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Enkephalin Analogs Containing 4,4-Difluoro-2-aminobutyric Acid: Synthesis and Fluorine Effect on the Biological Activity[1]

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Abstract: Analogs of Met-enkephalin and [D-Pen^2, D-Pen^5]enkephalin (DPDPE) containing the partially fluorinated amino acid 4,4-difluoro-2-aminobutyric acid (DFAB) in the 2- or 3-position of the peptide sequence were synthesized and their opioid activities and receptor selectivities were determined *in vitro*. The linear fluorinated [D-DFAB^2, Met^5-NH$_2$]enkephalin showed μ and δ agonist potencies comparable to those of natural [Leu^5]enkephalin. The partially fluorinated DPDPE analogs behaved differently as compared with their non-fluorinated correlates. While L-amino acid substitution in position 3 of DPDPE usually resulted in higher δ agonist potency than D-amino acid substitution, [D-DFAB^3]DPDPE turned out to be a more potent δ agonist than [L-DFAB^3]DPDPE. Furthermore, [D-DFAB^3]DPDPE showed over 100-fold higher δ agonist potency than [D-Abu^3]DPDPE (Abu = 2-aminobutyric acid), indicating that the fluorine substituents interact favorably with a δ opioid receptor subsite. © 1998 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: enkephalins; DPDPE; opioid agonists; δ-receptor; fluorine containing amino acid

INTRODUCTION

The opioid receptors, which belong to the superfamily of G-protein coupled receptors, are distributed throughout the mammalian central and peripheral nervous system and can be classified at least into three different types (μ, δ, κ) with various possible subtypes. Their endogenous peptide ligands, e.g. enkephalins, are involved in the regulation of different CNS pathways [1,2]. However, besides their analgesic activity, addictive potential and other side-effects known to be produced by opiates are often also observed with opioid peptides. Morphine-like non-peptide compounds and the endomorphins show high preference for μ opioid receptors. δ Opioid receptors are selectively activated by enkephalin-like peptides, in particular Leu-enkephalin, while dynorphin-like peptides have high affinity and some preference for κ opioid receptors.

The replacement of Gly^2 in Met-enkephalin with D-5,5,5-trifluoronorvaline (D-TFNV) results in an enhancement of the *in vivo* analgesic activity by five orders of magnitude [3]. However, the 'fluorine effect' of this substitution contributes only about half an order of magnitude to the activity enhancement, because the substitution of glycine in position 2 by D-norvaline already increases the activity by factor 17500. This increase of *in vivo* activity is not primarily the result of improved receptor binding, but mainly seems to be a consequence of reduced metabolism due to the presence of the D-amino acid residue. Furthermore, improved transport properties due to the incorporation of a more lipophilic amino acid residue may also be a factor [3].

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Conformationally constrained peptide analogs have considerably contributed to the up-to-date knowledge on the nature and role of the opioid receptors, because some of them retain physiological activity and are highly selective towards either μ or δ opioid receptors [4,5]. In these cases, the three-dimensional structure of the ligand in solution and in the receptor-bound state are likely to be quite similar. Hence, based on the conformational analysis of such constrained analogs, a 3D pharmacophore model may be constructed.

The peptide [D-Pen^2, D-Pen^5]enkephalin (DPDPE) is a conformationally constrained, δ selective opioid peptide analog characterized by side-chain to side-chain disulfide cyclization [6]. According to recent studies DPDPE is selective towards the δ_1 receptor subtype, while the deltorphins are δ_2 selective [7]. Interactions between δ-opioid receptors and morphine-preferring μ-opioid receptors have been suggested. It was found that selective δ-opioid agonists may also require μ-opioid receptor occupancy for full efficacy. Hence, the μ-opioid receptor seems to be necessary for DPDPE induced analgesia [8]. DPDPE is enzymatically stable and excreted extensively into bile. It has been suggested that an active transport system in hepatocytes mediated the rapid disappearance of DPDPE from the systemic circulation [9]. A peptide carrier system may also play a role in the ability of DPDPE to penetrate the blood–brain barrier (BBB) to a limited extent [10].

