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Enkephalin Analogues with 2′,6′-Dimethylphenylalanine Replacing Phenylalanine in Position 4

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Abstract—Four Leu-enkephalin (Enk) analogues containing 2′,6′-dimethyphenylalanine (Dmp) in position 4 were prepared and tested for their receptor binding and biological activities. Among the analogues prepared, [2′, 6′-dimethyltyrosine[1], D-Dmp[4]]Enk was found to be an antagonist toward μ and δ opioid receptors with pA_2 values of 6.90 and 5.57, respectively.

Introduction

Two aromatic amino acids, Tyr[1] and either Phe[3] or Phe[4] are important structural elements in opioid peptides that interact with the opioid receptors.[1] Recent structure–activity studies of opioid peptides have demonstrated that the introduction of 2′,6′-dimethyltyrosine (Dmt) in place of Tyr[1] produces compounds with vastly improved opioid receptor binding affinities.[2–16] Via the combination of Dmt with 1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid (Tic), some δ opioid receptor selective antagonists, including Dmt-Tic-OH,[13,14] *N*,*N*′-diMe-Dmt-Tic-OH,[15] DIPP-NH_2[10] and DIPP[ψ],[12] have been developed. Conformational studies of these peptides have revealed that the topology of the two aromatic rings induced by the two unusual amino acid residues and increased hydrophobicity imparted by the Dmt residue are critical to the high activity and δ receptor selectivity.[9,11,14] Very recently, we also demonstrated that [Dmt[1]]Leu-enkephalin is an exceedingly potent opioid agonist[16] and that the modification of the Tyr[1] aromatic nucleus by 2′,6′-dimethylation imparts a high enzymatic stability to the peptides.[16] From these findings, modification of a Phe aromatic nucleus at position 3 or 4 of an opioid peptide by 2′,6′-dimethylation can be considered to be another interesting approach in the design of opioid mimetics with unique biological activity. Nevertheless, there has been no conclusive demonstration that the phenyl ring-methylated phenylalanine is incorporated into opioid peptides but only a few into other biologically active peptides.[17,18] We present here novel Leu-enkephalin (Enk) analogues containing 2′,6′-dimethylphenylalanine (Dmp) in position 4, and their opioid receptor binding and biological activities in comparison with the parent molecules.

Chemistry

Scheme 1 illustrates the synthetic route to Dmp. For the synthesis of a key intermediate, 2-iodo-*m*-xylene (**7**), commercially available **5** was reacted with sodium trimethylstannane according to Yamamoto et al.,[19] followed by reaction with iodine according to the method of Ohno et al.[20] The reaction of **7** with methyl 2-acetamidoacrylate, Dygos's method,[21] led to **8**, followed by saponification to yield **9**. The catalytic hydrogenation of **9** then yielded racemic Ac-Dmp (**10**). For the optical resolution, **10** was converted to its dipeptide derivatives, Ac-DL-Dmp-Arg-OMe (**11**), which were easily separated into each diastereoisomer by preparative HPLC.[22] Acid hydrolysis and neutralization of each isomer yielded the L- (**12**) and D-Dmp (**13**), which were converted to their N^{α}-Fmoc derivatives (**14** and **15**).[23] The synthesis of all Enk analogues[24] (**1**–**4** in Table 1) was performed via a solid-phase method using Fmoc chemistry, as previously described.[16,25]

Biological Results and Discussion

Table 1 shows the results of receptor binding affinity of the analogues, which were determined using rat brain

