Development of Potent μ-Opioid Receptor Ligands Using Unique Tyrosine Analogues of Endomorphin-2

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Six analogues of tyrosine, which contained alkyl groups at positions 2′, 3′, and 6′, either singly or in combination on the tyramine ring, were investigated for their effect on the opioid activity of [Xaa[1]]endomorphin-2 (EM-2). The opioid analogues displayed the following characteristics: (i) high μ-opioid receptor affinity [$K_i(\mu) = 0.063-2.29$ nM] with selectivity [$K_i(\delta)/K_i(\mu)$] ranging from 46 to 5347; (ii) potent functional μ-opioid agonism [GPI assay ($IC_{50} = 0.623-0.924$ nM)] and with a correlation between δ-opioid receptor affinities and functional bioactivity using MVD; (iii) intracerebroventricular administration of [Dmt[1]]- (**14**) and [Det[1]]EM-2 (**10**) produced a dose−response antinociception in mice, with the former analogue more active than the latter; and (iv) a marked shift occurred from the *trans*-orientation at the Tyr[1]-Pro[2] bond to a *cis*-conformer compared to that observed previously with [Dmt[1]]EM-2 (**14**) (Okada et al. *Bioorg. Med. Chem*. **2003**, *11*, 1983−1984) except [Mmt[1]]EM-2 (**7**). The active profile of the [Xaa[1]]EM-2 analogues indicated that significant modifications on the tyramine ring are possible while high biological activity is maintained.

Introduction[1]

Despite the structural diversity among endogenous mammalian opioid ligands, the enkephalins,[2] endorphins,[3] dynorphins,[4] Tyr-W-MIF-1,[5] and endomorphins,[6] as well as the exogenous amphibian skin opioid peptides,[7,8] an N-terminal Tyr residue is a common structural element. According to the concept of the message and address domains,[9] the Tyr residue acts as part of the message domain to anchor the opioid peptide within the receptor. Since μ-opioid receptors mediate the pharmacological effects associated with morphine, the high selectivity for these receptor sites by the endogenous opioid peptides endomorphin-1 (EM-1, H-Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (EM-2, H-Tyr-Pro-Phe-Phe-NH_2)[6] may be important model peptides in the search for new analgesics.

The introduction of 2′,6′-dimethyl-L-tyrosine (Dmt) into various opioid ligands provided evidence that a single residue can elicit substantial changes in the activity profile of an opioid peptide.[10] The most dramatic representation of this effect involved the development of derivatives containing the Dmt-Tic pharmacophore that displayed extremely potent δ-opioid receptor antagonism.[10] Similarly, structure−activity studies on a variety of opioid ligands containing Dmt revealed a dramatic alteration in their activity through the elevation of affinity, modification of receptor selectivity, and change in the spectrum of their bioactivity profile.[11,12] Recent studies detailed the effects of [Dmt[1]]EM-2 (**14**) and its C-terminally modified analogues, which were characterized with potent μ-opioid receptor binding affinity, μ-receptor agonism, or mixed μ-/δ-opioid agonism and δ-opioid antagonism properties in some of the C-terminally modified analogues.[13] Interestingly, the formation of opioid mimetic compounds containing two N-termini of Dmt separated by either an unbranched alkyl diamine[14] or 3,6-diaminoalkylpyrazinone[15] exhibited unique μ-opioid receptor agonism in vitro and potent morphine-like analgesia in vivo.

Rationale

Determination of the structure−activity profile of opioid peptides has been approached largely through the modification of side chains or alterations on various functional groups of many residues including Tyr. For example, *N*-alkylation of Tyr with bulky groups reduced the affinity of the Dmt-Tic pharmacophore for the δ-opioid receptor,[10c] although methylation exhibited minimal effects on δ-opioid receptor affinity and bioactivity.[10a] The increase in functional activity may be due to increased resistance to enzymatic degradation[16] as well as providential hydrophobic interactions within the receptor.[10f] On the other hand, *O*-methylation of Tyr eliminated activity[17] as well as the introduction of various types of functional groups (CO_2H, $CONH_2$, CH_2OH).[18] Further, introduction of I, OCH_3, NO_2, NH_2,[19] or CH_3[20] in the 3′-position of Tyr induced negative consequences. Substitution of the hydroxyl group with NO_2,[20] its deletion,[21] or merely shifting its position in the ring from the 4′- to the 3′-position[20] completely

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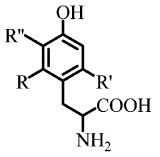


R	R'	R''		
Me	H	H	Mmt	(2'-Monomethyltyrosine)
Me	Et	H	Emt	(2'-Ethyl-6'-methyltyrosine)
Me	*i*-Pr	H	Imt	(2'-Isopropyl-6'-methyltyrosine)
Et	Et	H	Det	(2',6'-Diethyltyrosine)
i-Pr	*i*-Pr	H	Dit	(2',6'-Diisopropyltyrosine)
Me	Me	Me	Tmt	(2',3',6'-Trimethyltyrosine)

Figure 1. Structure of tyrosine analogues and substituents.

