A Tetrameric Enkephalin Analog for the Putative Multivalent Interaction with Opioid Receptors

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Based on increased affinity and selectivity of dimeric enkephalin analogs for opioid receptors, divalent interactions have been demonstrated between opioid peptides and receptors. To explore the receptor multivalency suggested by fluorescent microscopic studies, a novel type of tetrameric enkephalin analog was designed and synthesized. The tetrameric enkephalin comprises four [D-Ala^2, Leu^5]enkephalin molecules coupled with the amino groups of the *gem*-diamino derivative of cystine, which was derived from cysteinamide by a Hofmann-type rearrangement, causing an amide-to-amine conversion. By the reduction of the *gem*-cystine disulfide bond in the tetramer, two dimeric analogs with a free or *p*-methoxybenzyl-protected mercapto group were also prepared. The receptor binding characteristics of these tetramer and dimers for the δ and μ receptors were analyzed in an NG108-15 cell and rat-brain membranes. The affinity of the tetrameric analog for the δ receptors was about equal to that of the dimers, but 2—4-fold lower for the μ receptors. The dimers were 5—8-fold δ-selective, and, thus, the tetramer exhibited a high selectivity for the δ receptors. A divalent interaction mechanism was considered between the tetrameric ligand and the δ opioid receptors.

The primary structures of the opioid receptors have recently been clarified by cDNA cloning techniques.[1—4] These sequences suggest that the opioid receptors, like other neuropeptide receptors, might have a structure of seven transmembrane domains, although they gave no information about their molecular organization in biomembranes. Studies on the fluorescence microscopic analyses of opioid receptors have revealed that they cluster to microaggregate on specific membrane sites.[5,6]

Our synthetic studies on opioid peptides have shown that the dimerization of enkephalins can strongly enhance the binding affinity and selectivity for both the δ and μ opioid receptors, depending upon the size and/or amino acid sequences to be dimerized.[7] Such effects of ligand dimerization on receptor interaction imply that the two opioid receptors may contain at least two equivocal binding sites that are very close to each other.[8—10] On the other hand, the results indicating the microaggregation of opioid receptors have revealed the possibility of the simultaneous interaction of well-structured multivalent ligands.

In the present study we designed and synthesized a tetrameric analog of [D-Ala^2, Leu^5]enkephalin. Four enkephalin molecules were cross-linked at their *C*-terminus with the *gem*-diamine derivative of cystine, (*gem*-Cys)$_2$[11] (Fig. 1), in which the amino group replaced the carboxyl group of the cysteines. It was highly expected

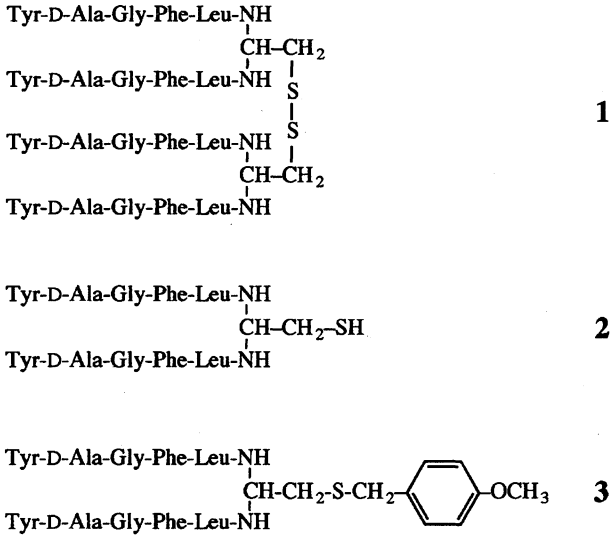

Fig. 1. Structures of dimeric and tetrameric enkephalin analogs.

that the resulting tetramer would simultaneously cross-link more than two receptors. The affinity for the opioid receptors was compared with those of dimeric derivatives obtained by a reduction of the disulfide bond of the tetramer, in order to evaluate the effect of multiattachment of enkephalins in one molecule. The binding characteristics of the tetramer and dimers were assessed by using rat brain and NG108-15 hybrid cell membranes.[12]

