Degradation of Deltorphins and Their Analogs by Rat Brain Synaptosomal Peptidases[1)]

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To obtain metabolically stable peptide ligands with high affinity and selectivity for the δ-opioid receptor, the enzymatic stability of deltorphins and their analogs was examined by using a crude rat brain synaptosomal membrane fraction. It was found that deltorphin (DLT: Tyr–D-Met–Phe–His–Leu–Met–Asp–NH_2) was easily degraded, producing DLT (1-4) and DLT (1-5) as the major degradation products, whereas [D-Ala^2]deltorphin II (DL-II: Tyr–D-Ala–Phe–Glu–Val–Val–Gly–NH_2) was very stable. Experiments with some enzyme inhibitors strongly suggested that the degradation of DLT was initiated by cleavage of the Leu^5–Met^6 bond by a metalloendopeptidase. On the other hand, stability experiments on DL-II analogs demonstrated that the presence of amino acids branched at the β-carbon atom or with a bulky side chain as residue 5 is of importance for the enzymatic stability. Based on these lines of evidence, some enzyme-resistant DLT analogs were synthesized.

Keywords deltorphin analog; [D-Ala^2]deltorphin II analog; enzymatic stability; degradation process; receptor binding assay; *in vitro* bioactivity

Most naturally occurring opioid peptides are labile to degradative enzymes, resulting in loss of bioactivities. Metabolically stable analogs are desirable for *in vivo* and *in vitro* biological studies.[2)] Deltorphins (DLT: Tyr–D-Met–Phe–His–Leu–Met–Asp–NH_2, DL-I: Tyr–D-Ala–Phe–Asp–Val–Val–Gly–NH_2 and DL-II: Tyr–D-Ala–Phe–Glu–Val–Val–Gly–NH_2), are naturally occurring peptides having high affinity and selectivity for the δ-opioid receptor.[3)] Recent structure–activity studies of deltorphins have revealed that hydrophobic residues at positions 5 and 6 are important for δ-affinity and selectivity,[4)] and in particular, Val^5 in DL-II is a key residue for δ-receptor selectivity.[4c)] On the other hand, it has recently been suggested that many δ-receptor agonists, including deltorphins, have varying degrees of stability to degradation enzymes *in vitro*.[5)] These observations prompted us to examine the enzymatic stability of two deltorphins, DLT and DL-II, and their analogs. In the present study, we observed high lability of DLT in the crude rat brain synaptosomal membrane fraction and the importance of the residue at position 5 in the two deltorphins for the enzymatic stability. Based on the degradation processes of DLT, some new enzyme-resistant analogs of DLT were synthesized and their opioid receptor-binding properties and *in vitro* bioactivities were examined.

Experimental

Enzymatic Stability of Peptides Peptides (100 nmol) were incubated with a crude rat brain synaptosomal (P2) membrane fraction[6)] (1.87 mg protein/ml, 300 μl) in a total volume of 500 μl containing 10 mM Tris–HCl buffer at pH 7.6. Aliquots of 100 μl were taken after appropriate incubation times and heated to 98 °C for 5 min, then 10 μl of 1 N HCl was added. After centrifugation at 0 °C for 10 min (10000 rpm), the supernatant was subjected to reversed-phase HPLC or examined in an amino acid analyzer (Hitachi model 835). For the peptidase inhibitory activity experiments, the membrane fraction was preincubated with the inhibitor in 10 mM Tris–HCl buffer for 2 min and then peptide (DLT) was added. HPLC was carried out on a YMC column (ODS-AM, AM-302-10, 4.6 × 150 mm) using the following solvent systems: A, 0.06% TFA; B, 0.06% TFA in 80% acetonitrile. A linear gradient from 15 to 50% B over 40 min was used at a flow rate of 1 ml/min. The eluate was monitored at 220 nm. For analyses of degradation products, the eluate corresponding to peaks was collected, hydrolyzed with 6 N HCl at 110 °C for 20 h and examined in an amino acid analyzer or a pico-tag amino acid analysis system (Waters). The degradation rate was estimated from the relative peak area of residual DLT to that of DLT at zero incubation time on HPLC using a Chromatocorder 12 integrator (System Instruments).

Peptide Synthesis All peptides used in this study were synthesized by the solid-phase technique using the DIPCDI/HOBt-mediated Boc strategy according to the method reported previously.[7)] No synthetic difficulty was observed with the DLT analogs presented here (Table I), including **4**, which has a sterically hindered Tle residue.[7)] Homogeneity of peptides was checked by TLC and HPLC and all analogs were at least 95% pure as judged from the HPLC profiles. The physico-chemical data of peptides are summarized in Table I.