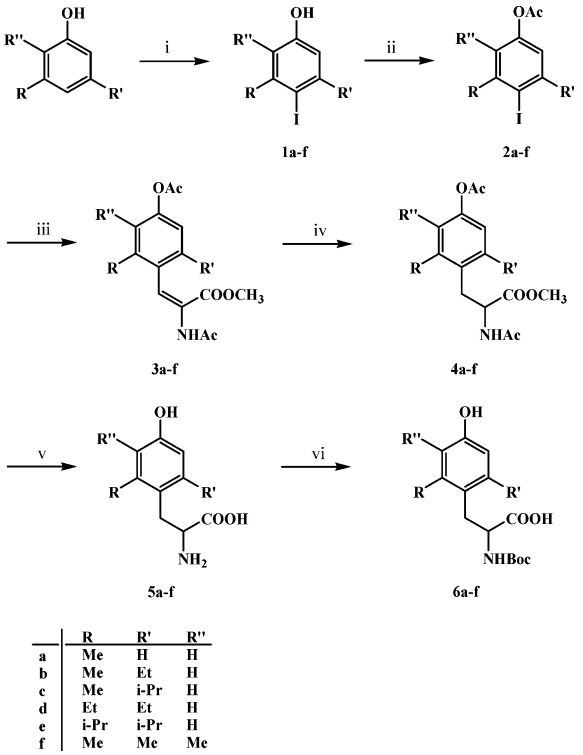
abolished activity. On the other hand, the bisubstituted Dmt-containing peptides[11−15] and those having a single methyl group at the 2′-position of the Tyr residue in DALDA[22] and DPDPE[19] maintained or increased receptor affinity and functional bioactivity. Furthermore, the replacement of Tyr with Dmp in endomorphin ([Dmp[1]]-EM-2)[23] and dermorphin[24] demonstrated that high biological activity was surprisingly maintained without the important hydroxyl group on the tyramine ring.

The enhancement of opioid activity upon inclusion of Dmt[11b] and Dmp[23,24] in the sequence of opioid peptides provided the impetus to develop analogues with systematic modifications at the 2′- and 6′-positions of the Tyr aromatic ring and investigate the impact on the activity of EM-2. In the present study, six tyrosine analogues containing different alkyl groups were prepared, namely, 2′-monomethyltyrosine (Mmt), 2′,3′,6′-trimethyltyrosine (Tmt), 2′-ethyl-6′-methyltyrosine (Emt), 2′-isopropyl-6′-methyltyrosine (Imt), 2′,6′-diethyltyrosine (Det), and 2′,6′-diisopropyltyrosine (Dit) (Figure 1) in order to produce [Xaa[1]]EM-2 analogues and compare them against [Dmt[1]]EM-2. The EM-2 analogues were analyzed for their interactions with μ- and δ-opioid receptors in vitro and in vivo, and the conformation around the Xaa[1]-Pro[2] bond was analyzed using ^{1}H NMR to investigate the effect of alkyl groups on the cis/trans isomerization of the EM-2 analogues.

Chemistry

Although two procedures exist for the stereoselective synthesis of L-Dmt, developed independently by Dygos et al.[25] and Hruby et al.,[26] preparation of the Tyr analogues in this investigation followed the method of Dygos et al. (Scheme 1).[25] The starting materials, 3-ethyl-5-methylphenol,[27] 3,5-diethylphenol,[28] and 3,5-diisopropylphenol,[29] were prepared in our laboratory according to procedures in the literature, and the other phenol derivatives were commercially available. Briefly, the alkylphenols were first iodinated in the 4-position (**1a**−**f**) and the phenolic hydroxyl group was protected with an acetyl group (**2a**−**f**). It was followed by a Heck reaction[30] to construct the dehydroamino acids (**3a**−**f**). Compound **2a**−**d** and **2f** proceeded well by the Heck reaction with exception of the 3,5-diisopropyl-4-iodophenyl acetate (**2e**), because of the bulky steric hindrance under the conditions described by Dygos et al.[25] The desired compound **3e** was obtained by using a relatively small ligand, $(C_6H_5)_3P$, and reaction at higher temperature, 130 °C. In the case of **3c**, we could not remove the starting material 3-isopropyl-5-methylphenol from **1c** at the iodination step, due to the identical R_f values of these two compounds. Thus, a sample of **1c** containing a small amount of starting material was used directly for acetylation and the impurity was removed after Heck reaction. The obtained dehydroamino acids (**3a**−**f**) were reduced enantioselectively in the presence of the chiral catalyst, [Rh(1,5-COD)(*R*,*R*-DIPAMP)BF_4] to give **4a**−**f**; the protecting groups were removed with 12 mol/L HCl to yield **5a**−**f**. The synthetic amino acids had ee values ranging from 75% to 96%. To obtain optically pure amino acids, the crude products were recrystallized from 5 mol/L HCl and analyzed by chromatography on a chiral column to yield enantiomeric purity > 95% ee (Table 1). The amino group of the Tyr analogues was protected with a Boc group (**6a**−**f**) before use in peptide synthesis.

