Biological Properties of Opioid Peptides Replacing Tyr at Position 1 by 2,6-Dimethyl-Tyr[1)]

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Received June 7, 1999; accepted June 25, 1999

To understand the effect of the replacement of Tyr residue at position 1 in opioid peptides by 2,6-dimethyl-Tyr (Dmt) on the biological property, chiral (D or L) Dmt[1] analogs of Leu-enkephalin (Enk) and Tyr-D-Arg-Phe-βAla-NH_2 (YRFB) were synthesized and their enzymatic stabilities, *in vitro* bioactivities and receptor binding affinities compared with those of parent peptides. [L-Dmt[1]]Enk (1) exhibited 4-fold higher stability against aminopeptidase-M and possessed dramatically increased activities in guinea pig ilium (GPI) (187-fold) and mouse vas deferens (MVD) (131-fold) assays, and in rat brain receptor binding assays (356-fold at μ receptor and 46-fold at δ receptor) as compared to Enk. [L-Dmt[1]]YRFB (3) also exhibited increased activities in GPI (46-fold) and MVD (177-fold) assays, and in the binding assays (69-fold at μ receptor and 341-fold at δ receptor) as compared to the parent peptide. [D-Dmt[1]]Enk (2) and [D-Dmt[1]]YRFB (4) exhibited activities with diminished or lesser potency than the parent peptide in all assays. These results indicate that there is a tendency for μ affinity to be enhanced more than δ affinity with introduction of L-Dmt into δ ligand peptide (Enk), and for δ affinity to be enhanced more than μ affinity in case of μ ligand peptide (YRFB), resulting in reduced receptor selectivities at the receptors.

Key words 2,6-dimethyltyrosine; opioid peptide analog; receptor affinity; opioid activity; peptide synthesis; enzymatic stability

Tyrosine residue or tyramine moiety at position 1 of opioid peptides is a primary structural element essential for opioid activity. Despite numerous structure–activity relationship (SAR) studies few reports have focussed on the tyramine moiety, especially the phenol ring. Hansen *et al.*[2)] first reported enkephalin analogs when they introduced alkyl groups onto the Tyr[1] aromatic ring, which produced a dramatic increase in opioid and analgesic activities. The 2,6-dimethyl-Tyr (Dmt)[1] replacement was particularly remarkable in its effect on the receptor binding.[2)] Recent SAR studies of enkephalin and deltorphin short analogs containing Dmt residue at position 1 also showed that the introduction of Dmt[1] is a promising choice to improve opioid potency and/or receptor affinity,[3)] and developed some specific δ antagonists,[3e,3f,4)] but little is known about the effect of Dmt[1] replacement on metabolic stability and opioid receptor selectivity. The present paper describes the effects of Dmt used in place of Tyr[1] in Leu-enkephalin (Enk)[5)] and Tyr-D-Arg-Phe-βAla-NH_2(YRFB),[6)] which are known to be a δ agonist and a potent μ agonist, respectively, on the metabolic stability, opioid receptor binding and *in vitro* biological activities.

Results and Discussion

Racemic Dmt was prepared by the method of Abrash and Niemann.[7)] To resolve the racemic Dmt, Boc-DL-Dmt was derivatized to Boc-DL-Dmt-Arg-OMe, which was readily separated into each diastereomer by a medium pressured HPLC. After the acid hydrolysis, direct Boc-derivatization of amino acid mixture using $(Boc)_2O/Na_2CO_3$ reagents and simple extraction with EtOAc gave chiral Boc-Dmt-OH. The chirality of Dmt was comfirmed by optical rotations, TLC using a chiral plate and HPLC using a chiral column. The Dmt[1] analogs of Enk and YRFB were synthesized by the solid phase method based on Fmoc chemistry. [L-Dmt[1]]Enk (**1**) and [D-Dmt[1]]Enk (**2**) were synthesized using the chiral Boc-Dmt-OH at the final coupling stage because the use of racemic Boc-Dmt-OH resulted in incomplete separation of diastereomers on HPLC. For the synthesis of [L-Dmt[1]]YRFB (**3**) and [D-Dmt[1]]YRFB (**4**), racemic Boc-DL-Dmt-OH was used and the diastereomeric peptides were isolated separately by preparative HPLC. Analytical data of all synthetic peptides are shown in Table 1.

