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Original article

Synthesis and in vitro opioid activity profiles of DALDA analogues

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Abstract – The tetrapeptide DALDA (H-Tyr-D-Arg-Phe-Lys-NH_2) is a polar and selective μ agonist showing poor penetration of the placental and blood–brain barriers. In an effort to enhance the potency of DALDA, analogues containing 2′,6′-dimethyltyrosine (Dmt), *N*,2′,6′-trimethyltyrosine (Tmt), 2′-methyltyrosine (Mmt) or 2′-hydroxy,6′-methyltyrosine (Hmt) in place of Tyr^1, or Orn or α,γ-diaminobutyric acid (A_2bu) in place of Lys^4, were synthesized. All compounds displayed high μ receptor selectivity in the rat and guinea pig brain membrane binding assays and most of them were more potent μ agonists than DALDA in the μ receptor-representative guinea pig ileum assay, with [Dmt^1]DALDA showing the highest potency. Because of its extraordinary μ agonist potency, high μ selectivity, polar character (charge of 3+) and metabolic stability, [Dmt^1]DALDA has potential for use in obstetrical or peripheral analgesia. © 2000 Éditions scientifiques et médicales Elsevier SAS

μ opioid agonists / DALDA / DALDA analogues / [Dmt^1]DALDA / opioid receptor binding assays / GPI and MVD bioassays / opoid peptides

1. Introduction

Centrally acting μ opioid agonists (e.g. morphine) are still the most frequently used analgesics for the relief of severe pain, but their usefulness is limited by a number of well known side-effects, including tolerance, physical dependence, respiratory depression, adverse gastrointestinal effects, etc. Therefore, current efforts to develop centrally acting opioid analgesics are aimed at developing compounds with different opioid activity profiles (e.g. δ agonists or mixed μ agonist/δ antagonists (for a review, see ref. [1]). However, very hydrophilic μ opioid agonists that are unable to cross certain biological barriers still have considerable therapeutic potential for some analgesic applications. For example, μ opioid compounds that cannot penetrate the blood–brain barrier (BBB) may elicit peripheral analgesic effects without producing centrally mediated side effects. Also, some μ opioid agonists with polar character may not be able to cross the placental barrier (PB) and, therefore, may have potential for use in obstetrical analgesia.

In general, opioid peptides are more hydrophilic than non-peptide opiates. Opioid peptides with high μ receptor binding affinity and high μ receptor selectivity include several enkephalin analogues as well as morphiceptin and its analogues (for a review, see ref. [2]). The putative endogenous μ opioid receptor ligands endomorphin-1 and endomorphin-2 are also potent and very selective μ agonists [3], but are subject to enzymatic degradation [4, 5]. Among various ana-

Abbreviations: A_2bu, α,γ-diaminobutyric acid; BBB, blood–brain barrier; Boc, *tert*-butyloxycarbonyl; $(Boc)_2O$, di-tert-butyl dicarbonate; DALDA, H-Tyr-D-Arg-Phe-Lys-NH_2; DAMGO, H-Tyr-D-Ala-Gly-N^{α}MePhe-Gly-ol; DIC, 1,3-diisopropylcarbodiimide; DIEA, diisopropylethylamine; DMAP, 4-dimethylaminopyridine; 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; FAB-MS, fast atom bombardment mass spectrometry; Fmoc, (fluoren-9-ylmethoxy)-carbonyl; GPI, guinea pig ileum; Hmt, 2′-hydroxy,6′-methyltyrosine; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; Mmt, 2′-methyltyrosine; MVD, mouse vas deferens; Orn, ornithine; PB, placental barrier; Tmt, N,2′,6′-trimethyltyrosine; TAPS, H-Tyr-D-Arg-Phe-Ser-OH; TEA, triethylamine; TFA, trifluoroacetic acid; THF, tetrahydrofuran; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; Tic(OH), 7-hydroxy,1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TLC, thin layer chromatography; U69,593, (5α,7α,8β)-(—)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide.

