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Studies on the structure–activity relationship of 2′,6′-dimethyl-L-tyrosine (Dmt) derivatives: bioactivity profile of H–Dmt–NH–CH_3

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Abstract—The 2′,6′-dimethyl-L-tyrosine (Dmt) enhances receptor affinity, functional bioactivity and in vivo analgesia of opioid peptides. To further investigate its direct influence on these opioid parameters, we developed a series of compounds (H–Dmt–NH–X). Among them, H–Dmt–NH–CH_3 showed the highest affinity ($K_i\mu$ = 7.45 nM) equal to that of morphine, partial μ-opioid agonism (E_{max} = 66.6%) in vitro and a moderate antinociception in mice.

The common N-terminal amino acid of endogenous opioid peptides (enkephalins,[1] endorphins,[2] dynorphins,[3] endomorphins,[4] deltorphins,[5] dermorphins,[6] and so on) is Tyr, except Phe in nociceptin,[7] and is considered an important pharmacophore to interact with opioid receptors according to the message–address concept.[8] Recent studies verified that Dmt (2′,6′-dimethyl-L-tyrosine)[9] can readily replace Tyr in opioid substances and dramatically enhances receptor affinity, in vitro functional bioactivity and in vivo analgesic action.[10] For example, affinity increased by several hundredfold for enkephalin derivatives, such as [Leu^5]enkephalin[11] and DPDPE (*cyclo*[D-Pen^{2,5}]enkephalin),[12] the dermorphin analogue DALDA (H-Tyr-D-Arg-Phe-Lys-NH_2),[13] deltorphin,[14] and endomorphin-2 (EM-2)[15] as well as the Tic-containing di- and tripeptide DOR (δ-opioid receptor) antagonists.[10,16] A study on [Dmt^1]EM-2 deletion analogues also revealed that H–Dmt–Pro–NH_2 exhibited moderate affinity ($K_i\mu$ = 41.7 nM) in contrast to the inactive Tyr derivative.[15] Furthermore, the design and synthesis of the novel opioid ligands, H–Dmt–NH–X–NH–Dmt–H, in which the Dmt pharmacophores were linked by either diaminoalkane or 3,6-bis-(aminoalkyl)-5-methyl-2(1*H*)pyrazinone, produced analogues that had higher MOR (μ-opioid receptor) affinities and greater functional bioactivities than those containing single or multiple Tyr residues.[17] This report presents comprehensive data on the development of an opioid compound library using Dmt as the single core amino acid (Scheme 1).

Compounds were synthesized as shown in Scheme 1. The NH_2–CH_2–NH–Boc was prepared from Boc–Gly–NH_2 by Hofmann rearrangement using bis-(trifluoroacetoxy)-iodobenzene.[18] It was reported that although diaminomethane is unstable, mono-protected diaminomethane is comparatively stable.[19] The NH_2–CH_2–NH–Boc obtained here was identified by TLC, NMR, and MS.[20] The NH_2–C_2H_4–NH–Boc was prepared from commercially available NH_2–C_2H_4–NH_2[21] and the other amino components were purchased from commercial sources. Dmt was prepared according to the Dygos' method.[9]

