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# Synthesis of Carboxyl-Terminal Extension Analogs of Dermorphin and Evaluation of Their Opioid Receptor-Binding and Opioid Activities[1)]

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**Eight dermophin (DM) analogs extended at the C-terminus, based on the sequence of prepro-DM deduced from the cDNA, were synthesized by a simultaneous solid-phase method in which a pair of peptides was synthesized in a single vessel. Six peptides (three pairs) were obtained in satisfactory yields (17—37%). In the opioid receptor-binding assay using a rat brain homogenate, the $\mu$-affinities correlated well to the net positive charges of peptides, and DM–Gly–Glu–Ala–Lys–Lys–Ile–Lys–Arg–$NH_2$ showed the highest $\mu$-affinity, 115-fold that of DM. But, in the guinea pig ileum assay, extension analogs with net positive charge of 2 to 4 exhibited potencies comparable to or slightly lower than that of DM, even though they possessed 2- to 115-fold higher $\mu$-affinities than DM in the receptor-binding assay.**

**Keywords** dermorphin extension analog; simultaneous solid-phase synthesis; receptor-binding assay; guinea pig ileum assay

A group of opioid peptides has been discovered in the skin of South America frogs belonging to *Phyllomedusa* (*Ph.*).[2)] They are heptapeptides with the common amino-terminal sequence Tyr–D-Xaa–Phe, in which D-Xaa is either D-alanine or D-methionine. The first peptide isolated from frog skin was dermorphin (DM, Tyr–D-Ala–Phe–Gly–Tyr–Pro–Ser–$NH_2$), which has high affinity and selectivity for $\mu$-opioid receptors, and a potent opioid activity in several mammalian bioassays. By using a cDNA library from skin of *Ph. sauvagei*, the amino acid sequences of several DM precursor polypeptides were elucidated.[3)] One DM precursor was shown to have a repetitive pattern of five highly homologous sequences of 35 amino acids, one of which contained the deltorphin (or dermenkephalin[4)]) sequence, while the other four contained the DM sequence.

In a previous paper, we have reported that a DM-containing pentadecapeptide based on the structure of DM precursor sequence, Tyr–D-Ala–Phe–Gly–Tyr–Pro–Ser–Gly–Glu–Ala–Lys–Lys–Ile–Lys–Arg, is half and twice as potent as DM in terms of $\mu$- and $\delta$-opioid receptor affinities, respectively.[5)] To elucidate further the effect of peptide chain extension of DM on the biological activity, we have synthesized eight DM-containing peptides extended systematically at the C-terminus (Fig. 1) and examined their opioid receptor-binding properties and *in vitro* bioactivity for the inhibition of electrically evoked contraction of guinea pig ileum (GPI). All peptides were synthesized as the C-terminal amide form to protect them from carboxypeptidases during biological assays, because enzymatic degradation from the C-terminus would complicate the biological assay results.

Various methods of simultaneous solid-phase peptide synthesis (SPPS) have been developed; *e.g.*, the tea-bag method,[6)] polyethylene rod method,[7)] and coupling a mixture of amino acids to a single batch of resin.[8)] For the synthesis of this series of analogs, a simultaneous solid-phase method in a single vessel was employed to save time and reduce cost. Two peptides were simultaneously constructed on a benzhydrylamine (BHA) resin as shown in Fig. 2. Combinations of target peptides were chosen based on differences of net charge and/or possible hydrophilicity, *i.e.* I and VII, II and VI, III and V. After construction of protected peptides on the resin, the peptides were cleaved from the resin and deprotected with HF–anisole mixture (Fig. 3), followed by purification by medium-pressure HPLC or a combination of CM-cellulose column chromatography and medium-pressure HPLC. Yields and physicochemical data of synthetic peptides are shown in Table I. Simultaneously synthesized peptides were obtained in satisfactory yields (17—37%). These results suggest that this synthetic strategy is useful for C-terminally extended analogs, with certain limitations in that the combinations of two or more peptides should have adequate net charge differences for easy purification.