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logues of the N-terminal tetrapeptide segment of dermorphin, the compounds TAPS (H-Tyr-D-Arg-Phe-Ser-OH) [6] and DALDA (H-Tyr-D-Arg-Phe-Lys-NH_2) [7] were reported to be potent and selective μ agonists.

Among these selective μ agonist peptides, DALDA has the most polar character because it carries a net positive charge of 3 + at physiological pH. DALDA was shown to be metabolically stable and to have very limited distribution across the BBB [8]. The results of a recent study indicated that DALDA did not cross the PB to a significant extent in the pregnant sheep model [9]. In agreement with this finding, DALDA had no effect on fetal cardiorespiratory parameters when given to pregnant sheep [10]. Furthermore, DALDA produced only a transient, minor increase in blood pressure and did not affect the maternal respiratory pattern. Taken together, these results suggest that DALDA or DALDA analogues may have potential for use in obstetrical analgesia or peripheral analgesia.

In the present paper we describe a series of DALDA analogues that were designed with the goal to develop highly polar μ opioid agonists with increased potency. The design was mainly based on structural modification of the Tyr^1 residue. Tyr^1 was replaced with 2′,6′-dimethyltyrosine (Dmt), *N*,2′,6′-trimethyltyrosine (Tmt), 2′-methyltyrosine (Mmt) or 2′-hydroxy,6′-methyltyrosine (Hmt). Analogues containing an α,ω-diamino acid with a shorter side chain (ornithine [Orn] or α,γ-diaminobutyric acid [A_2bu]) in place of Lys^4 were also prepared.

2. Chemistry

Peptides were synthesized by the solid-phase method using *tert*-butyloxycarbonyl (Boc)-protected amino acids and 1,3-diisopropylcarbodiimide (DIC)/1-hydroxybenzotriazole (HOBt) as coupling agents. Boc-Tmt was prepared as described elsewhere [11]. Mmt was first synthesized by hydrogenolysis of 7-hydroxy,1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid [Tic(OH)] using Pd-black as catalyst and a reaction temperature of 90 °C [12]. Under these conditions extensive racemization occurred. Van Betsbrugge prepared 2′-methylphenylalanine from 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic) with minimal racemization (8%) by carrying out the hydrogenolysis at 60 °C using 10% Pd/C and 4 atm H_2 pressure [13]. We used the latter conditions to prepare Mmt from Tic(OH) in good yield (48%) and with minimal racemization (< 10%). D,L-Hmt was synthesized in racemic form essentially as described in the literature [14]. The bis-Boc derivative of Mmt and the tris-Boc derivative of D,L-Hmt were prepared by reaction with di-*tert*-butyl dicarbonate in the presence of triethylamine (TEA) and 4-dimethylaminopyridine (DMAP). The tris-Boc derivative of D,L-Hmt was incorporated into the peptide in racemic form. Peptides were cleaved from the resin by HF/anisole treatment in the usual manner. Peptide purification and separation of the diasteromeric peptides in the case of the Hmt^1-analogue were achieved by preparative reversed-phase HPLC. Analytical data of the peptides are presented in *table I*.

Table I. Analytical parameters of DALDA analogues.

No.	Compound	TLC		HPLC	FAB-MS
		R_f (II)	R_f (III)	k'^a	$(M+H)^+$
1	H-Tyr-D-Arg-Phe-Lys-NH_2 (DALDA)	0.097	0.470	1.60	612
2	H-Dmt-D-Arg-Phe-Lys-NH_2 ([Dmt^1]DALDA)	0.111	0.482	3.34	640
3	H-Dmt-D-Arg-Phe-Orn-NH_2	0.118	0.482	3.05	626
4	H-Dmt-D-Arg-Phe-A_2bu-NH_2	0.111	0.487	2.87	612
5	Tmt-D-Arg-Phe-Lys-NH_2	0.112	0.465	3.63	654
6	H-Mmt-D-Arg-Phe-Lys-NH_2	0.115	0.476	2.33	626
7a	H-Hmt-D-Arg-Phe-Lys-NH_2 I	0.125	0.465	2.21	642
7b	H-Hmt-D-Arg-Phe-Lys-NH_2 II	0.125	0.467	2.02	642

[a] Capacity factor.