Keywords: Dmt; μ-Opioid agonist; Structure–activity relationship.
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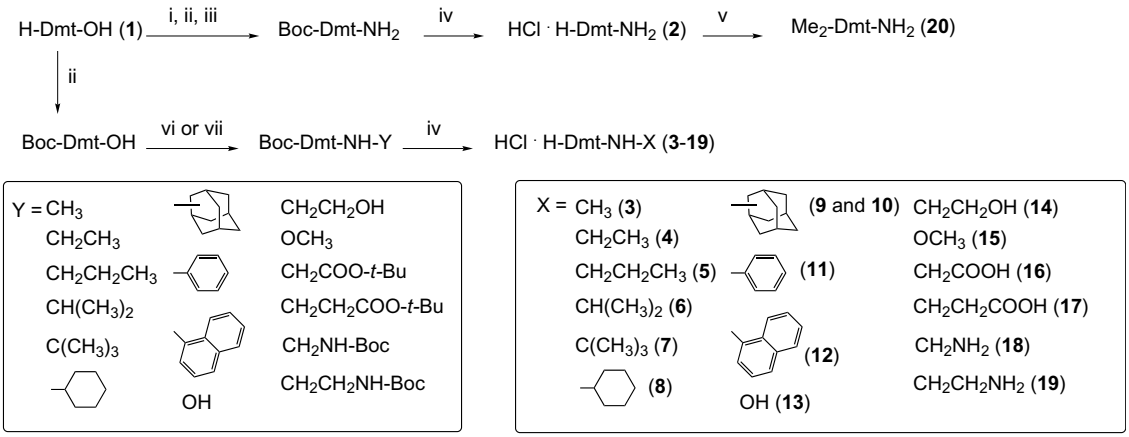

Scheme 1. Synthetic scheme for the Dmt compound library. Reagents and conditions: (i) $SOCl_2/CH_3OH$; (ii) $(Boc)_2O$ and Et_3N; (iii) NH_3/CH_3OH; (iv) HCl/dioxane; (v) HCHO and $NaBH_3CN$; (vi) PyBop, *N*,*N*-diisopropylethylamine and NH_2–Y; (vii) isobutyl chloroformate, Et_3N, and NH_2–Y.

The competitive displacement assay[15] was performed using [^{3}H]DAMGO (H-Tyr-D-Ala-Gly-N$^{\alpha}$MePhe-Gly-ol) and [^{3}H]DPDPE for MOR and DOR, respectively (Table 1). The data for Tyr derivatives are not shown due to negligible affinities (K_i = 3.4–56 μM). Although the free amino acid H–Dmt–OH (**1**) was almost inactive toward both opioid receptors, amidation of the carboxyl group (**2**) greatly enhanced receptor affinity, particularly toward MOR. Since the C-terminal substituent influences the degree of binding affinity and selectivity between MOR and DOR,[10] the carboxyl moiety was further modified.

Alkylation of the carboxylamide group of H–Dmt–NH_2 further increased the affinity toward both opioid receptors: H–Dmt–NH–CH_3 (**3**) exhibited high MOR affinity (K_i = 7.45 nM), nearly equivalent to morphine[22] and approximately one-tenth of EM-2,[4] however, increasing the size of the alkyl group decreased MOR affinity in the series of CH_3 (**3**) > C_2H_5 (**4**) > *n*-propyl (**5**) > *iso*-propyl (**6**) > *tert*-butyl (**7**). Moreover, the introduction of bulky hydrophobic residues, such as cyclohexyl (**8**) and adamantyl (**9**, **10**), and aromatic residues, such as phenyl (**11**) and naphthyl (**12**), decreased MOR affinity and increased DOR affinity, such that adamantyl (**9**, **10**), phenyl (**11**), and naphthyl (**12**) exhibited DOR selectivity. The introduction of a polar functional group such as an OH group (**13**, **14**) decreased MOR affinity in comparison to substances **2** and slightly decreased compared with compound **4**, respectively. Furthermore, compound **15**, in which the OH group was masked as OCH_3, showed higher receptor affinity than that of **13**.