Scheme 1. Synthetic Scheme for Six Tyr Analogues[a]



	R	R'	R''
a	Me	H	H
b	Me	Et	H
c	Me	i-Pr	H
d	Et	Et	H
e	i-Pr	i-Pr	H
f	Me	Me	Me

[a] Reagent and conditions: (i) KI/KIO_3, HCl/MeOH; (ii) Ac_2O/pyridine, 50 °C; (iii) 2-acetamidoacrylate, $Pd(OAc)_2$, $(2\text{-}MeC_6H_4)_3P$, Et_3N/MeCN, reflux, 12 h. For **3e**: 2-acetamidoacrylate, $Pd(OAc)_2$, $(C_6H_4)_3P$, Et_3N, 130 °C, 20 h; (iv) H_2 (60 psig), [Rh(1,5-COD)(*R*,*R*-DIPAMP)]BF_4, 60 °C; (v) 12 mol/L HCl, reflux, 6 h; (vi) $(Boc)_2O$, Et_3N.

Table 1. Analytical Data of the Tyrosine Analogues

	Mmt	Emt	Imt	Det	Dit	Tmt
t_R (C18)[a]	9.8	11.65	12.47	12.55	13.88	11.92
t_R (WH)[b]						
D analogue	16.9[c]	22.4[c]	22.5[c]	26.2[c]	30.4[c]	49.0[d]
L analogue	32.2	29.1	32.3	35.4	49.7	51.6
ee (recrystallization)	96.3	96	96.2	98	94.5	>99.0

[a] Retention time with COSMOSIL 5C18-AR-II, 95:5 to 10:90 (30 min). [b] Retention time with CROWNPAK WH. [c] 50 °C, 1.5 mL/min, 1 mM $CuSO_4$:MeOH = 90:10. [d] 50 °C, 1.5 mL/min, 0.5 mM $CuSO_4$:MeOH = 95:5.

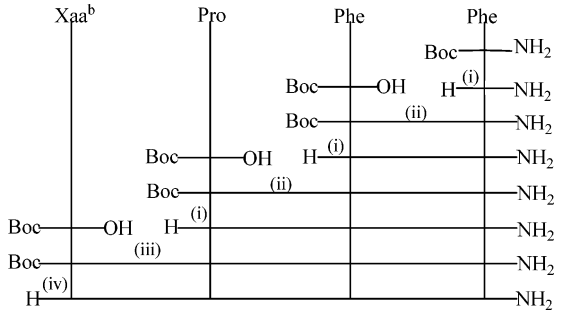
As shown in Scheme 2, the EM-2 analogues were synthesized in solution using Boc-protection methods. After deprotection of Boc-Pro-Phe-Phe-NH_2[13a] with HCl/dioxane, the resulting amino component was condensed with Boc-protected Tyr analogues (**6a**−**f**) using PyBop as the coupling reagent. The final Boc protecting group was removed with TFA in the presence of anisole, and the resulting peptides were precipitated and purified when necessary by semipreparative RP-HPLC. The identification and purity of the final compounds were verified using MS, NMR, analytical HPLC, and elemental analysis. The compounds exhibiting greater than

Table 2. Physicochemical Properties of the [Xaa^1]EM-2 Analogues

compd	peptide	$[\alpha]_D$ (deg)	c in H_2O	TLC[a] R_f	tof-mass [M + 1]	HPLC t_R (min)[b]
7	H-Mmt-Pro-Phe-Phe -NH_2	−14.2	0.44	0.56	586. 7	14.98
8	H-Emt-Pro-Phe-Phe-NH_2	−4.57	0.41	0.68	614. 8	15.89
9	H-Imt-Pro-Phe-Phe-NH_2	−7.92	0.36	0.74	628. 8	16.6
10	H-Det-Pro-Phe-Phe-NH_2	−2.70	0.36	0.72	628. 8	16.55
11	H-Dit-Pro-Phe-Phe-NH_2	−15.26	0.43	0.78	656. 8	17.85
12	H-Tmt-Pro-Phe-Phe-NH_2	−2.05	0.51	0.70	614. 8	16.12

[a] Solvent: *n*-BnOH:HOAc:H_2O = 4:1:5 (upper layer). [b] A:B = 90:10 to A:B = 10:90 for 30 min.

Scheme 2. Synthetic Scheme for [Xaa^1]EM-2 analogues (**7**−**12**)[a]



[a] (i) HCl/dioxane; (ii) IBCF, Et_3N, THF/DMF; (iii)PyBop, DIPEA, DMF; (iv) TFA, anisole. [b]Xaa: **7**, Mmt; **8**, Emt; **9**, Imt; **10**, Det; **11**, Dit; **12**, Tmt.