3. In vitro bioassays and opioid receptor binding assays

For the determination of their in vitro opioid activities, analogues were tested in bioassays based on inhibition of electrically evoked contractions of the guinea pig ileum (GPI) and the mouse vas deferens (MVD). The GPI assay is usually considered as being representative for μ opioid receptor interactions, even though the ileum does also contain κ opioid receptors. In the MVD assay opioid effects are primarily mediated by δ opioid receptors but μ and κ receptors also exist in this tissue. Binding affinities for μ and δ receptors were determined by displacing, respectively, [^{3}H]DAMGO and [^{3}H]DSLET from rat brain membrane binding sites, and κ receptor affinities were measured by displacement of [^{3}H]U69,593 from guinea pig brain membrane binding sites.

4. Results and discussion

DALDA (**1**) is a full μ agonist in the μ receptor representative GPI assay (*table II*). It has high μ receptor binding affinity and very weak binding affinities for δ and κ receptors (*table III*). Therefore, DALDA has very high selectivity for μ receptors vs. δ and κ receptors with selectivity ratios of $K_i^\delta/K_i^\mu = 11400$ and $K_i^\kappa/K_i^\mu = 2500$.

Replacement of Tyr^1 in DALDA with Dmt produced a 180-fold potency enhancement in the GPI assay and a somewhat less pronounced (34-fold) potency increase in the MVD assay. In the rat brain membrane binding assay, [Dmt^1]DALDA (**2**) showed 12 times higher μ receptor affinity than its parent compound and nine times higher affinity for δ receptors, thus retaining very high μ vs. δ selectivity ($K_i^\delta/K_i^\mu = 14700$). These data confirm previous

Table II. Guinea pig ileum (GPI) and mouse vas deferens (MVD) assay of DALDA analogues and related compounds.

No.	Compound	GPI	MVD	MVD/GPI
		IC_{50} [nM][a]	IC_{50} [nM][a]	IC_{50} ratio
1	H-Tyr-D-Arg-Phe-Lys-NH_2 (DALDA)	254 ± 27	781 ± 146	3.07
2	H-Dmt-D-Arg-Phe-Lys-NH_2 ([Dmt^1]DALDA)	1.41 ± 0.29	23.1 ± 2.0	16.4
3	H-Dmt-D-Arg-Phe-Orn-NH_2	2.08 ± 0.38	30.3 ± 4.6	14.6
4	H-Dmt-D-Arg-Phe-A_2bu-NH_2	7.56 ± 1.12	126 ± 32	16.7
5	Tmt-D-Arg-Phe-Lys-NH_2	7.86 ± 0.88	55.3 ± 15.3	7.04
6	H-Mmt-D-Arg-Phe-Lys-NH_2	10.9 ± 0.8	21.2 ± 1.5	1.94
7a	H-Hmt-D-Arg-Phe-Lys-NH_2 I	189 ± 25	>10000	>52.9
7b	H-Hmt-D-Arg-Phe-Lys-NH_2 II	1120 ± 140	>10000	>8.93
	H-Tyr-Pro-Phe-Phe-NH_2	7.71 ± 1.47	15.3 ± 1.8	1.98
	Morphine	29.3 ± 2.2	155 ± 31	5.29
	[Leu^5]enkephalin	246 ± 39	11.4 ± 1.1	0.0463

[a] Mean of three determinations ± SEM.

Table III. Opioid receptor binding affinities of DALDA analogues and related compounds.