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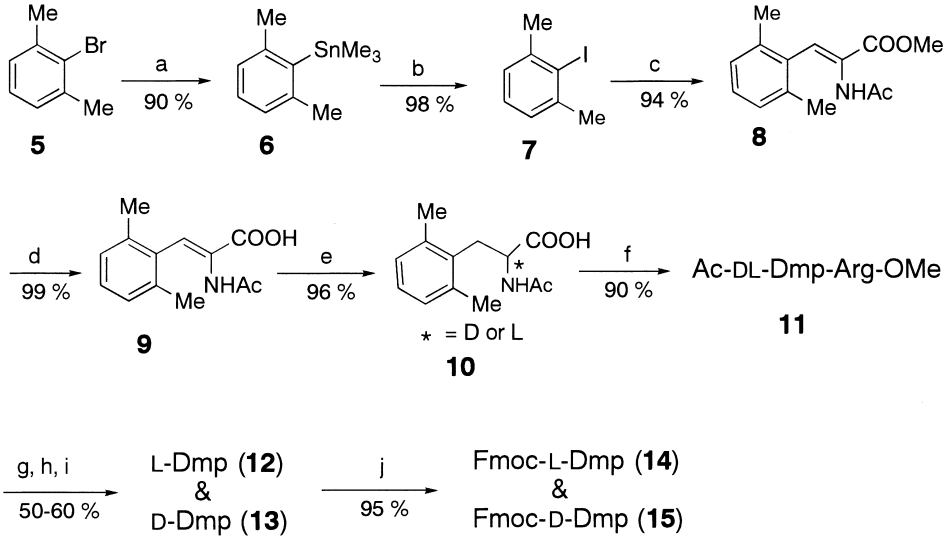
synaptosomes, as previously reported.[25] Replacement of Phe[4] in Enk by L-Dmp led to formation of **1** with a μ affinity comparable to Enk but with an approximately 12-fold reduced δ affinity, suggesting that this process of displacement is detrimental to δ receptor affinity, but not to μ receptor affinity. The D-Dmp[4] replacement of Enk (**2**) induced a drastic reduction in binding affinity to both receptors, a finding in accordance with some reports that L-configuration at the fourth residue in Enk is an important factor for high receptor affinity and opioid activity.[26] On the other hand, a combination of L-Dmp[4] and Dmt[1] replacements produced **3** with markedly improved affinities for both receptors. This analogue exhibited 40-fold and 110-fold higher affinities than **1** for the μ and δ receptors, respectively, but a 5-fold lower affinity than [Dmt[1]]Enk[16] for both receptors, possibly due to slight changes of an active conformation by the simultaneous dimethylations of two aromatic nucleuses. It should be noted that the other combination of D-Dmp[4] and Dmt[1] replacements produced **4**, which retained a μ affinity nearly equivalent to that of Enk and with a modest δ affinity, showing again



Scheme 1. Synthetic route to Fmoc-L-Dmp (**14**) and Fmoc-D-Dmp (**15**): (a) Me_3SnNa, DME, ice-salt bath, 2 h; (b) I_2/THF, rt, 3 h; (c) methyl 2-acetamidoacrylate/$Pd(OAc)_2$/Et_3N/MeCN, $(2\text{-}MeC_6H_4)_3P$, reflux, 24 h; (d) 1 M NaOH/dioxane, rt, 2 h; (e) H_2 (4 kgf/cm^2)/10% Pd-C/AcOH, 70 °C, 48 h; (f) HCl·Arg-OMe/Et_3N/DCC/HOBt/DMF, 0 °C to rt, 5 h; (g) preparative HPLC; (h) concd HCl, reflux, 8 h; (i) pH 4–6/H_2O; (j) Fmoc-OSu/Na_2CO_3/aq MeCN.

Table 1. Opioid receptor binding affinity of Dmp replacing Enk analogues

Peptide	Binding affinity, K_i±S.E. (nM)		Selectivity
	μ[a]	δ[b]	μ/δ
Tyr-Gly-Gly-Phe-Leu (Enk)	2.42 ± 0.93	1.43 ± 0.71	1.69
[Dmt[1]]Enk[c]	0.0068 ± 0.0030	0.031 ± 0.011	0.22
[L-Dmp[4]]Enk (**1**)	1.25 ± 0.29	17.7 ± 4.2	0.07
[D-Dmp[4]]Enk (**2**)	2505 ± 169	8924 ± 4098	0.28
[Dmt[1], L-Dmp[4]]Enk (**3**)	0.030 ± 0.011	0.158 ± 0.034	0.19
[Dmt[1], D-Dmp[4]]Enk (**4**)	5.61 ± 0.59	40.9 ± 11.5	0.14

[a]Versus [^{3}H]DAMGO.
[b]Versus [^{3}H]deltorphin II.
[c]Data cited from ref 16.