Enzymatic stability of analogs against aminopeptidase-M (AP-M) and in rat brain synaptosomal fractions[8)] was examined and the results are shown in Table 2. In an AP-M solution, Enk was degraded completely within 10 min under the conditions used in this study. Analog **1** showed improved stability with a half lifetime of 16 min, which is 4-fold more stable than Enk. The half lifetime of D-Dmt[1]-Enk (**2**) was 45 min, which is 10-fold more stable than Enk. Analog **1** also

Table 1. Analytical Data of Synthetic Dmt[1]-Analogs

Analog	$[\alpha]_D$[a)] (°)	TLC[b)] (Rf^2)	HPLC[b)] (t_R)	FAB-MS $(M+H)^+$	Amino acid analysis					
					Dmt[c)]	Gly	Phe	Leu	D-Arg	βAla[d)]
L-Dmt-Gly-Gly-Phe-Leu (**1**)	+47.1	0.70	32.57	584	1.02	1.98	1	0.95	—	—
D-Dmt-Gly-Gly-Phe-Leu (**2**)	−72.0	0.67	32.62	584	1.06	1.92	1	0.96	—	—
L-Dmt-D-Arg-Phe-βAla-NH_2 (**3**)	+40.2	0.42	19.95	583	1.06	—	1	—	1.10	0.95
D-Dmt-D-Arg-Phe-βAla-NH_2 (**4**)	−42.9	0.35	20.40	583	1.01	—	1	—	1.08	0.92

a) Optical rotation was measured in 10% AcOH (c=0.50) at 22 °C. *b*) See Experimental. *c*) Dmt was eluted at position of Lys. *d*) βAla was eluted a time just before Phe.

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showed a 3-fold higher stability than Enk in rat brain extracts, while Dmt analogs of YRFB (**3** and **4**) had high stability against AP-M and more than 90% of the peptide remained intact even after 20 h. This outstanding stability of YRFB analogs may be mainly due to the presence of D-amino acid (Arg) at position 2 in its molecule as reported previously.[9)]

In vitro bioactivity of synthetic analogs was evaluated using isolated longitudinal muscle strips of guinea pig ileum (GPI) and mouse vas deferens (MVD). The former tissue contains μ receptor while the latter contains δ receptor.[10)] As shown in Table 3, **1** showed dramatically increased activity, over two orders of magnitude more potent than Enk, in both GPI and MVD assays. The GPI/MVD ratio of **1** was almost the same as Enk, suggesting that this analog behaves as an excellent Enk mimic at the peripheral receptors. Interestingly, the D-Dmt analog, **2**, still showed increased activities, 6- and 3-fold more potent than Enk in GPI and MVD assays, respectively, despite the fact that D-Tyr^1 replacement of [Met^5]Enk reportedly causes a great loss of MVD activity.[11)] On the other hand, [L-Dmt^1]YRFB (**3**) showed marked increases in MVD (177-fold) and GPI (46-fold) assays while the potency of its D-Dmt analog (**4**) was significantly unchanged as compared to the parent peptide. The high potency of **3** in MVD assay caused a decrease of GPI/MVD ratio by 4-fold less than that of the parent peptide.

The receptor binding affinity was determined using rat brain synaptosomes by competitive binding assays with [^{3}H]DAMGO and [^{3}H]deltorphin II for μ and δ receptors, respectively, and is shown in Table 4. Analog **1** showed a 356-fold increased μ affinity and a 46-fold increased δ affinity compared to Enk. The value of δ receptor selectivity of **1** was 0.22, which indicates a change of receptor selectivity from δ to μ and it become 8-fold less selective than Enk. On the contrary, its D-Dmt analog (**2**) decreased affinity slightly to both receptors without alteration of receptor selectivity. Analog **3** showed a 69-fold increased μ affinity and a 341-fold increased δ affinity, while its D-Dmt analog (**4**) showed a decrease in μ affinity and a slight increase in δ affinity as compared to the parent peptide. The μ selectivity of both YRFB analogs was less than that of the parent peptide due to a greatly enhanced δ affinity (**3**) or poor μ affinity (**4**). It should be noted that **1** exhibited a higher μ affinity than that of YRFB as expected from the GPI assay.