No.	Compound	K_i^μ [nM][a]	K_i^δ [nM][a]	K_i^κ [nM][a]	K_i^δ/K_i^μ	K_i^κ/K_i^μ
1	H-Tyr-D-Arg-Phe-Lys-NH_2(DALDA)	1.69 ± 0.25	19200 ± 2000	4230 ± 360	11400	2500
2	H-Dmt-D-Arg-Phe-Lys-NH_2([Dmt^1]DALDA)	0.143 ± 0.015	2100 ± 310	22.3 ± 4.2	14700	156
3	H-Dmt-D-Arg-Phe-Orn-NH_2	0.151 ± 0.018	1240 ± 240	26.3 ± 2.4	8210	174
4	H-Dmt-D-Arg-Phe-A_2bu-NH_2	0.803 ± 0.076	6130 ± 850	47.5 ± 2.6	7630	59.2
5	Tmt-D-Arg-Phe-Lys-NH_2	0.192 ± 0.043	543 ± 75	46.1 ± 9.0	2830	240
6	H-Mmt-D-Arg-Phe-Lys-NH_2	0.222 ± 0.028	2320 ± 360	193 ± 43	10500	869
7a	H-Hmt-D-Arg-Phe-Lys-NH_2 I	1.76 ± 0.02	>10000	1840 ± 160	>5680	1050
7b	H-Hmt-D-Arg-Phe-Lys-NH_2 II	11.3 ± 0.9	>10000	9220 ± 1470	>885	816
	H-Tyr-Pro-Phe-Phe-NH_2	2.06 ± 0.19	3940 ± 460	>10000	1910	>4850
	Morphine	1.00 ± 0.04	32.6 ± 3.7	217 ± 49	32.6	217
	[Leu^5]enkephalin	9.43 ± 2.07	2.53 ± 0.35	4570 ± 550	0.268	485

[a] Mean of three to six determinations ± SEM.

observations indicating that substitution of Dmt for Tyr[1] in opioid peptides generally resulted in a higher increase in μ receptor affinity than in δ receptor affinity [1, 15]. The fact that the potency increase of this Dmt[1]-analogue in the GPI assay as compared to DALDA was much higher than expected on the basis of its μ receptor binding affinity enhancement may be due to differences between central and peripheral μ receptors with regard to their structural requirements for ligand binding. Interestingly, [Dmt[1]]DALDA also displayed 190-fold increased κ receptor binding affinity as compared to DALDA but still exhibited pronounced μ vs. κ selectivity ($K_i^{\kappa}/K_i^{\mu} = 156$).

Shortening of the side chain of Lys[4] in analogue **2** by one methylene group resulted in a compound, H-Dmt-D-Arg-Phe-Orn-NH_2 (**3**), with slightly reduced agonist potency in the GPI assay and with slightly lower μ receptor binding affinity. It retained high μ vs. δ and μ vs. κ receptor selectivities similar to those of analogue **2**. An analogue with an even shorter side chain in the 4-position of the peptide sequence, H-Dmt-D-Arg-Phe-A_2bu-NH_2 (**4**), was a somewhat less potent μ agonist in the GPI assay and bound somewhat less tightly to μ receptors. Compound **4** still showed high preference for μ receptors over δ receptors but lower preference for μ receptors over κ receptors.

Methylation of the N-terminal amino group of [Dmt[1]]DALDA led to a compound, Tmt-D-Arg-Phe-Lys-NH_2 (**5**), which was an about 5 times less potent μ agonist in the GPI assay as compared to [Dmt[1]]DALDA and was about half as potent in the MVD assay. In comparison with [Dmt[1]]DALDA, the Tmt[1]-analogue showed slightly lower μ receptor binding affinity, four times higher δ receptor affinity and about half the κ receptor affinity in the receptor binding assays. Consequently, compound **5** displayed lower μ vs. δ selectivity than [Dmt[1]]DALDA but higher μ vs. κ selectivity.