Receptor Binding Assay Receptor binding properties of DLT analogs were examined according to essentially the same method as reported previously[4c,6)] by using radioligands, [^{3}H]DAGO and [^{3}H]DADLE for μ- and δ-receptors, respectively. Inhibition constants (K_i) were calculated from the IC_{50} value by the equation of Cheng and Prusoff,[8)]

TABLE I. Physico-Chemical Properties of Synthetic Peptides

Analog	Yield[a)] (%)	$[\alpha]_D$[b)] (°)	TLC[c)]		HPLC[d)]	FAB MS	Amino acid analysis[e)]					
			Rf^1	Rf^2	t_R (min)	$(M+H)^+$	Tyr	Met	Phe	His	Asp	Xaa^5
[Val^5]DLT (**1**)	35	−1.1	0.46	0.65	16.64	941	0.92	1.74	1.00	0.86	1.02	0.91
[Ile^5]DLT (**2**)	36	−1.1	0.50	0.69	18.81	955	0.93	1.82	1.00	0.91	0.85	1.03
[Mle^5]DLT (**3**)	41	−2.1	0.59	0.73	23.71	969	0.68	1.54	1.00	0.88	0.87	0.93
[Tle^5]DLT (**4**)	28	−1.3	0.49	0.65	18.30	955	0.84	1.64	1.00	1.00	0.89	1.00

a) Calculated based on the starting resin. *b*) Optical rotations were measured in 50% methanol ($c=0.5$) at 23 °C. *c*) Solvents: Rf^1, 1-BuOH–AcOH–H_2O (4 : 1 : 5, upper phase); Rf^2, 1-BuOH–AcOH–pyridine–H_2O (15 : 3 : 10 : 12). *d*) Retention time on a column of YMC AM-303-10 using a linear gradient from 16 to 44% acetonitrile in 0.06% TFA over 40 min at a flow rate of 1 ml/min. *e*) After 6 N HCl hydrolysis at 110 °C for 22 h.

$K_i = IC_{50}/(1 + L/K_d)$, where L is the concentration of the radioligand, and K_d represents the dissociation constant for [^{3}H]DAGO or [^{3}H]DADLE (0.861, 1.64, respectively).

***In Vitro* Bioactivity** The GPI and MVD assays were carried out as reported in detail previously[7] using isolated longitudinal muscle strips of Hartley strain guinea-pig (250—300 g) ileum and mouse *vas deferens* of male ddY strain mouse (25—35 g), respectively. In both assays, log dose–response curves were constructed and IC_{50} values (concentration causing a 50% reduction of the electrically induced twitches) were determined.

Results and Discussion

The enzymatic stability was examined by incubation of peptides with a crude rat brain synaptosomal membrane fraction at 37°C. As shown in Fig. 1, DLT was easily degraded (half-life: 45 min), producing DLT (1-4) and DLT (1-5) as the major degradation products. During the early incubation period (*ca.* 60 min), the quantities of

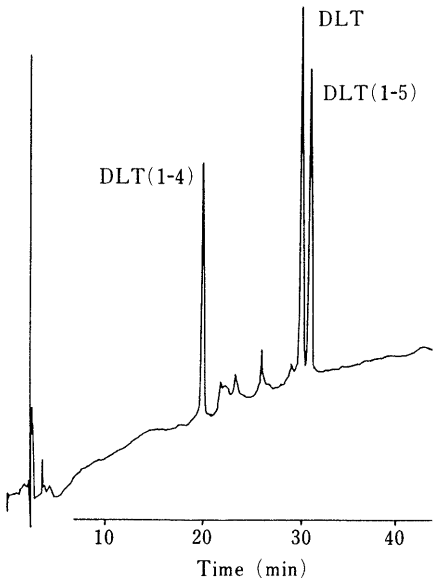

Fig. 1. HPLC Profile of the Degradation of DLT after Incubation for 1 h

See Experimental for HPLC conditions.