In summary, the present study demonstrates that L-Dmt^1 replacement of Enk and YRFB improves enzymatic stability and causes marked improvements in the *in vitro* bioactivity and receptor binding affinity, while the D-Dmt^1 replacement resulted in a diminished change in bioactivity or reduced binding affinity at both μ and δ receptors. Concerning the potency of L-Dmt^1 analogs, there are tendencies to enhance μ affinity more than δ affinity by introducing L-Dmt into δ ligand peptide (Enk) and to enhance δ affinity more than μ affinity in case of μ ligand peptide (YRFB), resulting in reduced receptor selectivities at the receptors. Such tendencies were also observed with other Dmt analogs of Enk[3b)] and deltorphins.[3d,3f,12)] Results of **1** and **3** in GPI and MVD assays coincided well with those of the binding data. The discrep-

Table 2. Enzymatic Stability of Enk and Dmt^1-Analogs[a)]

Peptide	Half life time (min) against	
	Aminopeptidase-M	Rat brain homogenate
Enk	4	50
[L-Dmt^1]Enk (**1**)	16	160
[D-Dmt^1]Enk (**2**)	45	>900
[L-Dmt^1]YRFB (**3**)	>1200	n.d.[b)]
[D-Dmt^1]YRFB (**4**)	>1200	n.d.[b)]

a) See Experimental for determination of half life-time. *b*) Not determined.

Table 3. *In Vitro* Bioactivities and Partition Coefficient of Peptides[a)]

Peptide	GPI		MVD		GPI/MVD	log *P*
	(IC_{50}, nM)	RP[b)]	(IC_{50}, nM)	RP[b)]		
Enk	103±30	1	22.2±4.3	1	4.64	−0.62
[L-Dmt^1] (**1**)	0.55±0.17	187	0.17±0.02	131	3.24	−0.26
[D-Dmt^1] (**2**)	16.1±3.6	6.4	7.73±0.97	2.9	2.08	−0.37
YRFB	1.57±0.35	1	70.9±15.0	1	0.022	n.d.[c)]
[L-Dmt^1] (**3**)	0.034±0.007	46.1	0.40±0.09	177	0.085	n.d.[c)]
[D-Dmt^1] (**4**)	1.31±0.35	1.2	27.3±4.7	2.6	0.049	n.d.[c)]

a) The values are the mean of 8—14 experiments±S.E.M. *b*) Relative potency to Enk orYRFB. *c*) Not determined.

Table 4. Opioid Receptor Binding Affinities of Peptides[a)]

Peptide	[^{3}H]DAMGO (μ)		[^{3}H]Deltorphin II (δ)		Receptor selectivity	
	(K_i, nM)	RP[b)]	(K_i, nM)	RP[b)]	$K_i(\mu)/K_i(\delta)$[c)]	$K_i(\delta)/K_i(\mu)$[d)]
Enk	2.42±0.93	1	1.43±0.71	1	1.69	—
[L-Dmt1] (**1**)	0.0068±0.0030	356	0.031±0.011	46.1	0.22	—
[D-Dmt1] (**2**)	16.1±3.8	0.15	5.08±1.99	0.28	3.17	—
YRFB	0.145±0.040	1	385±150	1	—	2655
[L-Dmt1] (**3**)	0.0021±0.0007	69.0	1.13±0.13	341	—	538
[D-Dmt1] (**4**)	0.934±0.124	0.16	105±27	3.67	—	112

a) The values are the mean of 4—6 experiments±S.E.M. *b*) Relative potency to Enk or YRFB amide. *c*) δ receptor selectivity. *d*) μ receptor selectivity.