Table 3. Opioid Receptor Binding Affinity of [Xaa^1]EM-2 Analogues

compd	peptide	$K_i(\mu)$ (nM)[a]	n[c]	$K_i(\delta)$ (nM)[b]	n[c]	$K_i(\delta)/K_i(\mu)$
7	[Mmt^1]EM-2	0.132 ± 0.008	3	528.6 ± 47	6	4005
8	[Emt^1]EM-2	0.063 ± 0.006	5	55.7 ± 6.2	3	884
9	[Imt^1]EM-2	0.15 ± 0.013	5	190 ± 10	3	1266
10	[Det^1]EM-2	0.084 ± 0.006	3	69.7 ± 5.3	3	830
11	[Dit^1]EM-2	2.29 ± 0.37	4	105 ± 16	3	46
12	[Tmt^1]EM-2	0.111 ± 0.002	3	593.5 ± 80	5	5347
13	EM-2[d]	0.69 ± 0.16		9230 ± 200		13400
14	[Dmt^1]EM-2[d]	0.15 ± 0.04		28.2 ± 8.1		188

[a] Versus [^{3}H]DAMGO. [b] Versus [^{3}H]DPDPE. [c] The number of independent repetitions used different synaptosomal preparations. [d] Data cited from ref 13a.

98% purity were used for all biological assays. The physicochemical constants are shown in Table 2 and given in more detail in Supporting Information.

Results and Discussion

Opioid Receptor Affinity. The opioid receptor binding affinities of the EM-2 analogues are summarized in Table 3. The μ-opioid receptor affinity increased 4.6-fold after replacement of the Tyr^1 by Dmt (**14**) relative to the parental peptide (**13**), as seen previously.[13a] Introduction of a third methyl group at the 3′-position in the Dmt aromatic ring (Tmt^1) (**12**) further increased the μ-opioid receptor affinity by nearly 50%, being 6.4-fold higher than that of EM-2 (**13**). Substitution of one or two methyl groups in Dmt by ethyl groups substantially enhanced the binding affinity of the peptide; namely, [Emt^1]EM-2 (**8**) and [Det^1]EM-2 (**10**) were about 11- and 8-fold more potent, respectively, than EM-2 (**13**), and about twice as active as [Dmt^1]EM-2 (**14**). These results suggested that the side chain of Emt and Det might be more accessible to residues within the binding pocket than even Dmt. On one hand, introduction of two isopropyl groups on the Tyr ring (Dit^1) (**11**) decreased binding affinity by one-third relative to that of EM-2 (**13**), suggesting that the bulky isopropyl group interfered with the binding to receptor. On the other hand, Imt^1 (**9**), which bears one bulky isopropyl and a methyl group, exhibited a binding affinity similar to that of Dmt^1 (**14**). While the affinity of Mmt^1 (**7**) was analogous to that of Dmt^1 (**14**), it had a 20-fold higher μ-opioid receptor selectivity.

The results indicated that all of the Tyr^1-substituted EM-2 analogues had higher μ- and δ-opioid receptor affinities than EM-2 (**13**), except for [Dit^1]EM-2 (**11**), and [Dmt^1]EM-2 (**14**) exhibited the highest δ-opioid receptor affinity (Table 3). Of these analogues, [Mmt^1]EM-2 (**7**) and [Tmt^1]EM-2 (**12**) had the weakest δ-affinities and subsequently the highest μ-receptor selectivities [$K_i(\delta)/K_i(\mu)$ = 4005 and 5347, respectively], suggesting that either the reduced lipophilicity of the aromatic ring (**7**) or steric hindrance of the 3′-methyl group (**12**) was unfavorable for ligand−receptor interaction at the δ-opioid receptor. This also demonstrated a subtle difference in the composition of the binding sites between μ- and δ-receptors.

One model representing the possible receptor−ligand interaction of [Imt^1]EM-2 (**9**) implies that one side of the aromatic side chain could be inserted into a binding pocket, which could accommodate a relatively small alkyl group on the aromatic ring, such as methyl or ethyl, but is insufficient for a bulky isopropyl group. In other words, the portion of the aromatic ring containing the isopropyl group might protrude outside the binding pocket and therefore does not substantially interfere with ligand−receptor interaction. While the binding pocket accommodates either a methyl or an ethyl group, the higher affinity of [Det^1]EM-2 (**10**) and [Emt^1]EM-2 (**8**) (0.06 nM and 0.08 nM, respectively) suggested a preference for the ethyl derivative relative to the smaller methyl group. Furthermore, the [Xaa^1]EM-2 analogues containing Dmt (**14**), Tmt (**12**), Mmt (**7**), and Imt (**9**) exhibited comparable affinities (0.13−0.15 nM), but Dit (**11**), which has two bulky isopropyl groups, exhibited the weakest μ-opioid receptor affinity (2.29 nM).