The receptor-binding properties of the synthetic peptides were determined by displacement experiments with selective radioligands, [$^3$H]DAGO and [$^3$H]DPDPE, having $\mu$- and $\delta$-affinity, respectively, and the results are shown in Table II. All analogs synthesized in this study showed

dermorphin: Tyr–D-Ala–Phe–Gly–Tyr–Pro–Ser–$NH_2$
I: ——————————Gly–$NH_2$
II: ——————————Gly–Glu–$NH_2$
III: ——————————Gly–Glu–Ala–$NH_2$
IV: ——————————Gly–Glu–Ala–Lys–$NH_2$
V: ——————————Gly–Glu–Ala–Lys–Lys–$NH_2$
VI: ——————————Gly–Glu–Ala–Lys–Lys–Ile–$NH_2$
VII: ——————————Gly–Glu–Ala–Lys–Lys–Ile–Lys–$NH_2$
VIII: ——————————Gly–Glu–Ala–Lys–Lys–Ile–Lys–Arg–$NH_2$

Fig. 1. Dermorphin and Synthetic Dermorphin-Containing Peptides

higher $\mu$-receptor preferences. The C-terminal extension of DM led to analogs I—IV with comparable or reduced $\mu$-affinity and selectivity as compared to DM and the reduction reached maximum with analog III which has the net charge 0 owing to incorporation of the $Glu^9$ residue. Further extension to $Arg^{15}$ led to analogs V—VIII with increased $\mu$-affinity and selectivity, and in particular, analog VIII, which has not charge +4, showed extremely high $\mu$-affinity and selectivity, being 115- and 8.8-fold those of DM, respectively. As shown in Fig. 4, the $\mu$-receptor affinities correlated well to the net positive charges of peptides. As far as the effect of net charge on the $\mu$-affinity is concerned, these results are consistent with the membrane compartment concept,[9] *i.e.*, $\mu$-receptor may be located in an anionic membrane environment to which positively charged ligands would be attracted by electrostatic forces.

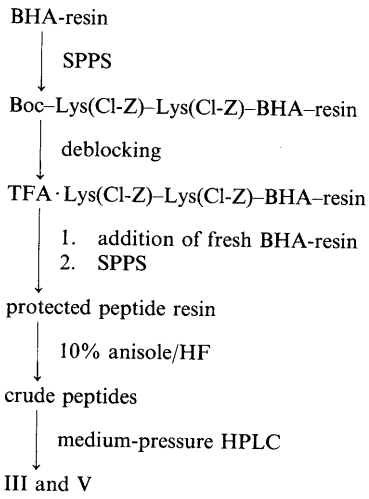


Fig. 2. Simultaneous Synthesis of III and V

In the GPI assays (Table II), analogs with net charge 0 (II and III) showed 2- to 5-fold lower potencies than DM (net charge +1). All analogs with higher net positive charges than DM (V—VIII) showed equal or slightly reduced potencies as compared to DM. These results are quite unexpected in view of the receptor-binding assay data, since analogs VII and VIII have 7- and 115-fold higher $\mu$-affinities than DM, respectively. Analog VIII showed good stability in a GPI homogenate mixture: only about 10% was degraded within 1 h incubation, while [$Lue^5$]enkephalin was completely degraded under the same conditions. From these results, susceptibility to degradative enzymes can be excluded as a cause of the low GPI potency. DM and VIII showed $K_e$ values for naloxone as an antagonist of $3.04 \pm 0.47$ and $2.28 \pm 0.61$ nM, respectively, in the GPI assay. Such low $K_e$ values are typical for the $\mu$-receptor interaction.[10] Analog VIII showed a very low $\kappa$-affinity ($IC_{50} > 10000$ nM) when assessed in terms of the inhibition of [$^3$H]U-69593 binding to guinea pig brain membrane. These results suggest that the effect of VIII is mediated by $\mu$-receptor in this tissue preparation. Additionally, in the GPI assay, the fifty

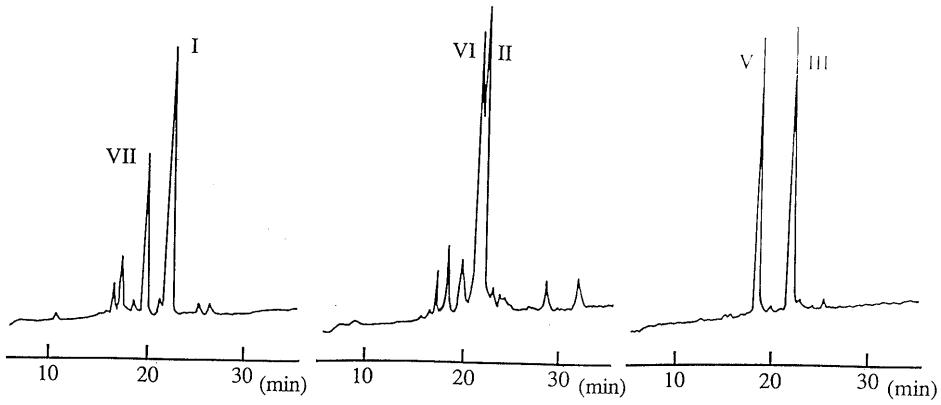


Fig. 3. HPLC Patterns of the Crude Product Mixture from Simultaneous Peptide Synthesis
Column, YMC-303-10 (4.6 × 250 mm); eluent, a linear gradient from 15 to 50% acetonitrile in 0.06% TFA over 40 min (see Experimental for other conditions).