Results and Discussion

Synthesis of Tetrameric Enkephalin Analog. A tetrameric enkephalin was synthesized by two different procedures, starting from cysteine (procedure A) or cystine (procedure B). Both procedures were performed by the conventional solution method. Figure 2 shows a synthetic route for dimeric and tetrameric enkephalin analogs starting from *S*-(*p*-methoxybenzyl)cysteine, Cys(MBzl), (procedure A). A mixed anhydride obtained from Boc–Cys(MBzl)–OH and isobutyl chloroformate (IBCF) was treated with ammonia in dioxane (1 : 5, by volume) to afford the amide derivative Boc–Cys(MBzl)–NH_2 (**4**). The Boc group of **4** was removed by a treatment with trifluoroacetic acid (TFA), and the resulting amine was coupled with Boc–Phe–Leu–OH by using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 1-hydroxybenzotriazole (HOBt). The resulting Boc–Phe–Leu–Cys(MBzl)–NH_2 (**5**) was treated with [bis(trifluoroacetoxy)iodo]benzene (BTIB) to convert the *C*-terminal amide group into an amino group.[13,14] This Hofmann-type rearrangement reaction, which caused an amide-to-amine conversion, created the *gem*-diamine derivative of cysteine, *gem*-Cys, with the backbone structure of Boc–Phe–Leu–NHCH(CH_2SMBzl)–NH_2 (**6**). 2-Substituted *gem*-diamines derived from amino acid amides have been successfully utilized by us for cross-linking in peptide dimerization.[15] The newly generated amino group of the *gem*-Cys moiety in **6** was coupled with another molecule of Boc–Phe–Leu–OH by the EDC–HOBt method, affording a dimeric dipeptide **7**, (Boc–Phe–Leu–)$_2$[–NHCH(CH_2SMBzl)NH–]. After removing the Boc groups, the dimer was elongated by coupling with Boc–Tyr–D-Ala–Gly–OH, yielding the Boc-pentapeptide dimer **8**. Dimeric enkephalin analog **3** containing *gem*-Cys(MBzl), (H–Tyr–D-Ala–Gly–Phe–Leu–)$_2$[–NHCH(CH_2SMBzl)NH–], was obtained from compound **8**. To prepare a tetrameric enkephalin analog [(H–Tyr–D-Ala–Gly–Phe–Leu–)$_2$[–NHCH(CH_2S–)NH–]]$_2$ (**1**), compound **3** was first treated with liquid HF in order to remove the *S*-MBzl group, and was then oxidized to form a disulfide bond between the *gem*-cysteines (Fig. 2). A dimeric enkephalin analog containing a free mercapto group, (H–Tyr–D-Ala–Gly–Phe–Leu–)$_2$[–NHCH(CH_2SH)NH–] (**2**), was obtained by a reduction of this disulfide bond with zinc powder in 50% AcOH.

The tetrameric enkephalin analog was also prepared by another method (procedure B) starting from cystine (Fig. 3). The starting material (Boc–Cys–OH)$_2$ was