Table 2. In vitro biological activity of Dmp replacing Enk analogues

Peptide	GPI (μ)		MVD (δ)		
	IC_{50} (nM)[a]	pA_2	IC_{50} (nM)[a]	pA_2	GPI/MVD
Tyr-Gly-Gly-Phe-Leu (Enk)	103 ± 30	—	22.2 ± 4.3	—	4.64
[Dmt[1]]Enk[b]	0.55 ± 0.17	—	0.17 ± 0.02	—	3.24
[L-Dmp[4]]Enk (**1**)	808 ± 101	—	624 ± 103	—	1.29
[D-Dmp[4]]Enk (**2**)	>10,000	—	>10,000	—	—
[Dmt[1], L-Dmp[4]]Enk (**3**)	2.00 ± 0.51	—	1.45 ± 0.26	—	1.38
[Dmt[1], D-Dmp[4]]Enk (**4**)	>10,000	6.90[c]	>10,000	5.59[d]	—

[a]Values are the mean of 4–8 experiments ± S.E.
[b]Data cited from ref 16.
[c]Against endomorphin 2 as an agonist.
[d]Against deltorphin II as an agonist.

the effectiveness of Dmt[1] replacement for maintaining high receptor affinity.

In vitro biological activity was evaluated using isolated guinea pig ileum (GPI) and mouse vas deferens (MVD) tissue samples, as previously reported.[27] The GPI tissue contains predominantly μ receptors, while MVD includes δ receptors. Analogue **1** showed reduced activity, and was 8- and 30-fold less potent than Enk in the GPI and MVD assays, respectively. Analogue **2** was devoid of activity in both assays as expected from the binding data. Consistent with the binding data, **3** had a high potency in both assays. Notably, analogue **4** was also devoid of activity in both assays despite the fact that this analogue showed potent μ affinity and modest δ affinity. This analogue turned out to be a potent μ antagonist and weak δ antagonist. The pA_2 values of **4** were 6.90 against endomorphin 2, as a μ agonist in the GPI assay, and 5.57 against deltorphin II, as a δ agonist, in the MVD assay. No significant antagonist activity was observed with **2** in either assay. The results of **4** are of interest in light of recent observations that Dmt-D-Phe-NH_2 and its C-terminally extended analogue are μ receptor antagonists.[28] However, it is possible that the antagonist action of **4** is attributable to the presence of the two dimethylated aromatic amino acids at positions 1 and 4 because the two newly synthesized peptides, Dmt-D-Dmp-NH_2 and Dmt-Gly-Gly-D-Phe-Leu, have no antagonist activity, but did show a weak agonist activity in the GPI assay (our unpublished results). In conclusion, the present study demonstrated that Dmp is useful as a Phe surrogate in the design of opioid mimetics with unique biological activity. Analogue **4** may lead to more potent and selective novel μ receptor antagonists. Further studies are now in progress.

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22. Medium-pressure HPLC was performed on a Develosil LOP ODS column (3×30 cm, Nomura Kagaku) using a CH_3CN–0.06% TFA solvent system.
23. An aqueous solution of the HC1 hydrolysate was adjusted to pH 4–6 with Na_2CO_3 yielding L- or D-Dmp as a colorless precipitate. Determination of the L and D configurations was carried out using L-amino acid oxydase according to a previously described method (Toth, G.; Lebl, M.; Hruby, V. *J Chromatogr.* **1990**, *504*, 450). The result showed that Dmp derived from the dipeptide analogue, which eluted later in the preparative HPLC, turned out to have an L-configuration, and the other analogue, which eluted more quickly, was a D-antipode. L-Dmp (**12**): $[\alpha]_D$ +75.1° (c=0.73, 0.1 N HCl), R_f 0.56 on a chiral TLC plate (Macherey-Nagel, Germany, 5×20 cm, CH_3CN:H_2O:MeOH=4:1:1). D-Dmp (**13**): $[\alpha]_D$ −69.0° (c=0.73, 0.1 N HCl), R_f 0.43. Both L- and D-Dmps were converted to Fmoc derivatives using Fmoc-OSu, following a standard method. Fmoc-L-Dmp (**14**): mp 196–198 °C, $[\alpha]_D$ −26.8° (c=0.5, MeOH). Fmoc-D-Dmp (**15**): mp 195–197 °C, $[\alpha]_D$ +34.8° (c=0.5, MeOH).
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