The amino acid side-chain requirements and the influence of the chirality at position 3 of DPDPE analogs have been investigated with respect to the opioid activity profile [11] and the three-dimensional structure of the compound has been studied both in the crystalline state and in solution [12]. [L-Ala^3]DPDPE was found to possess much higher δ-receptor binding selectivity compared with DPDPE itself. However, it antagonizes the antinociceptive effects of DPDPE at the δ-receptors in the brain [12].

We were interested in the effects on biological activity of the incorporation of amino acids with partially fluorinated side-chains [13], for example, of the non-natural amino acids D-and L-4,4-difluoro-2-aminobutyric acid [14] (Figure 1) into enkephalin analogs, especially into derivatives of DPDPE. Side-chain fluorination may result in enhanced local lipophilicity (enhancement of absorption and transport rates).

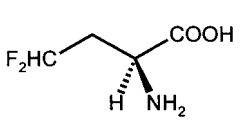

Figure 1 L-4,4-Difluoro-2-aminobutyric acid.

MATERIALS AND METHODS

D- and L-4,4-difluoro-2-aminobutyric acid were synthesized according to a previously published procedure [14]. Other protected amino acid derivatives, resins (Rink amide MBHA resin, 2-chlorotrityl resin) and TBTU (1-[Bis(dimethylamino)methyliumyl]-1H-benzotriazol-3-oxide tetrafluoroborate) were purchased from Novabiochem (Läufelfingen, Switzerland). HATU (1-[Bis(dimethylamino)methyliumyl]-1H-1,2,3-triazolo[4,5-*b*]pyridin-3-oxide hexafluorophosphate) as coupling reagent was from PerSeptive Biosystems (Framingham, USA). Solvents were dried and purified prior to use by common procedures. Analytical HPLC was performed on a Merck-Hitachi (Darmstadt, Germany) LaChrom system fitted with a Nucleosil 100 C18, 5 μm column (4 × 250 mm). Eluents: H_2O/CH_3CN/TFA: A (95:5:0.1), B (5:95:0.1); gradient: 7% B for 2 min, 7–45% B in 15 min, then isocratic; flow: 1 mL/min; diode array detection. Semi-preparative HPLC was performed on a Pharmacia LKB system (Uppsala, Sweden) using a Pharmacia SuperPep-S™ C2/C18, 15 μm column (22.5 × 250 mm) at 15 mL/h flows (eluents and gradient see above). Mass spectra were recorded on a Voyager-DE™RP MALDI-ToF mass spectrometer (PerSpective Biosystems) in the reflection mode using α-cyano-4-hydroxycinnamic acid as matrix and mass calibration standard.

Peptide Synthesis

Peptides were synthesized using an ACT 90 peptide synthesizer (Advanced Chem Tech, Louisville, USA) on a 0.2 mmol scale. A Fmoc protocol was followed for peptide synthesis both for the linear and the cyclic analogs.

The linear analogs were assembled on Rink amide MBHA resin as solid support, using a three-fold excess of DIPEA (*N*,*N*-diisopropylethylamine) as base and TBTU as coupling reagent. The Tyr side-chain hydroxyl group was protected as the *tert*-butyl ether. DMF was used as solvent throughout the syntheses. N$^{\alpha}$-Fmoc temporary protection was removed by treatment with 2% DBU, 2% piperdine in DMF (v/v). Cleavage from the resin with 95% TFA