In general, affinity of opioid ligands to the receptors increased when Tyr was substituted by Dmt,[11] suggesting that the side chain of Dmt fits quite tightly and precisely into a hydrophobic cavity situated within the receptor. In addition, we conclude, on the basis of the data from the [Xaa^1]EM-2 analogues (**7**−**12**, **14**) and other studies,[13b] including those dealing with C-terminally extended analogues of Dmt-Tic,[10a,c,e,f] that the μ-opioid receptor apparently accommodates a bulkier ligand than the δ-opioid receptor, which has different requirements for ligand interaction.[31]

Functional Bioactivity in Vitro. The in vitro biological activities were evaluated using isolated guinea

Table 4. Functional Bioactivity of [Xaa^1]EM-2 Analogues

		GPI assay		MVD assay		
compd	peptide	$IC_{50} \pm SE$ (nM)	n^a	$IC_{50} \pm SE$ (nM)	n^a	antagonism^b
7	[Mmt^1]EM-2	0.924 ± 0.431	6	28.7 ± 12.7	17	++
8	[Emt^1]EM-2	0.623 ± 0.113	5	1.08 ± 0.31	7	+
9	[Imt^1]EM-2	10.6 ± 2.7	11	601 ± 168	13	+
10	[Det^1]EM-2	0.903 ± 0.326	5	47.1 ± 17.9	13	+
11	[Dit^1]EM-2	299 ± 62	8	>10000	-	nd
12	[Tmt^1]EM-2	2.31 ± 0.74	12	46.4 ± 11.1	16	++
13	EM-2	7.96 ± 0.76	33	344 ± 93	14	+
14	[Dmt^1]EM-2	0.261 ± 0.038	10	0.59 ± 0.182	7	+

^{a} The number of independent repetitions used different isolated tissue preparations. ^{b} Antagonism by CTAP (200 nM) with the percent recovery of electrically evoked contraction: ++, >50%; +, <50%; nd, none detected.

pig ileum (GPI) for μ-opioid receptors and mouse vas deferens (MVD) for δ-opioid receptors (Table 4). The GPI tissue contains predominantly μ-opioid receptors, while MVD includes δ-opioid receptors. The analogues [Mmt^1]- (**7**), [Emt^1]- (**8**), [Det^1]- (**10**), and [Tmt^1]EM-2 (**12**) exhibited high GPI potencies (IC_{50} = 0.924, 0.623, 0.903, and 2.31 nM, respectively), although their activities were less than that of [Dmt^1]EM-2 (**14**). In the MVD assay, most analogues except for [Imt^1]EM-2 (**9**) and [Dit^1]EM-2 (**11**) exhibited an unexpectedly higher potency than that based on the δ-opioid receptor affinity. To confirm receptor preferences, the MVD activity of these analogues was examined using a specific μ-opioid receptor antagonist, CTAP.[32] As shown in Table 4, the MVD activity of the analogues and even that of parent peptide EM-2 was effectively inhibited by CTAP; the most effective antagonism was observed with [Tmt^1]EM-2 (**7**) and [Mmt^1]EM-2 (**12**), which have relatively low δ-opioid receptor affinity, suggesting that the MVD activity of these two analogues was, for the most part, elicited via a μ-opioid receptor which coexists in the MVD tissues. The activity of the other analogues [Emt^1]- (**8**), [Det^1]- (**10**), [Dmt^1]- (**14**), and EM-2 (**13**) was also inhibited by CTAP, suggesting that their MVD potencies were mediated in part by the μ-opioid receptor in MVD tissues. A similar phenomenon was also observed with other high-affinity μ-opioid receptor ligands.[23,24] The very high MVD potency of [Dmt^1]EM-2 (**14**) and [Emt^1]-EM-2 (**8**) may then be attributable to the concomitant μ-opioid receptor in MVD tissues as well as a relatively high δ-opioid receptor affinity of their own (Table 1). It should be noted that [Dit^1]EM-2 (**11**) has unexpectedly low GPI and MVD potencies while it still retained high receptor affinity for both μ- and δ-opioid receptors, implying that this analogue may have a mixed agonist/antagonist property for both receptor types. As a consequence, the antagonism experiments using the MVD tissues strongly suggest that this series of analogues are potent μ-opioid receptor agonists.