Introduction of only one methyl group at the 2′-position of the Tyr[1] aromatic ring of DALDA led to a compound, H-Mmt-D-Arg-Phe-Lys-NH_2 (**6**), which was 23 times more potent than DALDA and 6 times less potent than [Dmt[1]]DALDA in the GPI assay. In comparison with DALDA, compound **6** displayed about half as much binding affinity for μ receptors, similar affinity for δ receptors and 15-fold reduced κ affinity. Its μ vs. δ receptor selectivity is similar to that of the DALDA parent peptide, whereas its μ vs. κ selectivity is lower.

Substitution of D,L-Hmt for Tyr[1] in DALDA resulted in one diastereoisomer (I) (**7a**) which showed about the same μ agonist potency as DALDA in the GPI assay and a similar opioid receptor binding profile. Diastereoisomer II (**7b**) exhibited substantially lower μ agonist potency in the GPI assay and lower μ and κ receptor binding affinities than **7a**. Since it is well known that L-configuration at the Tyr[1] residue of opioid peptides is generally required for high opioid activity, we assume that diastereoisomer I (**7a**) represents the L-Hmt[1]-analogue.

5. Conclusions

Most of the prepared DALDA analogues displayed higher μ receptor binding affinity and higher μ agonist potency than the DALDA parent peptide and they were all μ receptor-selective. The most potent μ agonist was [Dmt[1]]DALDA (**2**), which also showed highest μ receptor binding affinity, highest μ vs. δ receptor selectivity and substantial preference for μ receptors over κ receptors. This compound was 5 times more potent in the GPI assay and had 14 times higher μ receptor binding affinity than endomorphin-2 (*tables II* and *III*). In comparison with morphine, it turned out to be a 20 times more potent μ agonist in the functional in vitro assay and displayed 7 times higher μ receptor binding affinity. Both DALDA and [Dmt[1]]DALDA carry a net positive charge of $3+$. Inspection of the capacity factors k' determined by HPLC (*table I*) revealed that [Dmt[1]]DALDA is still a quite hydrophilic compound, even though it is not quite as polar as the DALDA parent peptide due to the presence of the two additional methyl groups in the Dmt[1] residue. Therefore, [Dmt[1]]DALDA is also likely to have very limited distribution across the BBB or the PB. The much more lipophilic peptide endomorphin-2 did not elute from the column under the isocratic conditions used for the determination of the k' values of the DALDA analogues. The recently reported tetrapeptide amide H-Dmt-D-Arg-Phe-β-Ala-NH_2 [16] also contains an N-terminal Dmt residue and carries a positive charge of $2+$, thus being somewhat less polar than the DALDA compounds. This compound was reported to have extraordinary μ agonist potency and quite high μ vs. δ receptor selectivity; however, its κ receptor binding affinity had not been determined. Since [Dmt[1]]-DALDA is structurally very similar to its parent

DALDA, it can be expected to have the same high stability against enzymatic degradation. On the other hand, endomorphin-1 and -2 were reported to produce only a short-lasting analgesic effect after intrathecal (i.th.) administration due to rapid breakdown in the spinal cord [17] and there is evidence to indicate that the endomorphins are degraded by dipeptidyl peptidase IV [4, 5]. Because of its extraordinary μ agonist potency, high μ receptor selectivity, polar character and stability against enzymatic degradation, [Dmt[1]]DALDA is of potential interest for use in obstetrical analgesia (e.g. i.th. administration) or in peripheral analgesia.