Table 1 Synthesized Enkephalin Analogs

Sequence	Compound		RP-HPLC[a] t_R (min)	m/z MALDI ToF MS Calcd.	Found
H-Tyr-D-DFAB-Gly-Phe-Met-NH_2	[D-DFAB, Met^5-NH_2]enkephalin	**1**	14.72	637.12	637.10
H-Tyr-*c*[D-Pen-Gly-Phe-D-Pen]-OH	DPDPE	**2**	16.36	646.24	646.24
H-Tyr-*c*[D-Pen-Abu-Phe-D-Pen]-OH	[L-Abu^3]DPDPE	**3**	17.45	674.29	674.31
H-Tyr-*c*[D-Pen-D-Abu-Phe-D-Pen]-OH	[D-Abu^3]DPDPE	**4**	17.41	674.29	674.29
H-Tyr-*c*[D-Pen-Leu-Phe-D-Pen]-OH	[L-Leu^3]DPDPE	**5**	20.48	702.30	702.26
H-Tyr-*c*[D-Pen-D-Leu-Phe-D-Pen]-OH	[D-Leu^3]DPDPE	**6**	19.59	702.30	702.29
H-Tyr-*c*[D-Pen-DFAB-Phe-D-Pen]-OH	[L-$DFAB^3$]DPDPE	**7**	19.43	710.24	710.20
H-Tyr-*c*[D-Pen-D-DFAB-Phe-D-Pen]-OH	[D-$DFAB^3$]DPDPE	**8**	18.31	710.24	710.21

[a] Column: Nucleosil 100 C18, 5 μm, 250 mm × 4 mm; H_2O/CH_3/TFA: A (95:5:0.1), B (95:5:0.1); gradient elution.

yielded crude products (purities: 80–85%, yields: 75–80%) which were purified by HPLC (acetonitrile/water/TFA gradient) and lyophilized to give the peptides as trifluoro-acetate salts in ≥98% purity.

The DPDPE analogs were synthesized with Fmoc protected amino acids on 2-chlorotrityl resin, except for the last coupling which was performed with Z-Tyr(Bzl)-OH. Furthermore, the use of S-benzyl protected D-penicllamine allowed for the simultaneous reductive cleavage (Na, liquid ammonia) of all permanent protective groups at the end of the synthesis of the linear precursor peptide. HATU was used as coupling reagent in three-fold excess throughout the synthesis. Other reagents and solvents were used as described for the linear peptides. After cleavage of the protected linear precursor peptide from the resin with 0.5% TFA in CH_2Cl_2, the crude products (purities: ~85%, yields: ~80%) were purified by HPLC. Subsequent treatment with sodium in liquid ammonia at −33°C for 10 min resulted in cleavage of all protective groups. After evaporation of the ammonia, the residue was acidified to pH 4.5 with glacial acetic acid and lyophilized. Cyclization via disulfide bond formation was achieved by slow addition of the free peptide in methanol (rate: 10 mg/mL·h) to a dilute aqueous solution of $K_3[Fe(CN)_6]$ as described in the literature [15]. The peptides were purified by semi-preparative RP-HPLC (resulting purities ≥98%) and characterized by NMR, RP-HPLC and MALDI-ToF MS (Table 1).

Biological Assays

The opioid activity profiles of the compounds were determined in the *guinea pig ileum* (GPI) and *mouse vas deferens* (MVD) assays, using protocols published elsewhere [16]. The GPI and MVD assays, based on the inhibition of electrically evoked contractions, are μ and δ receptor representative bioassays, respectively. The GPI assay is usually considered as being representative for interactions with μ receptors, whereas in the MVD assay opioid effects are primarily mediated by δ receptors. The results are summarized in Table 2 and Figures 2 and 3.

RESULTS AND DISCUSSION

Replacement of Gly in position 2 of [Met^5-NH_2]enkephalin by D-DFAB (D-amino acids are generally favored in this position) [3] resulted in a compound with μ and δ agonist potencies comparable with those of the reference peptide [Leu^5]enkephalin. This finding is in accordance with results published previously concerning the *in vitro* opioid activities of [D-$TFNV^2$, Met^5-NH_2]enkephalin [3]. As in the case of the latter compound, transport properties, decreased metabolism, and, hence, higher *in vivo* activity can be expected for [D-$DFAB^2$, Met^5-NH_2]enkephalin.