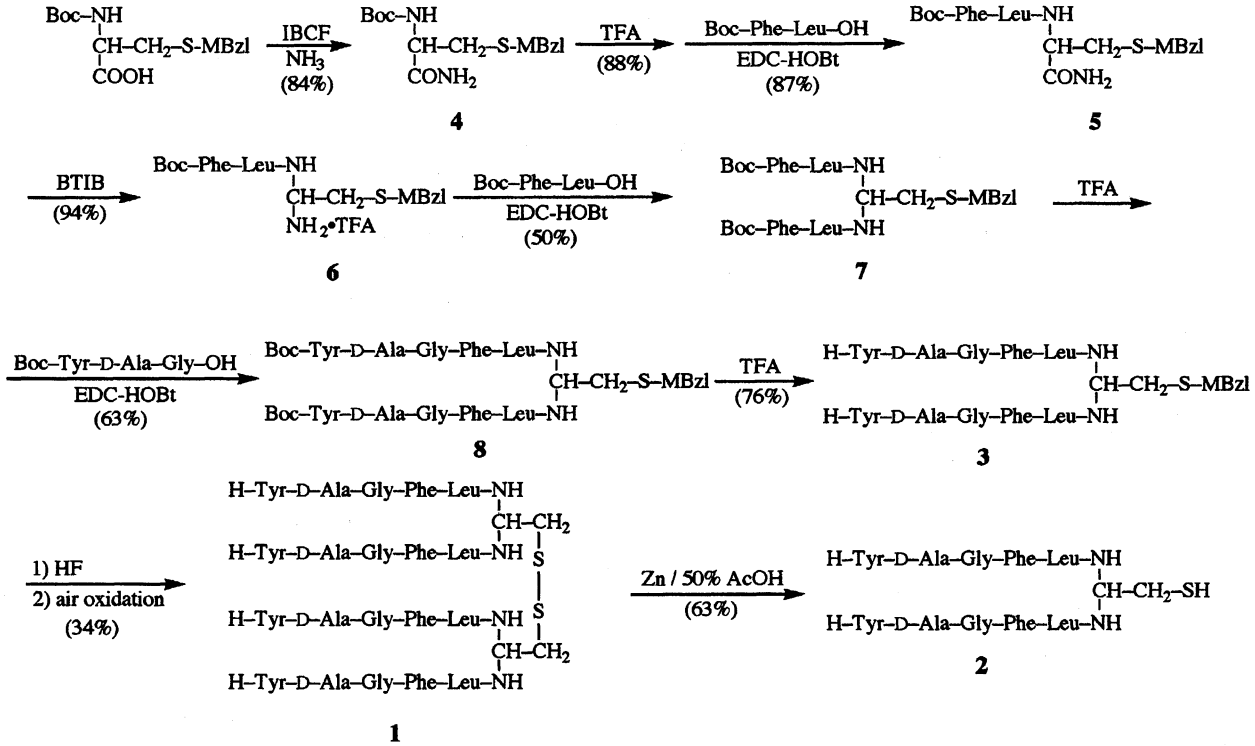

Fig. 2. Synthetic route for the tetrameric enkephalin analog (Procedure A).

converted into amide by the mixed anhydride method. After removing the Boc groups of the resulting amide **9**, Boc–Phe–Leu–OH was coupled by the EDC–HOBt method to obtain the dimeric tripeptide **10**. The *C*-terminal amide group of compound **10** was then converted into the amine by using BTIB, affording the *gem*-cystine moiety. The newly generated amino groups in peptide [Boc–Phe–Leu–NHCH(CH_2S–)NH_2]$_2$·2TFA (**11**) were coupled with Boc–Phe–Leu–OH, resulting in the formation of the tetrameric dipeptide **12**, [(Boc–Phe–Leu–)$_2$[–NHCH(CH_2S–)NH–]]$_2$. Compound **12** was further elongated with Boc–Tyr–D-Ala–Gly–OH to give a protected tetrameric pentapeptide enkephalin analog **13**. Compound **13** was treated with TFA to attain the final product as a tetrameric enkephalin analog **1**.

The homogeneity of the synthetic peptides was verified by TLC, HPLC, paper electrophoresis, and amino acid analysis. The elution profile of the SH-containing dimer (**2**) on HPLC, however, has revealed that a small amount of tetramer (6.4%) remains, even after purification by gel filtration (data not shown). The contaminating tetramer was presumably produced by air oxidation during purification. The number of reaction steps (8 steps) in procedure B was less than that (10 steps) in procedure A. Moreover, the total yield (4.9%) in the latter was almost two fold (10.1%) compared to the former. One of the important advantages in procedure B is the omission of the step using liquid HF, which resulted in a considerably lower recovery of the tetramer (34%) in procedure A. However, the disadvantage of procedure B is the poor solubility of almost all the intermediates. This caused a difficulty in purifying the products.

Receptor Binding Activities. Table 1 summarizes the results from radio-labeled receptor binding assays using neuroblastoma × glioma hybrid (NG108-15) cell and rat brain membranes. [^{3}H]–[D-Ala^2, D-Leu^5]-enkephalin ([^{3}H]DADLE) and [^{3}H]–[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin ([^{3}H]DAGO) were utilized as specific tracers for the δ and μ receptors, respectively. Although [^{3}H]DADLE binds to both the δ and μ receptors, and its selectivity for the δ receptors is not so high, we can evaluate the binding affinity for the δ receptors by using this [^{3}H]DADLE together with NG108-15 cells. NG108-15 cells contain predominantly the δ receptors.