6. Experimental protocols

6.1. Chemistry

6.1.1. General methods

Precoated plates (silica gel 60 F_{254}, 250 μm; Merck, Darmstadt, Germany) were used for ascending TLC in the following systems (all v/v): (I) $CHCl_3$/AcOH/MeOH (85:10:15); (II) *n*-BuOH/AcOH/H_2O (4:1:1); (III) *n*-BuOH/pyridine/AcOH/H_2O (15:10:3:12). A Varian VISTA 5500 liquid chromatograph was used for the purification and the purity control of the peptides. Preparative reversed-phase HPLC was performed on a Vydac 218-TP column (22 × 250 mm) with a linear gradient of 10–40% MeOH in 0.1% TFA at a flow rate of 7 mL/min. Analytical reversed-phase HPLC was carried out on a Vydac 218-TP column (10 × 250 mm) with a linear gradient of 10–40% MeOH in 0.1% TFA at a flow rate of 1.5 mL/min. The same column was also used for the determination of the capacity factors k' under isocratic conditions (15% MeOH in 0.1% TFA at a flow rate of 2 mL/min). Proton magnetic resonance spectra were recorded at 25 °C on a Varian VXR-400S spectrometer using tetramethylsilane as internal standard. Molecular weights of compounds were determined by FAB mass spectrometry on an MS-50 HMTCTA mass spectrometer interfaced to a DS-90 data system (Dr M. Evans, Department of Chemistry, University of Montreal).

6.1.2. Amino acids and derivatives

Amino acids and Boc amino acid derivatives were purchased from Bachem Bioscience. Boc-L-Dmt-OH was obtained from RSP Amino Acid Analogues, Inc.

6.1.2.1. Preparation of 2′-methyltyrosine (Mmt)

500 mg (2.76 mmol) of Tic(OH), 65 mL of AcOH and 1.0 g of Pd/C (10%) were placed into a Parr stainless steel mini-reactor equipped with a mechanical stirrer. After purging with argon the reactor was closed, the H_2 pressure was adjusted to 5 atm and the temperature was raised to 60 °C [13]. The reaction was continued for 24 h under vigorous stirring. At the end of the reaction the catalyst was removed by filtration and the AcOH was evaporated in vacuo. The oily residue was dissolved in 10 mL H_2O and the resulting solution was decolorized with charcoal, filtered and lyophilized to yield 415 mg (82%) Mmt: TLC R_f 0.42 (I). To assess the extent of racemization, the compound was run on an HPTLC-Chir plate (Merck) using the solvent system (v/v) acetonitrile/MeOH/H_2O (6:1:1): L-Mmt, R_f 0.77, D-Mmt, R_f 0.82 (faint spot, < 10% of total product).

6.1.2.2. Preparation of Boc-Mmt(Boc)-OH

To a solution of H-Mmt-OH (309 mg, 1.6 mmol) in 150 mL of dioxane/H_2O (2:1, v/v) were added 870 mg (4 mmol) of di-*tert*-butyl dicarbonate [(Boc_2O)]. The pH of the solution was adjusted to 9 with 1.0 N NaOH and the reaction was carried out for 16 h under stirring. A second portion of ($Boc)_2O$ (870 mg, 4 mmol) was then added and the reaction was continued for another 24 h. After evaporation of the dioxane, the pH of the aqueous phase was adjusted to 2 with 1N HCl and three extractions with 50-mL portions of EtOAc were performed. The combined extracts were dried over $MgSO_4$. After filtration and solvent evaporation the residue was crystallized from EtOAc/hexane to yield 550 mg (85%) Boc-Mmt(Boc)-OH: TLC R_f 0.80 (I); ^{1}H-NMR ($CDCl_3$) δ: 1.40 (m, 9H, $C(CH_3)_3$), δ: 1.56 (s, 9H, $C(CH_3)_3$), δ: 2.26 (s, 3H, CH_3 ar), δ: 2.83–3.00 (m, 2H, CH_2), δ: 4.00–4.06 (m, 1H, CH), δ: 6.53 (dd, 1H, ar), δ: 6.60 (d, 1H, ar), δ: 7.02 (d, ar), δ: 7.18 (d, 1H, NH); FAB-MS *m*/*e* 410.