As can be seen from Table 2 and Figures 2 and 3, the DPDPE analogs containing non-fluorinated L-configured amino acids in position 3 are more potent than their correlates with the corresponding D-configured amino acid in the same position. Moreover, the IC_{50} values for the [D-Xaa^3]DPDPE analogs increase in the series Xaa = Ala < Ser < Abu [11]. Hence, it has been postulated that L-amino acids in position 3 are highly favored over D-amino acids. The side-chain of an L-amino acid in this position is supposed to fit into a complementary pocket of the δ receptor.

Most interestingly, the situation is reversed in the case of the [$DFAB^3$]DPDPE derivatives: [D-

Table 2 *In Vitro* Opioid Activity Profiles of Partially Fluorinated Enkephalin Analogs in Comparison with Structurally Related Non-fluorinated Compounds

Compound		GPI assay[a] IC_{50} (nM)	Relative potency[b]	MVD assay[a] IC_{50} (nM)	Relative potency[b]	IC_{50} ratio (GPI/MVD)
[Leu^5]enkephalin		246 ± 39	1	11.4 ± 1.1	1	21.6
[D-DFAB^2, Met^5-NH_2]enkephalin	**1**	186 ± 72	1.32 ± 0.51	17.5 ± 1.8	0.651 ± 0.067	10.6
DPDPE[c]		7300 ± 1700	0.0337 ± 0.0078	4.1 ± 0.5	2.78 ± 0.26	1800
DPDPE	**2**	$>10\,000$	<0.0246	5.30 ± 0.59	2.15 ± 0.24	>1890
[L-Ala^3]DPDPE[c]		$54\,000 \pm 3000$	0.0045 ± 0.0003	12 ± 1.6	0.95 ± 0.127	4500
[D-Ala^3]DPDPE[c]		$33\,000 \pm 820$	0.0074 ± 0.0002	570 ± 130	0.02 ± 0.0046	58
[L-Ser^3]DPDPE[c]		$39\,000 \pm 7200$	0.0063 ± 0.0012	250 ± 33	0.456 ± 0.0060	160
[D-Ser^3]DPDPE[c]		3% inh. at 100 μM		1300 ± 250	0.0088 ± 0.0017	>79
[L-Abu^3]DPDPE[c]	**3**	4900 ± 350	0.0502 ± 0.0036	85 ± 13	0.0088 ± 0.0017	57
[D-Abu^3]DPDPE[c]	**4**	$30\,000 \pm 2700$	0.0082 ± 0.0007	1950 ± 200	0.0058 ± 0.0006	15
[L-Leu^3]DPDPE	**5**	$>10\,000$	<0.0246	$>10\,000$	<0.00114	–
[D-Leu^3]DPDPE	**6**	$>10\,000$	<0.0246	$>10\,000$	<0.00114	–
[L-DFAB^3]DPDPE	**7**	$>10\,000$	<0.0246	155 ± 5.1	0.0734 ± 0.0024	>65
[D-DFAB^3]DPDPE	**8**	$>10\,000$	<0.0246	17.9 ± 2.1	0.673 ± 0.074	>559

[a] Mean of three determinations ± SEM.
[b] Potency relative to [Leu^5]enkephalin.
[c] Data taken from Reference [11].