TABLE I. Yields and Physicochemical Data of Synthetic Peptides

| Compounds | Yield[a] (%) | Purification method[b] | $[\alpha]_D$ (°) ($c$=0.5, 1% AcOH) | TLC[c] *Rf* | | Amino acid analysis | | | | | | | | | | HPLC-$t_R$[c] (min) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | A | B | Tyr | Ala | Phe | Gly | Pro | Ser | Glu | Lys | Ile | Arg | |
| I[d] | 36 | H | −5.57 | 0.48 | 0.79 | 1.92 | 1.00 | 0.98 | 1.92 | 1.10 | 0.81 | — | — | — | — | 17.0 |
| II[d] | 18 | C, H | −5.66 | 0.45 | 0.74 | 1.90 | 1.00 | 0.96 | 1.02 | 1.02 | 0.78 | 1.02 | — | — | — | 17.7 |
| III[d] | 37 | H | −10.10 | 0.41 | 0.73 | 1.84 | 2.01 | 0.97 | 1.93 | 0.99 | 0.79 | 1.02 | — | — | — | 16.9 |
| IV | 29 | H | −20.12 | 0.25 | 0.57 | 1.90 | 2.02 | 1.01 | 2.00 | 1.05 | 0.84 | 1.05 | 1.00 | — | — | 14.7 |
| V[d] | 30 | H | −26.25 | 0.18 | 0.43 | 1.83 | 2.00 | 0.98 | 1.93 | 1.02 | 0.80 | 1.02 | 1.88 | — | — | 13.5 |
| VI[d] | 17 | C, H | −31.86 | 0.22 | 0.56 | 1.73 | 2.02 | 1.04 | 1.97 | 0.92 | 0.80 | 1.02 | 1.94 | 1.02 | — | 16.6 |
| VII[d] | 17 | H | −34.62 | 0.18 | 0.46 | 1.60 | 2.00 | 1.03 | 1.97 | 0.83 | 0.88 | 1.02 | 2.80 | 0.99 | — | 14.3 |
| VIII | 35 | H | −47.80 | 0.07 | 0.45 | 1.92 | 2.12 | 1.12 | 2.08 | 1.00 | 0.86 | 1.10 | 2.95 | 0.97 | 1.03 | 13.2 |

*a*) Based on the starting resin. *b*) H, medium-pressure HPLC; C, CM-cellulose chromatography. *c*) See Experimental. *d*) Synthesized by simultaneous peptide synthesis.

TABLE II. Opioid Receptor Binding Assays and GPI Assay of Dermorphin-Containing Pepetides

| Compounds | [$^3$H]DAGO | | [$^3$H]DPDPE | | $K_i(\delta)/K_i(\mu)$ ratio | GPI $IC_{50}$[d] nM |
|---|---|---|---|---|---|---|
| | $K_i(\mu)$[a] nM | R.p.[b] | $K_i(\delta)$[a] nM | R.p.[c] | | |
| DAGO | 0.37±0.03 | 1.000 | 173±19 | 0.0027 | 468 | — |
| DPDPE | 321±54 | 0.001 | 0.459±0.065 | 1.0000 | 0.0014 | — |
| Dermorphin | 0.23±0.03 | 1.609 | 94±35 | 0.0049 | 409 | 0.99±0.15 |
| I | 0.32±0.11 | 1.156 | 181±67 | 0.0025 | 566 | 5.09±0.67 |
| II | 0.95±0.51 | 0.389 | 453±157 | 0.0010 | 477 | 2.24±0.85 |
| III | 2.94±1.63 | 0.126 | 156±70 | 0.0029 | 53 | 4.54±0.40 |
| IV | 0.72±0.59 | 0.154 | 199±64 | 0.0023 | 276 | 1.45±0.20 |
| V | 0.092±0.066 | 4.02 | 86±25 | 0.0053 | 935 | 1.24±0.14 |
| VI | 0.149±0.109 | 2.60 | 130±60 | 0.0035 | 915 | 1.53±0.47 |
| VII | 0.032±0.031 | 11.5 | 29.4±10.2 | 0.0156 | 919 | 1.69±0.23 |
| VIII | 0.0020±0.0010 | 185 | 7.20±0.40 | 0.0638 | 3600 | 1.37±0.39 |

*a*) The inhibition constants ($K_i$) were calculated from $IC_{50}$. Data represent means±S.E. of 4 to 10 experiments. *b*) Relative potencies to DAGO (1.00). *c*) Relative potencies to DPDPE (1.00). *d*) Data represent means±S.E. of 4 to 11 experiments.