In Vivo Biological Activity. The analgesic effect of [Dmt^1]EM-2 (**14**) and [Det^1]EM-2 (**10**) were determined by the tail-flick test (spinally mediated mechanism) and the hot-plate test (supraspinal effects) in comparison to the effect produced by EM-2 (**13**). The results in Figure 2 showed that all three compounds produced analgesia with qualitatively similar results in the tail-flick test; however, a stronger activity was observed with [Dmt^1]-EM-2 (**14**) based on the analysis of hot-plate tests. In summary, [Dmt^1]EM-2 (**14**) > [Det^1]EM-2 (**10**) > EM-2 (**13**), yielding activity ratios of 1.00:0.86:0.65 in the tail-flick test and 1.00:0.47:0.30 in the hot-plate tests. These results indicated that the methyl side chains located at

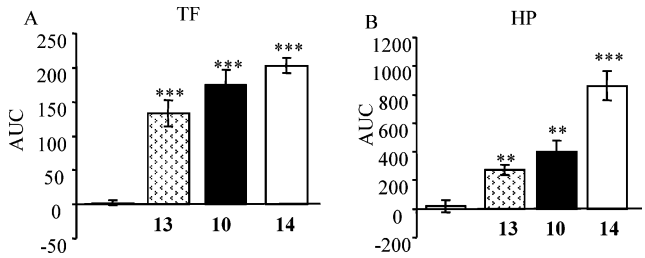


Figure 2. Effect of intracerebroventricularly injected EM-2 (**13**), [Det^1]EM-2 (**10**), and [Dmt^1]EM-2 (**14**) in the tail-flick (A) and hot-plate (B) tests. Each compound was administered at a dose of 3 μg/mouse and each point represents the mean ± SE determined with five or six mice. The asterisk denotes values that were significantly different than the control mice by the Student test (***, $p < 0.001$; **, $p < 0.01$).

Table 5. The Cis/Trans Conformer Ratio of [Xaa^1]EM-2

		cis/trans (DMSO-d_6)^a	
compd	peptide	cis	trans
7	[Mmt^1]EM-2	35	65
8	[Emt^1]EM-2	65	35
9	[Imt^1]EM-2	75	25
10	[Det^1]EM-2	75	25
11	[Dit^1]EM-2	90	10
12	[Tmt^1]EM-2	65	35
13	EM-2^b	1	2
14	[Dmt^1]EM-2^b	70	30

^{a} Determined by relative peak intensities from ^{1}H NMR analyses. ^{b} Data cited from ref 13a.

the 2′ and 6′-positions of Tyr represent the optimal alkyl groups for interaction with and activation of μ- and δ-receptors. Although [Dmt^1]EM-2 (**14**) was the most potent analogue in terms of the duration of the analgesic response [60 min in contrast to 20 min with EM-2 (**13**)], it was approximately 16% as effective as morphine (data not shown). Naloxone (10 mg/kg sc) blocked antinociception (data not shown), which supports the observation that opioid receptors are involved in producing analgesia.

Cis/Trans Conformation. With the presence of a proline residue in the second position of the peptide sequence, cis/trans isomerization readily occurs at the Xaa^1-Pro^2 peptide bond. The cis/trans ratio can be easily determined by ^{1}H NMR in DMSO as described by Okada et al.[13] The analysis revealed that [Xaa^1]EM-2 analogues **8**, **9**, **10**, and **12** potentially adopt the cis conformer with a cis/trans ratio of approximately 70:30 (Table 5), equivalent to that of [Dmt^1]EM-2 (**14**). Interestingly, [Dit]^{1}EM-2 (**11**), with two bulky alkyl groups in the aromatic ring, had a cis/trans ratio of 90:10, while [Mmt^1]EM-2 (**7**) was trans rich, similar to EM-2 (**13**). These observations suggest that alkyl groups at the 2′-

and 6′-positions of the tyramine moiety affected the cis/trans ratio of the EM-2 analogues and the bulkier groups produced greater cis isomers.

Our data revealed interesting observations concerning the cis/trans equilibrium: first, [Mmt^1]EM-2 (**7**) with a cis/trans ratio of 35:65 contrasts remarkably with [Dmt^1]-EM-2 (**14**), with a ratio of 70:30, yet both compounds had the same μ-opioid receptor affinities (0.13 and 0.15 nM, respectively) (Table 3); second, while [Dit^1]EM-2 (**11**) had the highest cis/trans ratio (90:10), it exhibited the lowest μ-opioid receptor affinity (Table 3) and weakest functional bioactivity (Table 4). Thus, the cis/trans ratio about the Xaa^1-Pro^2 bond may not be a predictor of biological activity.

Conclusions

Six L-tyrosine analogues were incorporated at the N-terminus of the EM-2 sequence. EM-2 containing Det (**10**), Emt (**8**), and Tmt (**12**) were the most potent analogues in the binding assays (Table 3), which signifies that the overall hydrophobicity of the peptides might be an important factor for ligand−receptor interaction; however, bulkier moieties, such as the isopropyl groups of Dit (**11**), substantially interfere with ligand−receptor interaction. Furthermore, the data suggest that the μ-opioid receptor can readily accommodate a bulkier N-terminal Tyr derivative than the δ-opioid receptor. The affinities of the EM-2 analogues, which are characteristic of μ-receptor selective ligands, were verified and supported by the functional bioactivity in vitro. In conclusion, the present study demonstrated that the Tyr analogues used in this research are useful as surrogate residues in the potential design of new opioid mimetics with unique biological properties.