6.1.2.3. Preparation of Boc-D,L-Hmt(Boc)$_2$-OH

To a solution of H-D,L-Hmt-OH [14] (1.669 g, 7.9 mmol) in 50 mL of THF/H_2O (1:1, v/v) were added ($Boc)_2O$ (6.024 g, 26.4 mmol), TEA (2.88 mL, 20.6 mM) and DMAP (214 mg, 1.78 mmol). The solution was stirred at room temperature for 18 h. After evaporation of the THF, the aqueous phase was acidified to pH 4 with a 5% $KHSO_4$ solution and was then extracted with three 50-mL portions of EtOAc. The combined extracts were washed with water and dried over $MgSO_4$. After filtration and solvent evaporation 3.78 g (94%) of Boc-

D,L-Hmt(Boc)$_2$-OH was obtained in pure form: TLC R_f 0.77 (II), ^{1}H-NMR ($CDCl_3$) δ: 1.38 (s, 9H, $C(CH_3)_3$), δ: 1.55 (s, 18H, $C(CH_3)_3$), δ: 2.62 (s, 3H, CH_3 ar), δ: 2.82–2.95 (m, 2H, CH_2), δ: 4.07–4.13 (m, 1H, CH), δ: 6.98 (d, 2H, ar); FAB-MS *m*/*e* 512.

6.1.3. Peptide synthesis

Peptide synthesis was performed by the manual solid-phase technique using a *p*-methylbenzhydrylamine resin (1% cross-linked, 100–200 mesh, 0.54 mequiv/g of titratable amine) obtained from Bachem Bioscience. Peptides were assembled using Boc-protected amino acids and Dic and HOBt as coupling agents. The side chains of Dmt, Tmt, Mmt, Hmt were also Boc-protected. Other side chain protection was as follows: tosyl (Arg), 2-chlorobenzyloxycarbonyl (Lys), (fluoren-9-yl-methoxy)-carbonyl (Fmoc) (Orn, A_2bu). The following steps were performed in each cycle: (1) addition of Boc amino acid in CH_2Cl_2 (2.5 equiv); (2) addition of HOBt (2.5 equiv); (3) addition of DIC (2.5 equiv) and mixing for 2–3 h; (4) washing with CH_2Cl_2 (3 × 1 min); (5) washing with EtOH (1 min); (6) monitoring completion of the reaction with the ninhydrin test; (7) Boc deprotection with 50% (v/v) TFA in CH_2Cl_2 (30 min); (8) washing with CH_2Cl_2 (5 × 1 min); (9) neutralization with 10% (v/v) DIEA in CH_2Cl_2 (2 × 5 min); and (10) washing with CH_2Cl_2 (5 × 1 min).

In the case of the Fmoc side chain protected Orn^4- and A_2bu^4-analogues, the Fmoc group was removed with 20% (v/v) piperidine in DMF after complete peptide assembly. After final deprotection with 50% (v/v) TFA in CH_2Cl_2 (30 min), the resin was washed with CH_2Cl_2 (3 × 1 min) and EtOH (3 × 1 min) and was dried in a desiccator. Peptides were cleaved from the resin by treatment with HF for 60 min at 0 °C (20 mL of HF plus 1 mL of anisole/g of resin). After evaporation of the HF, the resin was extracted three times with Et_2O and, subsequently, three times with glacial AcOH. The crude peptide was obtained in solid form through lyophilization of the acetic acid extract. Crude peptides were purified by preparative HPLC. Each peptide was at least 98% pure as assessed by analytical reversed-phase HPLC and by TLC. Molecular weights were confirmed by FAB-MS.

6.2. Pharmacological testing

6.2.1. GPI and MVD bioassays

The GPI [18] and MVD [19] bioassays were carried out as reported in detail elsewhere [20, 21]. 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 [22].

6.2.2. Opioid receptor binding assays

Opioid receptor binding studies were performed as described in detail elsewhere [20]. Binding affinities for μ and δ 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 binding 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 concentrations 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 obtained IC_{50} values by means of the equation of Cheng and Prusoff [23], 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.

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