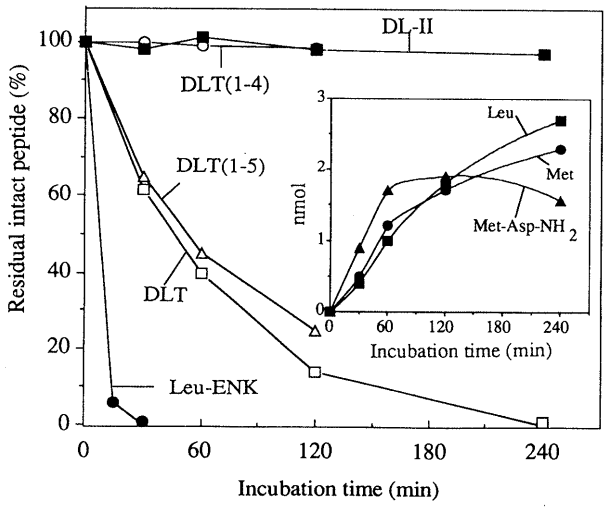

Fig. 2. Time Courses of Degradation of DLT, DLT (1-4), DLT (1-5), DL-II and Leu-ENK

The inset shows the release of Met–Asp–NH_2, Leu and Met from DLT (3.6 nmol) as detected by an amino acid analyzer.

Table II. Degradation of DL-II and Its Analogs in Rat Brain Synaptosomal Membrane Fraction

Peptide	Degradation rate[a] (%)	Major degradation products
DL-II	5>	N-Terminal tetrapeptide
[Ala^5]DL-II	51	N-Terminal pentapeptide
[Ala^6]DL-II	8	N-Terminal tetra-/pentapeptide (1.3 : 1)[b]
[Nva^{5,6}]DL-II	23	N-Terminal tetra-/pentapeptide (1.4 : 1)[b]
[Nle^{5,6}]DL-II	28	N-Terminal tetra-/pentapeptide (1.4 : 1)[b]
[Ile^{5,6}]DL-II	5>	N-Terminal tetrapeptide
[Leu^{5,6}]DL-II	17	N-Terminal tetra-/pentapeptide (1.1 : 1)[b]
[Tle^{5,6}]DL-II	—[c]	—
[Mle^{5,6}]DL-II	—[c]	—

a) Degradation rate was estimated from the residual intact peptide peak on HPLC after incubation for 2 h. *b*) Approximate ratio of tetra- and pentapeptide peak areas on HPLC. *c*) No metabolite peak(s) was detected on HPLC.

the two metabolite peaks on HPLC were approximately the same, but on prolonged incubation, the DLT (1-5) peak decreased while the DLT (1-4) peak increased, indicating further degradation of DLT (1-5) to DLT (1-4). The time course of the disappearance of DLT and various peptides in the incubation mixture is shown in Fig. 2. Leu-enkephalin was degraded completely within 30 min. The hydrolysis of DLT (1-5) to DLT (1-4) was confirmed by using authentic DLT (1-5). That is, the pentapeptide was degraded readily (half-life: 50 min), producing the N-terminal tetrapeptide, which is very stable under the above conditions. When the incubation mixture was analyzed with an amino acid analyzer (Fig. 2, inset), the C-terminal dipeptide, Met–Asp–NH_2, was released faster than Leu (*ca.* 120 min) and this fragment was degraded further, possibly by an aminopeptidase(s). The total release of Met–Asp–NH_2 and Met was in reasonable agreement with the theoretical amount (3.6 nmol). The C-terminal tripeptide, Leu–Met–Asp–NH_2, could not be detected by either HPLC or the amino acid analyzer. These results strongly suggest that the degradation of DLT was primarily initiated by cleavage of the Leu^5–Met^6 bond by an endopeptidase and that DLT (1-4) was derived from DLT (1-5) by hydrolysis of DLT (1-5) with a carboxypeptidase(s). The inhibition of the degradation of DLT by some peptidase inhibitors was examined by HPLC, with the following results: 10 μM phosphoramidon, 32%; 100 μM phosphoramidon, 65%; 100 μM thiorphan, 37%, 100 μM captopril, 2%; 1 mM EDTA, 82%; 500 μM *o*-phenanthroline, 98%. A relatively high inhibition was observed with phosphoramidon, an inhibitor of neutral endopeptidase (or endopeptidase-24.11).[9] Metal-chelating reagents strongly inhibited the hydrolysis, suggesting that the primary inactivation enzyme of DLT is a metalloendopeptidase. Table II shows the enzymatic stability of DL-II and its previously described analogs with aliphatic side chains at residues 5 and/or 6.[4c] In contrast to DLT, DL-II was very stable, in accordance with the reported result using a crude preparation of rat brain membranes.[3c] The two analogs, [*tert*-leucine (Tle)^{5,6}] and [γ-methyl-leucine (Mle)^{5,6}]DL-II, were also highly stable. Degradation rates of the other analogs varied from 8 to 51%, depending on the substitutes at positions 5 and/or 6, and the degradation products corresponded to the N-terminal tetra- or