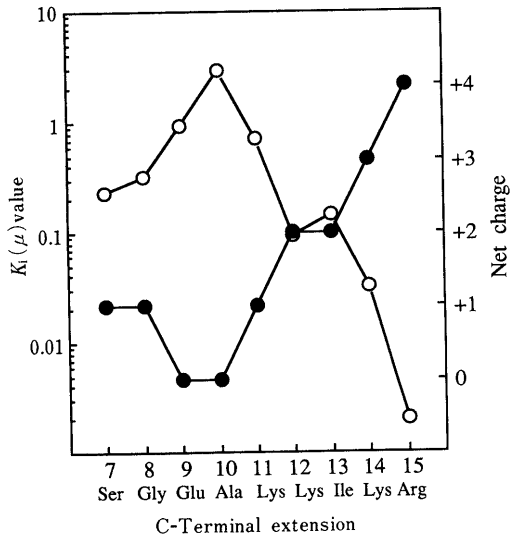


Fig. 4. Correlation between $\mu$-Receptor Affinity and Net Charge of Analogs

○, $K_i$ ($\mu$) value; ●, new charge.

percent inhibitory concentration ($IC_{50}$) values of DM in the presence and absence of VIII were statistically not significantly different ($IC_{50}$ was 1.44±0.14 nM in the presence of 0.5 nM VIII), suggesting that VIII has no antagonist property. At present we can only speculate that the unexpectedly low GPI potency of analogs with high net positive charges may be caused by differences between central and peripheral $\mu$-receptors.

In summary, we have demonstrated that some DM extension analogs have higher $\mu$-receptor affinities than DM for rat brain membrane preparation. Their $\mu$-receptor preferences were correlated well to the net positive charges of peptides. However, those analogs which showed much higher $\mu$-receptor binding affinity than DM unexpectedly showed low activities in the GPI assay.

## Experimental

Optical rotations were measured with a JASCO DIP-40 polarimeter. Amino acid analysis was performed using a Hitachi 835 amino acid analyzer after 6 N HCl hydrolysis of the peptide in an evacuated sealed tube at 110 °C for 20 h. Analytical HPLC was performed on a YMC octadecyl silica (ODS) column (AM-303-10, 4.6×250 mm) by gradient elution using the following solvent systems: A, 0.06% TFA; B, 80% acetonitrile containing 0.06% TFA. A linear gradient from 20% B to 50% B over 40 min at a flow rate of 1.2 ml/min was used and the eluate was monitored at 215 nm. Thin-layer chromatography (TLC) was performed on silica gel plates (Merck, Kiesel gel $60F_{254}$, 5×10 cm) with the following solvent systems: A, *n*-BuOH–AcOH–$H_2O$ (4:1:5, v/v, organic phase); B, *n*-BuOH–AcOH–pyridine–$H_2O$ (15:3:10:12, v/v).

**Peptide Synthesis** All peptides (Fig. 2) were synthesized by the SPPS method. Peptides were constructed on a BHA resin hydrochloride (0.6 meq/g, 1% cross link) using a Biosearch 9600 peptide synthesizer according to the schedule described previously.[5] Single coupling was employed for incorporation of each amino acid residue. $N^{\alpha}$-Boc-protected amino acids were used with the following side chain protections: Tos for Arg, Cl-Z for Lys, cHex for Glu, Bzl for Ser, and Br-Z for Tyr. Typically, for the synthesis of II and VI, FFA·Ala–Lys(Cl-Z)–Lys(Cl-Z)–Ile–BHA resin was synthesized, then fresh BHA resin was added and protected target peptides was constructed on the resin. The peptide resin was deprotected and peptides were cleaved from the resin with 10% anisole/HF at 0 °C for 60 min. After usual work-up, the resulting residue was treated with dilute $NH_4OH$ (pH 8.0) at 0 °C for 60 min to reverse any N→O shift[11] and then applied to a CM-cellulose column (2×10 cm) which was eluted with $H_2O$ (100 ml) and then 5% AcOH (100 ml). The former eluate contained II and the latter contained VI. Each crude peptide was purified by medium-pressure HPLC on a Develosil LOP ODS column (3×30 cm) which was eluted with a linear gradient from 18% to 40% acetonitrile in 0.06% TFA over 150 min at a flow rate of 3 ml/min. Fractions (6 ml/tube) 46—50 were pooled, evaporated, and lyophilized from $H_2O$ to yield II. Under the same elution conditions, VI was obtained from fractions 37—41. Yields and physico-chemical data of synthetic peptides are given in Table I.