Table 1. Competitive receptor affinities of opioid ligands with the single amino acid Dmt

	Compounds	Receptor affinity $K_i\mu$ (nM)[a]	Receptor affinity $K_i\delta$ (nM)[a]	$K_i\delta/K_i\mu$
1	H–Dmt–OH	145,300 ± 28,800	46,500 ± 11,800	1.55
	H–Dmt–NH–X			
2	X = H	112 ± 6.2	1,470 ± 123	13
3	CH_3	7.45 ± 0.4	460 ± 30	61.7
4	C_2H_5	15.6 ± 2.7	649 ± 119	41.6
5	*n*-Propyl	16.9 ± 0.56	166 ± 32	9.82
6	*iso*-Propyl	22.4 ± 0.92	492 ± 32	22
7	*tert*-Butyl	36 ± 2.5	531 ± 7.0	14.8
8	Cyclohexyl	115 ± 3.8	197 ± 2.0	1.71
9	1-Adamantyl	767 ± 26	208 ± 38	0.27
10	2-Adamantyl	746 ± 50	175 ± 29	0.23
11	Phenyl	1,730 ± 98	120 ± 18	0.069
12	Naphthyl	966 ± 87	52.8 ± 6.5	0.054
13	OH	1,700 ± 246	9,490 ± 2,650	5.58
14	C_2H_4OH	16.2 ± 3.0	455 ± 38	28.1
15	OCH_3	174 ± 15	4,580 ± 1,310	26.3
16	CH_2COOH	2,700 ± 214	1,150 ± 490	0.43
17	C_2H_4COOH	329 ± 2.45	328 ± 93	1
18	CH_2NH_2	180 ± 55	2,410 ± 640	13.4
19	$C_2H_4NH_2$	10.8 ± 1.85	3,650 ± 724	338
20	*N*,*N*-$(CH_3)_2$–Dmt–NH_2	216 ± 17	1,450 ± 270	6.71
Morphine[22]		6.55	217	33.1
EM-2[4]		0.69	9230	13400
H–Dmt–Tic–OH[16b]		3220	0.022	6.6×10^{-7}

[a] Values are means of four to seven experiments, K_i values as the mean ± SE.

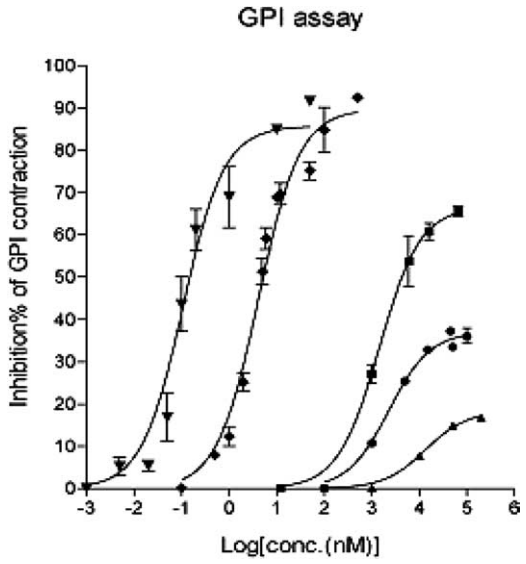

Figure 1. Dose–response curves in the GPI assay. Substances: ♦ EM-2 (E_{max} = 88.0%, IC_{50} = 5.79 nM); ▼ [Dmt^1]EM-2 (E_{max} = 85.7%, IC_{50} = 0.071 nM); ● H–Dmt–Pro-NH_2 (E_{max} = 36.9%); ▲ H–Dmt–NH_2 (E_{max} = 18.2%); ■ H–Dmt–NH–Me (E_{max} = 66.6%, IC_{50} = 6568 nM).