ancy in relative potencies between binding data and *in vitro* bioassay may reflect the difference of receptors in the brain and the periphery. For enhanced binding affinity and opioid activity of L-Dmt[1]-analogs, it is possible to conclude that chirality (L) at Cα of Dmt is of great importance to facilitate best fit of the peptides into the binding sites in both μ and δ receptors. The ligand-receptor interaction may be improved by the apparently increased hydrophobicity of Dmt conferred by dimethylation of the phenol ring (log *P*: −0.26 of **1** *vs.* −0.62 of Enk in Table 3) through hydrophobic forces with aliphatic or aromatic residues of receptors and/or by a properly oriented phenol ring arising from the dimethylation; this is true in receptor binding pockets in which possible binding partners for tyramine moiety are commonly conserved in μ and δ receptors, and is suggested by recent molecular modeling studies of opioid receptors.[13)]

Experimental

Melting points were determined on a Yanaco MP-S3 apparatus and are uncorrected. TLC was performed on silica gel plates (Merck, Kiesel gel $60F_{254}$, 5×10 cm) or chiral plates (CHIRALPLATE, Macherey-Nagel, Germany, 5×20 cm) with the following solvent systems: Rf^1, CH_3CN–H_2O–MeOH (4 : 1 : 1); Rf^2, *n*-BuOH–AcOH–H_2O (4 : 1 : 5, upper phase). Analytical HPLC used in the synthesis of all peptides and in determining enzymatic stability and partition coefficients was performed on a Wakopak column (Wakosil-II 5C18 AR, 4×150 mm) using the following solvent systems: A, 0.06% TFA; B, 0.06% TFA in 80% CH_3CN. A linear gradient elution from 5 to 50 B% over 40 min was used at a flow rate of 1 ml/min and the column eluate was monitored at 220 nm. Amino acid analysis was performed using a HITACHI L-8500 amino acid analyzer after 6 N HCl hydrolysis of peptide at 110 °C for 22 h. FAB-MS was run on a JEOL JMS-DX303 instrument.

Boc-L or D-Dmt-OH Racemic Dmt was synthesized according to the method of Abrash and Niemann[7)] and converted to Boc derivative. The Boc-DL-Dmt-OH was coupled with H-Arg-OMe·2HCl to yield Boc-DL-Dmt-Arg-OMe as usual. The racemic product (300 mg) was separated on a Develosil LOP ODS column (3×30 cm) which was eluted with a linear gradient of 25—55% solvent B (80% CH_3CN containing 0.06% TFA) over 180 min at a flow rate of 3 ml/min. Fractions of 6 ml each were collected and the fraction numbers of 38—42 and 46—52 were separately pooled and evaporated to dryness. The product eluted faster was found to be Boc-L-Dmt-Arg-OMe (106 mg) and the later one was Boc-D-Dmt-Arg-OMe (120 mg) as described below. Each product (250 mg) was hydrolyzed with 6 N HCl at 110 °C for 20 h, when the hydrolysate was evaporated to dryness, and then reacted with $(Boc)_2O/Na_2CO_3$ in CH_3CN–H_2O (1 : 1). Extraction of the desired product with EtOAc and work-up in the usual way for Boc-derivative yielded Boc-L-Dmt-OH (130 mg), mp 169—170 °C, $[\alpha]_D^{22}$ −9.7° (*c*=0.77, MeOH), FAB-MS *m/z*: 310 $(M+H)^+$. Salvadori *et al*. have recently reported the same compound to be an optical rotation, $[\alpha]_D^{20}$, of +20.0°,[3e)] although the solvent used is unclear. Boc-D-Dmt-OH was prepared in the same manner as described above, mp 168—170°, $[\alpha]_D^{22}$ +10.4° (*c*=0.77, MeOH), FAB-MS *m/z*: 310 $(M+H)^+$. A part of both products was deprotected to yield Dmt hydrochloride. L-Dmt·HCl: mp 241—245° (dec.) [lit.[14)] 250—252°], $[\alpha]_D^{22}$ +72.6° (*c*=1.0, 95% AcOH) [lit.[14)] +62.8° (*c*=1.01, AcOH)], Rf^1 0.61 on chiral TLC. D-Dmt·HCl: mp 246—248° (dec.), $[\alpha]_D^{22}$ −71.1° (*c*=1.0, 95% AcOH), Rf^1 0.49 on chiral TLC. The Dmt diastereomers were also analyzed by HPLC using a Sumichiral OA-6100 (4.6 mm×15 cm) column, which was eluted with 5% EtOH/95% 0.25 mmol $CuSO_4$ at a flow rate of 2 ml/min. D-Dmt was eluted faster than the L-isomer as reported in the literature.[14)]