DFAB^3]DPDPE (Figure 4) is not only nine times more potent than [L-DFAB^3]DPDPE, but also more δ-selective. The fluorine effect on the δ receptor interaction can be assessed by comparing [D-DFAB^3]DPDPE with [D-Abu^3]DPDPE. The D-DFAB^3 analog is at least two orders of magnitude more potent and about 40 times more δ selective than the D-Abu^3 analog and, in fact, it is also more potent and ten times more δ selective than [L-Abu^3]DPDPE. We conclude that the fluorine substituents of [D-DFAB^3]DPDPE interact favorably with a lipophilic δ receptor subsite or displace water molecules from receptor pockets. Furthermore, the fluorine substituents might act as hydrogen bond acceptors. Interestingly, the two [Leu^3]DPDPE analogs that contain two methyl groups at C^{γ} of the 3-position residue instead of the two fluorine substituents present in the [DFAB^3]DPDPE analogs were found to be completely inactive. A discussion on the 3D-structure based on conformationally relevant NMR data will be published elsewhere (the complete set of NMR data will be published there).

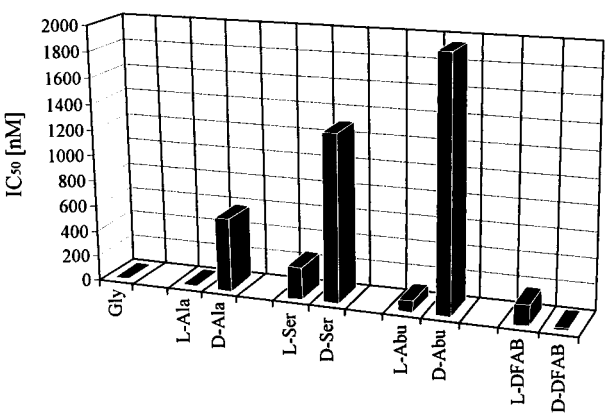

Figure 2 IC_{50} values of [Xaa^3]DPDPE derivatives determined in the MVD assay (Ala, Ser, Abu data from Reference [11]).

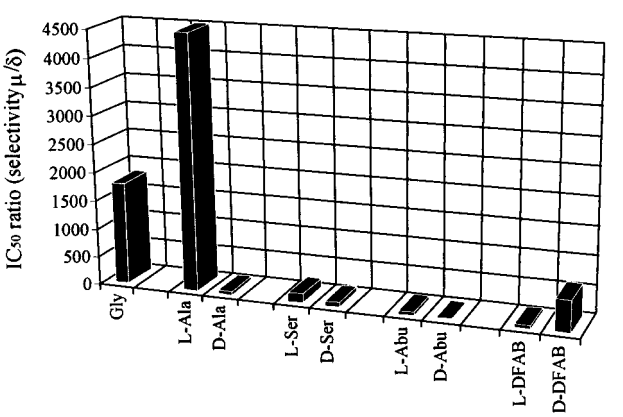

Figure 3 μ/δ Selectivity ratios of [Xaa^3]DPDPE derivatives (Ala, Ser, Abu data from Reference [11]).

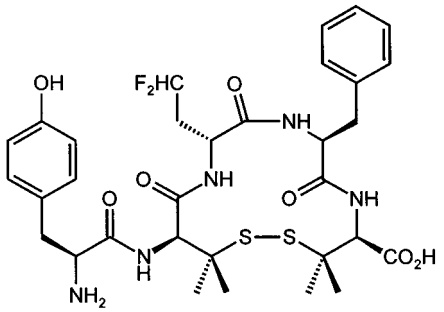

Figure 4 [D-DFAB^3]DPDPE.

CONCLUSIONS

A pronounced 'fluorine effect' is observed in the case of partially fluorinated DPDPE analogs: [D-DFAB^3]DPDPE possesses 100-fold higher δ agonist potency compared with [D-Abu^3]DPDPE. Furthermore, [D-DFAB^3]DPDPE is a more potent δ agonist than [L-DFAB^3]DPDPE, while usually the [L-Xaa^3]DPDPE derivatives are more active than the [D-Xaa^3] correlates. This favorable interaction between the fluorine substituents with the δ opioid receptor subsite may, for example, arise from additional hydrophobic contacts, hydrogen bridges with fluorine as a hydrogen bond acceptor, modified electrostatic properties due to the polarization of the CF bonds, and/or the displacement of water molecules from the receptor pocket, respectively.

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