Experimental Section

Mass spectra were measured with a KRATOS MALDI-TOF analyses (matrix-assisted laser desorption ionization time-of-flight mass spectrometry). Melting points were determined on a Yanagimoto micromelting point apparatus and are uncorrected. TLC was performed on precoated plates of silica gel F254 (Merk, Darmstadt, Germany). Optical rotations were determined with a DIP-1000 automatic polarimeter (Japan Spectroscopic Co.). Semipreparative RP-HPLC and analytic RP-HPLC used a Waters Delta 600 with COSMOSIL C18 column (20 mm × 250 mm) and COSMOSIL C18 column (4.6 mm × 250 mm), respectively. The solvents for analytical HPLC were as follows: A, 0.05% TFA in water; B, 0.05% TFA in CH_3-CN. The column was eluted at a flow rate of 1 mL/min with a linear gradient of 90% A to 10% A in 30 min; the retention time is reported as t_R (min). ^{1}H NMR spectra were measured on a Bruker DPX-400 spectrometer at 25 °C. Chemical shift values are expressed as ppm downfield from tetramethylsiliane.

Synthesis of Tyr Analogues (Mmt, Emt, Imt, Det, Dit, Tmt). The six unique L-Tyr analogues were synthesized according to Scheme 1. The detailed synthetic procedure for each Tyr analogue and the elemental analysis data are summarized in Table 1 and presented in the Supporting Information.

General Procedure for Synthesis of Boc-Xaa-Pro-Phe-Phe-NH_2 (Xaa = Mmt, Emt, Imt, Det, Dit, Tmt). Boc-Pro-Phe-Phe-NH_2[13a] (0.492 mmol) was treated with 8.8 mol/L HCl/dioxane (9.84 mmol) to remove the Boc group at room temperature for 30 min. The product was precipitated with ether, filtered, and dried at vacuum. The resulted hydrochloride salt was dissolved in DMF (10 mL) containing DIPEA (1.19 mmol), and to this solution were added Boc-Xaa-OH (0.541 mmol) and PyBop (0.595 mmol). The reaction mixture was stirred at 0 °C for 10 min and then room temperature for 4 h. After removal of the solvent, the residue was diluted with AcOEt. The dilution was washed with ice cold 10% citric acid (3 × 10 mL), 5% Na_2CO_3 (3 × 10 mL), and saturated NaCl (3 × 10 mL); dried over Na_2SO_4; and evaporated to dryness. The residue was purified by flash chromatograph. The compound was precipitated with hexane, filtered, and dried under vacuum. Elemental analysis data are summarized in the Supporting Information (Table 2) along with the data for yield, melting point, optical rotation, R_f, ^{1}H NMR, and ^{13}C NMR.

General Procedure for Synthesis of TFA-H-Xaa-Pro-Phe-Phe-NH_2 (7−12; Xaa = Mmt, Emt, Imt, Det, Dit, Tmt). Boc-Xaa-Pro-Phe-Phe-NH_2 (0.21 mmol) was treated with TFA (7 mmol) and anisole (0.46 mmol) for 1 h at room temperature. The reaction solution was diluted with hexane, and the solid was collected by filter, dried over KOH pellets, and purified by semipreparative RP-HPLC. The purified peptide was lyophilized to give amorphous powder. Elemental analysis data are summarized in the Supporting Information (Table 3) along with the data for yield, ^{1}H NMR, and ^{13}C NMR.

Opioid Receptor Binding Assays. Opioid receptor affinities were determined under equilibrium conditions [2.5 h at room temperature (23 °C)] in a competition assay using brain P_2 synaptosomal membranes prepared from Sprague−Dawley rats. Synaptosomes were preincubated to remove endogenous opioids, washed in excess ice-cold buffer containing protease inhibitor, and stored in a glycerol-containing buffer with protease inhibitor at −80 °C as described previously.[33] The δ- and μ-opioid receptors were radiolabeled with [^{3}H]DPDPE and [^{3}H]DAMGO, respectively,[13−15,33] and excess unlabeled peptide (2 μM) established the level of nonspecific binding. After incubation, the radiolabeled membranes were rapidly filtered on Whatman GF/C glass fiber filters presoaked in 0.1% polyethylenimine in order to optimize the signal-to-noise ratio, washed with ice-cold BSA buffer, and dried at 75 °C. Radioactivity was determined using EcoLume (ICN, Costa Mesa, CA). All analogues are analyzed in duplicate using five to seven peptide dosages and several synaptosomal preparations in independent repetitions (n values noted in Table 3) to ensure statistical significance. The affinity constants (K_i) were calculated according to Cheng and Prusoff.[34]

