as described elsewhere.[27,28] A dose–response curve was determined with [Leu^5]-enkephalin as standard for each ileum and vas preparation, and IC_{50} values of the compounds being tested were normalized according to a published procedure.[29] Opioid receptor binding studies were performed as described in detail elsewhere.[27] Binding affinities for μ and δ opioid receptors were determined by displacing, respectively, [^{3}H]DAMGO (Multiple Peptide Systems, San Diego, CA) and [^{3}H]DSLET (Multiple Peptide Systems) from rat brain membrane binding sites, and κ opioid receptor affinities were measured by displacement of [^{3}H]U69,593 (Amersham) from guinea pig brain membrane binding sites. Incubations were performed for 2 h at 0 °C with [^{3}H]DAMGO, [^{3}H]DSLET and [^{3}H]U69,593 at respective concentration of 0.72, 0.78 and 0.80 nM. IC_{50} values were determined from log–dose displacement curves, and K_i values were calculated from the IC_{50} values by means of the equation of Cheng and Prusoff,[30] using values of 1.3, 2.6 and 2.9 nM for the dissociation constants of [^{3}H]DAMGO, [^{3}H]DSLET and [^{3}H]U69,593, respectively.

Agonist potencies determined with compounds in the GPI assay are often significantly lower than their binding affinities measured in the μ opioid receptor binding assay.[1,31] This could be due to differences between central and peripheral μ receptors or to somewhat impeded receptor access of compounds in the tissue preparation (Kosterlitz, personal communication). Furthermore, compounds with high μ agonist potency tend to show higher agonist potency in the MVD assay than is expected on the basis of their δ receptor binding affinities determined in the binding assay. This is due to activation of μ receptors that are also present in the vas preparation, albeit at lower concentration than the predominant δ receptors.[32]

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