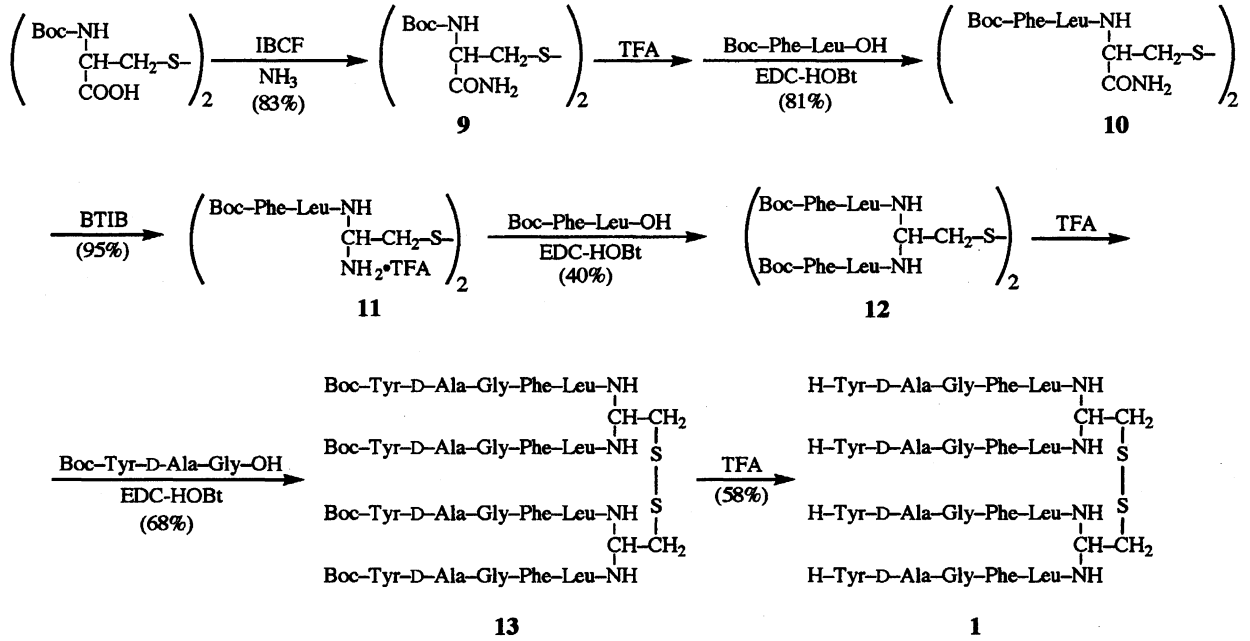

Fig. 3. Synthetic route for the tetrameric enkephalin analog (Procedure B).

Table 1. Receptor Binding Affinities of Tetrameric and Dimeric Enkephalin Analogs

Compounds		IC_{50} (nM) [^{3}H]DADLE NG108-15 cells	IC_{50} (nM) [^{3}H]DAGO rat brain	δ/μ-selectivity
DALEA monomer		1.50	1.46	0.97
gem-Cys tetramer	(**1**)	2.41	31.5	13
gem-Cys dimer	(**2**)	1.80	14.4	8.0
gem-Cys(MBzl) dimer	(**3**)	1.53	7.40	4.8

It is clear that both tetrameric and dimeric enkephalin analogs are highly potent to bind to the δ receptors in NG108-15 cells, showing small IC_{50} values (1.5—2.4 nM) (Table 1). Monomer [D-Ala^2, Leu^5]enkephalin amide (DALEA) also exhibited a high binding potency for the δ receptors.

When enkephalin analogs were tested in rat brain using [^{3}H]DAGO as a tracer, the tetramer exhibited a considerably reduced binding affinity (IC_{50}=31.5 nM), as compared to that in NG108-15 cells. Thus, the selectivity ratio of the δ/μ receptors was calculated to be 13, indicating that the tetramer binds to the δ receptors 13-fold more effectively than to the μ receptors. Although both dimers of compounds **2** and **3** were also δ-selective (δ/μ receptor selectivity ratio=5—8) (Table 1), the selectivity ratio of tetramer is definitely higher than those of the dimers. In contrast to the δ receptor preference of the tetramer and dimers, monomer DALEA was nonselective, as shown by its selectivity ratio, 0.97 (Table 1).