Biological Activity in Isolated Tissue Preparation. The myenteric plexus longitudinal muscle preparations (2−3 cm segments) from the small intestine of male Hartley strain guinea pigs (GPI) measured μ-opioid receptor agonism, and a single mouse vas deferens (MVD) was used to determine δ-opioid receptor agonism as described previously.[23] The isolated tissues were suspended in organ baths containing balanced salt solutions in a physiological buffer, pH 7.5. Agonists were tested for the inhibition of electrically evoked contraction, expressed as IC_{50} (nM) obtained from the dose−response curves. The IC_{50} values represent the mean ± SE of 5−33 separate assays. To measure whether μ-opioid receptor-mediated antagonism occurred in the MVD, the test compound was added to the tissue preparation at the IC_{50} dose value; after 5 min incubation, CTAP (200 nM) was added and the percent recovery (reversal rate) of electrically evoked contraction was then calculated.

Determination of Analgesia in Vivo. Tail-flick and hot-plate latencies in Swiss-Webster male mice measured the effect of icv administration of EM-2 (**13**), [Det^1]EM-2 (**10**), and [Dmt^1]-EM-2 (**14**) as follows: compounds were dissolved in physiological saline and injected into mice icv.[15] Tail-flick tests (spinal analgesia) were performed by applying radiant heat to the dorsal surface of the tail (Columbus Instruments, Columbus, OH). The latency period for removal of the tail, defined as the tail-flick latency (TFL), was adjusted between 2 and 3 s (preresponse time), and a cut-off time was set at 8 s to avoid external heat-related damage. The analgesic response was measured beginning at 10 min following icv administration of EM-2 (**13**), [Det^1]EM-2 (**10**), or [Dmt^1]EM-2 (**14**), respectively, and testing was terminated when TFL approached the preresponse time. The AUC (area under the curve) was obtained by plotting the response time (s) versus

(min) after administration of the compound. Morphine was used as a positive control and naloxone was used as a general opioid antagonist.

For the hot-plate test (supraspinal analgesia), mice were set on an electrically heated plate at 55 ± 0.1 °C (IITC INC., Woodland Hills, CA) following the same drug injection paradigm as above. Hot-plate latency (HPL) was measured as the interval between the placement of the mice on the hot plate and observing movements consisting of either jumping, licking, or shaking their hind paws with a baseline latency of 15 s and maximal cut-off time of 30 s. The area under the curve (AUC) was derived from data based on the response (mean ± SE) of five to seven mice per time point. The analgesic effect of EM-2 (**13**), [Det[1]]EM-2 (**10**), and [Dmt[1]]EM-2 (**14**) are relative to the saline control.

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Supporting Information Available: Experimental procedures and analytical data for the synthesis of Tyr analogues and their derivatives (**1a**−**f** to **6a**−**f**) are presented herein. Yield, melting point, $[\alpha]^{25}_D$, R_f, and NMR values of [Boc-Xaa[1]]-EM-2 as well as the yield, melting point, and NMR values of [Xaa[1]]EM-2 (**7**−**12**) are given, including the elemental analysis data of [Boc-Xaa[1]]EM-2 and [Xaa[1]]EM-2 (**7**−**12**). This material is available free of charge via the Internet at http://pubs.acs.org.

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(1) Abbreviations. In additional to the IUPAC−IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.* **1985**, *260*, 14−42), this paper uses the following symbols and abbreviations: Ac_2O, acetic anhydride; AcOEt, ethyl acetate; Boc, *tert*-butyloxycarbonyl; BSA, bovine serum albumin; CTAP, D-Phe-*cyclo*-(Cys-Tyr-D-Trp-Arg-Thr-Pen)-Thr-NH_2; DALDE, [D-Ala,[2]D-Leu[5]]enkephalin; DAMGO, [D-Ala[2],*N*-Me-Phe[4],Gly-ol[5]]enkephalin; DIPEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; Dmp, 2′,6′-dimethyl-L-phenylalanine; DMSO, dimethyl sulfoxide; DPDPE, *cyclo*-[D-Pen[2,5]]enkephalin; GPI, guinea-pig ileum; IBCF, isobutyl chloroformate; IC_{50}, concentration required for 50% inhibition of the electrically induced contraction in muscle derived from a dose−response curve; MVD, mouse vas deferens; NMR, nuclear magnetic resonance; $Pd(OAc)_2$, palladium(II) acetate; PyBOP, benzoltriazol-1-yloxytrispyrrolidinophosphonium hexafluorphosphate; [Rh(1,5-COD) (*R*,*R*-DIPAMP)]BF_4, (*R*,*R*)-(−)1,2-bis[(*O*-methoxyphenyl)(phenyl) phosphino]ethane(1,5-cyclooctadiene)-rhodium(I) tetrafluoroborate; RP-HPLC, reverse-phase high performance liquid chromatography; TFA, trifluoroacetic acid; TLC, thin-layer chromatography.

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