**Receptor-Binding Assays** The opioid receptor-binding assay was carried out by the modified method described previously.[12] [$^3$H]DAGO, [$^3$H]DPDPE, and [$^3$H]U-69593 were used as $\mu$-, $\delta$-, and $\kappa$-radioligands, respectively. Binding assays were carried out by incubating an aliquot of the crude rat brain synaptosomal fraction (600 $\mu$g protein/ml) in an assay mixture containing 500 $\mu$g of bovine serum albumin (BSA), 50 $\mu$g of bacitracin, 10 $\mu$g of bestatin, 20 $\mu$g of soybean trypsin inhibitor, and 2 nM radioligand in a final volume of 500 $\mu$l (in 50 mM Tris–HCl buffer at pH 7.4). The $\kappa$-receptor binding assay was done with guinea pig brain homogenate. Nonspecific binding was determined in the presence of excess (1 $\mu$M) unlabeled ligand. After incubation for 60 min at 25 °C, the contents of each tube were filtered through GF/B filters (presoaked in 0.1% polyethyleneimine) with 2×4 ml of ice-cold Tris–HCl buffer. Filters were counted in a Beckman liquid scintillation counter after overnight extraction with liquid scintillation fluid (3 ml). The $IC_{50}$ values were obtained from log dose–displacement curve. The values of inhibitory constant ($K_i$) of the synthetic peptides were calculated according to the equation of Cheng and Prusoff.[13] The $K_d$ values of [$^3$H]DAGO and [$^3$H]DPDPE used are 0.45 and 3.43 nM, respectively.

**GPI Assay** The myenteric plexus-longitudinal muscle was prepared from the ileum of anesthetized male Hartley guinea pigs weighing 250—300 g. The tissue was mounted in a 20 ml bath containing

oxygenated (95% $O_2$, 5% $CO_2$) Krebs–Henseleit solution at 37 °C. The tissue was transmurally stimulated (0.1 Hz, 0.5 ms, supramaximal voltage). The contractions of the longitudinal muscle were recorded by mean of isometric transducers. The $IC_{50}$ is the concentration of compound necessary to inhibit the amplitude of the electrically induced contraction by 50% (Table II). The $K_e$ values to characterize antagonist activity for naloxone were obtained from the ratio of $IC_{50}$ values in the presence and absence of a fixed naloxone concentration (20 nM).[14] For the antagonist study of VIII, VIII (0.5 nM) was added 10 min before the addition of DM as the agonist and the $IC_{50}$ value of DM was determined.

**Stability of VIII in GPI Homogenate** Guinea pig ileum obtained from male Hartley guinea pig was homogenized in 50 mM Tris–HCl buffer (pH 7.4) at 0 °C and diluted to 48.7 μg protein/ml. Analog VIII (40 nmol) was incubated with 300 μl of the above homogenate at 36 °C for 1 h. The reaction was stopped by boiling for 5 min. After centrifugation at 10000 rpm for 10 min, an aliquot of the supernatant was subjected to HPLC and the degradation rate was determined from the amount of residual intact peptide. Under the same conditions [Leu$^5$]enkephalin was completely degraded.

## References and Notes

1) Amino acids and peptides are of L-configuration unless otherwise noted. Abbreviations used are: Boc = *tert*-butoxycarbonyl, Tos = tosyl, Cl-Z = 2-chlorobenzyloxycarbonyl, cHex = cyclohexyl, Bzl = benzyl, Br-Z = 2-bromobenzyloxycarbonyl, TFA = trifluoroacetic acid, DAGO = [D-Ala$^2$, MePhe$^4$, Gly-ol$^5$]enkephalin, DPDPE = [D-Pen$^2$, D-Pen$^5$]enkephalin, U-69593 = (5α,7α,8β)-(−)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide, HPLC = high-performance liquid chromatography, CM-cellulose = carboxymethyl cellulose, SPPS = solid-phase peptide synthesis.
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