It has been reported that the dimers of pentapeptide, having full length of native enkephalins, are highly selective for the δ receptors, while the dimers of the *N*-terminal tetra- or tripeptide are selective for the μ receptors.[7,8] It should be noted that dimers **2** and **3** have the pentapeptide sequence of [D-Ala^2, Leu^5]-enkephalin and that tetramer **1** also possesses the same sequence. This is probably the main rationale for their preference for the δ receptors. The affinity of the tetramer was almost equal to those of the dimers for the δ receptors and 2—4 times lower for the μ receptors. Meanwhile, dimer **2** contained a small amount of tetramer. This contamination seemed to cause a reduction in the binding affinity for the μ receptors (Table 1). These results indicate that an enhanced δ-selectivity of the tetramer was not attained by its favorable interaction with the δ receptors, but by an unfavorable interaction with the μ receptors. The tetrameric structure appears not to be so advantageous to stimulate a further interaction with the δ receptors, but is apparently disadvantageous for interactions with the μ receptors.

The experimental evidence of cluster formation of the δ receptors in NG cells[5,6] has provided the idea that a single multivalent enkephalin analog might cross-link several receptors simultaneously. However, the tetramer synthesized in this study was not a case like this. The membrane organization of the δ receptors might not be multivalent to accept a number of ligands simultaneously. On the other hand, the tetramer has a molecular structure in which two dimers are bridged by a disulfide bond. The receptor selection of the tetramer is likely to be due to this dimeric nature of the tetramer. It is also possible that the length of the spacer between these dimers is not sufficient to cross-link more than two receptors simultaneously. Although the monomer DALEA showed almost the same IC_{50} value for the δ receptors as did the tetramer and dimers (Table 1), several lines of kinetic studies[8,9] have demonstrated that well-structured dimers of pentapeptide enkephalin interact with the δ receptors bifunctionally. The bifunctional interaction between the tetramer and the δ receptors should also be proven. In addition to such pharmacokinetic and structural studies, it might also be important to carry out experiments to examine whether the receptor structure which accepts a dimeric ligand is constructed by single or multiple receptor molecules.

Experimental

General. The melting points are uncorrected. TLC was carried out on Silica gel 60 GF_{254} (Merck) with the following solvent systems (by volume): R_f^1, $CHCl_3$–MeOH (9:1); R_f^2, $CHCl_3$–MeOH (5:1); R_f^3, $CHCl_3$–MeOH–AcOH (8:1:1); R_f^4, $CHCl_3$–MeOH–AcOH (95:5:1). For high-performance thin layer chromatography (HPTLC), the following solvent systems were used: R_f^5, $CHCl_3$–MeOH (9:1); R_f^6, $CHCl_3$–MeOH–AcOH (8:1:1); R_f^7, *n*-BuOH–AcOH–H_2O (4:1:5, upper phase); R_f^8, *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2). Paper chromatography was performed using Toyo Roshi No. 50 paper with the following solvent systems: R_f^9, *n*-BuOH–AcOH–H_2O (4:1:5, upper phase); R_f^{10}, *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2). Paper electrophoreses were carried out on Toyo Roshi No. 51A paper at pH 1.8 with a solvent mixture of HCOOH–AcOH–MeOH–H_2O (1:3:6:10). The electrophoretic migrations were measured with respect to lysine, and the ratio (R_{Lys}) was calculated. Compounds containing the mercapto group were detected by spraying 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and those containing the disulfide bond were spotted by spraying $NaBH_4$ followed by DTNB. Optical rotations were measured on a JASCO J-20 spectropolarimeter. For amino acid analyses, peptides were hydrolyzed in 6 M HCl (1 M=1 mol dm^{-3}) for 24 h at 110 °C in evacuated tubes, and analyzed on a JEOL JLC-300 Amino Acid Analyzer. Mass-spectrum measurements were obtained on a JEOL JMS-DX 300 equipped with JMZ-3100. HPLC analysis was performed on Wakosil-5CII$_{18}$ (0.4×25 cm, Wako Pure Chemical Ind., Osaka), eluted with a linear gradient of 5—95% acetonitrile in 0.05% TFA for 60 min. The retention times are indicated as R.T. values. BTIB was fleshly prepared by reported method.[13,14]