TABLE III. Enzymatic Stability, Receptor Binding and *in Vitro* Bioassays of DLT Analogs

Peptide	Degradation rate (%)[a]	K_i (nM)		$K_i(\mu)/K_i(\delta)$	IC_{50} (nM)		GPI/MVD
		[^{3}H]DAGO(μ)	[^{3}H]DADLE(δ)		GPI	MVD	
DLT	65	226±61	0.21±0.11	1076	664±48	1.06±0.08	626
[Val^5]DLT (**1**)	24	169±34	0.24±0.10	704	719±40	2.29±0.79	314
[Ile^5]DLT (**2**)	21	187±23	0.15±0.06	1247	892±51	1.93±0.16	462
[Mle^5]DLT (**3**)	10	203±29	0.39±0.11	521	831±23	2.01±0.13	413
[Tle^5]DLT (**4**)	—[b]	339±40	10.9±3.9	31	1268±129	2570±790	0.5

a) After 2 h incubation with a different lot of enzyme preparation from that used for the experiments in Fig. 2 and Table II. *b*) No metabolite peak(s) was detected on HPLC.

pentapeptide. These data suggested that the degradation processes of this series of analogs are similar to that of DLT. It should be noted that [Ala^6]DL-II showed good stability while [Ala^5]DL-II was readily degraded, indicating the importance of the Val^5 residue in DL-II for enzymatic stability. These results suggest that the enzymatic lability of [Ala^5]DL-II at least partly accounted for its lower δ-selectivity than [Ala^6]DL-II[4c] since this analog degraded easily, producing the N-terminal pentapeptide having a very low δ-affinity (data not shown). It seems likely that sterically hindered amino acids with branching at the β-carbon atom or a bulky side chain (*i.e.* Val, Ile, Tle, Mle) at position 5 in DL-II analogs are of key importance for the enzymatic stability. Based on these lines of evidence, we synthesized four DLT analogs with sterically hindered side chains at residue 5 (Table III) to confirm the importance of the residue at position 5 for enzymatic stability. As expected, all the analogs synthesized in this study showed enhanced enzymatic stabilities as compared to DLT itself, and most of the analogs possessed similar receptor binding and *in vitro* bioactivity profiles to those of DLT. The replacement of Leu by Tle (**4**) exceptionally resulted in a remarkable reduction in δ-receptor affinity and MVD bioactivity as was observed in similarly modified DL-II analogs.[4c,7] This is possibly due to the apparently high steric hindrance of the Tle residue, which disrupts the required conformation of the molecule at the δ-receptor, *e.g.*, by disturbing the β-turn conformation involving residues 4 to 7.[10] These results support the view that a proper lipophilic and topographic structure is necessary in this region of DLT to maximize interaction with the δ-receptor.[4a,11] Interestingly, analog **4** showed an antagonist activity against DLT as an agonist in the MVD assay. Details of the antagonist properties and further studies of this series of analogs are in progress.

In conclusion, the present study has demonstrated that the Leu^5–Met^6 bond of DLT is labile to brain enzymes and the presence of an amino acid with a branch at the β-carbon atom or a bulky side chain at position 5 in deltorphins is advantageous in terms of enzymatic stability. The DLT analogs presented here (**1**, **2**, **3**) may serve as useful lead compounds in studies to find more stable DLT derivatives.

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References and Notes

1) Amino acids and peptides are of L-configuration unless otherwise noted. Symbols for amino acids and peptides used are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature in *Eur. J. Biochem.*, **138**, 9 (1984). Other abbreviations used are: DAGO=Tyr–D-Ala–Gly–MePhe–Gly–ol, DADLE=Tyr–D-Ala–Gly–Phe–D-Leu, ENK=enkephalin, Tle=*tert*-leucine(C$^\alpha$-*tert*-butylglycine), Mle=γ-methyl-leucine, Nva=norvaline, Nle=norleucine, Boc=*tert*-butoxycarbonyl, DIPCDI=diisopropylcarbodiimide, HOBt=1-hydroxybenztriazole, FAB=fast atom bombardment, TFA=trifluoroacetic acid.
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