Peptide Synthesis Peptides were synthesized by a DIC/1-hydroxybenztriazole(HOBt)-mediated Fmoc strategy according to the schedule described previously,[15)] starting with Fmoc-Leu-SAL-resin[1)] for Enk analogs and Fmoc-NH-SAL-resin for YRFB analogs. The side chain of D-Arg was protected with Pmc[1)] group and that of Dmt remained unprotected. For the synthesis of Enk analogs (**1** and **2**), Boc-L or D-Dmt-OH was used for final coupling reactions, while for the synthesis of **3** and **4** Boc-DL-Dmt-OH was used. The protected peptide resin was cleaved from the resin and deprotected by treatment with Reagent K[16)] at room temperature for 1—1.5 h. The crude peptides were purified on a Develosil LOP ODS column as described above. Analogs **3** and **4** were successfully separated on the column with a linear gradient from 5—30% solvent B (80% CH_3CN containing 0.06% TFA) over 150 min. Analog **3** eluted faster than **4**. Purity of all peptides was >95% as analyzed on HPLC. Analytical data of synthetic peptides is shown in Table 1.

Enzymatic Stability Peptide (1 mg/ml, 150 μl) was incubated with a solution of AP-M (1 mg/ml, 80 μl) or a rat bain synaptosomal fraction[8)] (protein content : 2.0 mg/ml, 400 μl) in 10 mM Tris·HCl buffer (pH 7.60, 500 μl for AP-M or 200 μl for rat brain enzymes) at 37 °C for appropriate times. A part of the mixture was withdrawn and 0.2 N HCl (20 μl) was added. After centrifugation at 5 °C for 10 min (4000 rpm), the supernatant was analyzed by HPLC. The degradation rate was estimated from the relative peak area of residual intact peptide to that of the peptide at zero time on HPLC using a Chromatocorder 12 integrator (System Instruments). The half lifetime was obtained from the time–course curve of the degradation rate.

***In Vitro* Bioactivity Assay** The GPI and MVD assays were performed as reported in detail previously[17)] using isolated longitudinal muscle strips of Hartley strain guinea pig (250—300 g) ileum and vas deferens of ddY strain mouse (25—35 g), respectively. In both assays, log–dose response curves were constructed and IC_{50} values were determined.

Receptor Binding Assay The opioid receptor-binding assay was performed by the method as described previously.[15,18)] [^{3}H]DAMGO and [^{3}H]deltorphin II were used as μ- and δ-radioligands, respectively. The values of inhibitory constant (K_i) of peptides were calculated according to the equation of Cheng and Prusoff.[19)] The K_d values of [^{3}H]DAMGO and [^{3}H]deltorphin II used were 0.35 and 0.56, respectively.

Partition Coefficients The partition coefficients of **1**, **2** and Enk in the *n*-octanol/water system were determined on HPLC according to the method of Leo *et al*.[20)]

References and Notes

1) Amino acids and peptides are of L-configuration unless otherwise noted. Amino acids and peptides used are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature in *Eur. J. Biochem*., **139**, 9 (1984). Other abbreviations used are: Enk=Leu-enkephalin, YRFB=Tyr-D-Arg-Phe-βAla-NH_2, DAMGO=[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin, Dmt=2,6-dimethyltyrosine, Boc=*tert*-butoxycarbonyl, DIC=diisopropylcarbodiimide, Fmoc=*N*-9-fluorenylmethyloxycarbonyl, Pmc=2,2,5,7,8-pentamethyl-chroman-6-sulfonyl, AP-M=aminopeptidase M, TFA=trifluoroacetic acid, SAL-resin=4-(2′,4′-dimethoxyphenyl-hydroxymethyl)phenoxy resin, Fmoc-NH-SAL-resin=4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin, t_R=retention time.

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