The incorporation of COOH (**16**, **17**), which introduces a negative charge, also clearly demonstrated a decrease in MOR affinity since it might repel ionic site of MOR;[23] although both compounds had weak affinities, they were either slightly DOR selective (**16**) or nonselective (**17**). On the other hand, the incorporation of NH_2 to the C-terminus of H–Dmt–NH–C_2H_5 (**4**) to produce H–Dmt–NH–C_2H_4–NH_2 (**19**), which enhanced MOR affinity and decreased DOR affinity, resulting in the highest MOR selective ligand ($K_i\delta/K_i\mu$ = 338), so far examined. In summary, these data shown in Table 1 confirmed the principle that opioid ligands containing a C-terminal hydrophobic, aromatic, or negatively charged group are selective for DOR.[10,13,17,23–25] The alkylation of the amino group, *N*,*N*-dimethylation (**20**), had a negative effect on opioid receptor interaction, and produced a compound with a very weak DOR affinity even though N^{α}-alkylation is known to enhance receptor affinity to DOR sites.[23,26] The C-terminal stepwise deletion of amino acids from [Dmt^1]EM-2 decreased MOR affinity and the final dipeptide (H–Dmt–Pro–NH_2) exhibited moderate MOR affinity (K_i = 41.7 nM) that was, however, two orders of magnitude weaker than that of the parent analogue.[15] Interestingly, although the molecular dimension of H–Dmt–NH–CH_3 (**3**) is somewhat smaller than that of H–Dmt–Pro–NH_2, it exhibited a 5.6-fold higher MOR affinity suggesting that the Pro residue interfered with alignment of the ligand in the receptor pocket.

Preparation of GPI (guinea-pig ileum) and MVD (mouse vas deferens) tissues and stimulation tests were performed as described previously.[15] The results of functional bioactivity in vitro are illustrated in Figure 1. The agonist potency was ranked in the order of H–Dmt–NH–CH_3 (**3**) > H–Dmt–Pro-NH_2[15] > H–Dmt–NH_2 (**2**) and a similar trend was noted with the receptor binding data (Table 1). The maximum effect (E_{max}) value of inhibiting electrical induced twitches in GPI assay by both H–Dmt–Pro–NH_2 and H–Dmt–NH_2 (**2**) were below 50%. H–Dmt–NH–CH_3 (**3**), however, produced a significant activity with E_{max} of 66.6%, although its potency was three orders of magnitude lower than that of EM-2 and [Dmt^1]EM-2. From these results, we can deduce that these compounds might be weak MOR antagonists at high doses. Neither H–Dmt–NH_2 (**2**) nor H–Dmt–NH–CH_3 (**3**) exhibited agonism in the MVD assay (IC_{50} > 10,000 nM) and therefore the data are not shown.

Table 2. Analgesia of Dmt compounds relative to morphine in mice

	Compounds	MED (nmol)	HP test (%)	TF test (%)	Antagonism (%)
2	H–Dmt–NH_2	200	0.26	0.48–0.55	100
3	H–Dmt–NH–CH_3	30	0.64–0.85	1.3–1.7	73

The analgesia was determined by hot-plate (HP) and tail-flick (TF) tests after icv administration. Antagonism was evaluated using naloxone.

Antinociception of intracerebroventricularly (icv) administered H–Dmt–NH_2 (**2**) and H–Dmt–NH–CH_3 (**3**) was measured by the tail-flick (TF) and hot-plate (HP) tests in mice as described previously.[17b] While H–Dmt–NH–CH_3 (**3**) exhibited a 6-fold stronger response than that of H–Dmt–NH_2 (**2**), it was only 1.3–1.7% and 0.64–0.85% as effective as morphine in TF and HP tests, respectively; in both cases naloxone blocked the response (Table 2), which verifies that the compound interacted with opioid receptors.[17b] These data are consistent with the data of functional bioassays in GPI suggesting that the **2** and **3** recognizes similar opioid receptors in in vitro and in vivo described above to the same degree.

In summary, our data demonstrate for the first time that a single C-terminally hydrophobically modified amino acid selectively interacts with MOR. These results substantiate the flood of data that supports the observation that Dmt is the key residue responsible for the enhancement of affinity to MOR in opioid ligands which bear hydrophobic C-terminal substituents.[10,16b,17,23–25] In addition these results provided a clue to develop MOR antagonists. The presence of Dmt was responsible for the transmission of opioid mimetic substances through the blood–brain barrier[16b,17] and the further application of Dmt in the synthesis of opioid peptides will contribute to the development of additional compounds that can readily pass through membrane barriers.

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