Bos–Cys(MBzl)–NH$_2$ (4). To a solution of Boc–Cys(MBzl)–OH (529 mg, 15.5 mmol) and *N*-methylmorpholine (NMM) (1.70 ml, 15.5 mmol) in THF (15 ml), isobutyl chloroformate (IBCF) (2.01 ml, 15.5 mmol) was added at −10 °C. After being stirred for 10 min, an aqueous ammonia in dioxane (1:5 by volume, 100 ml) was added to the reaction mixture, and the stirring was continued for 3 h at room temperature. The reaction mixture was evaporated, and the residue was dissolved in $CHCl_3$, and washed successively with 0.5 M (1 M=1 mol dm^{-3}) $NaHCO_3$, 10% citric acid and H_2O. The organic phase was dried over Na_2SO_4 and the filtrate was evaporated in vacuo. The solid residue was recrystallized from $CHCl_3$–diethyl ether to yield compound **4**; yield, 4.42 g (84%); mp 143—146 °C; $[\alpha]_D^{20}$ −18.7° (*c* 1.0, *N*-methylpyrrolidone (NMP)); R_f^1, 0.55; MS *m/z* 340 (Calcd 340). Found: C, 56.26; H, 7.02; N, 8.20%. Calcd for $C_{16}H_{24}N_2O_4S$: C, 56.45; H, 7.11; N, 8.23%.

Bos–Phe–Leu–Cys(MBzl)–NH$_2$ (5). Boc–Cys-

(MBzl)–NH_2 (**4**) (3.53 g, 10.4 mmol) was dissolved in TFA (10 ml) at 0 °C. After 30 min, the solution was evaporated to leave an oil, which was solidified by the addition of dry diethyl ether to yield H–Cys(MBzl)–NH_2·TFA, 3.24 g (88%); $R_f{}^9$, 0.64. This TFA salt (3.24 g, 9.13 mmol), Et_3N (1.28 ml, 9.13 mmol) and Boc–Phe–Leu–OH (3.46 g, 9.13 mmol) were dissolved in *N*,*N*-dimethylformamide (DMF) (25 ml); to this solution HOBt (1.48 g, 11.0 mmol) and EDC·HCl (1.93 g, 10.0 mmol) were added at −10 °C. The reaction mixture was stirred for 2 h at 0—10 °C and overnight at room temperature. After the solution was evaporated in vacuo, ice-water was added to the residue to give a solid precipitate. The solid collected was successively washed with 0.5 M $NaHCO_3$, 10% citric acid and H_2O. Dried material was purified on a silica-gel column (3.5×75 cm) eluted with $CHCl_3$–MeOH (9:1); yield, 4.77 g (87%); mp 183—185 °C; $[\alpha]_D^{20}$ −20.9° (*c* 1.0, NMP); $R_f{}^3$, 0.33; $R_f{}^5$, 0.57. Found: C, 61.75; H, 7.38; N, 9.17%. Calcd for $C_{31}H_{44}N_4O_6S$: C, 61.98; H, 7.38; N, 9.17%.

Boc–Phe–Leu–NHCH(CH_2SMBzl)NH_2·TFA (6). To a solution of Boc–Phe–Leu–Cys(MBzl)–NH_2 (**5**) (1.57 g, 2.62 mmol) in DMF–H_2O (1:1 by volume, 12 ml), BTIB (2.41 g, 6.41 mmol) was added at 0 °C. After 10 min, pyridine (1.03 ml, 12.8 mmol) was added and the solution was stirred for 4 h at room temperature. After evaporation, ice-water was added to the residue to precipitate a solid. Collected solid was washed with H_2O and then with diethyl ether to remove BTIB; yield, 1.40 g (94%); $R_f{}^1$, 0.50; $R_f{}^3$, 0.43. Obtained material was used for the next reaction without further purification.

(Boc–Phe–Leu–)$_2$[–NHCH(CH_2SMBzl)NH–] (7). To a solution of compound **6** (1.27 g, 2.22 mmol), NMM (0.26 ml, 2.22 mmol) and Boc–Phe–Leu–OH (0.84 g, 2.22 mmol) in DMF (10 ml) were added EDC·HCl (0.47 g, 2.44 mmol) and HOBt (0.33 g, 2.44 mmol) at 0 °C. The solution was stirred for 1 h at 0 °C and for 3 d at room temperature. After the residue obtained by evaporation was solidified by ice-water, the solid collected was successively washed as described for compound **5**. Purification was carried out by gel filtration on a Sephadex LH-20 column (3.6×110 cm) eluted with DMF and finally the compound was obtained by recrystallization from DMF–$CHCl_3$–diethyl ether; yield, 1.06 g (50%); mp 214—215 °C; $[\alpha]_D^{20}$ +5.1° (*c* 1.0, NMP); $R_f{}^1$, 0.50; $R_f{}^4$, 0.32. Found: C, 62.82; H, 7.56; N, 8.75%. Calcd for $C_{50}H_{72}N_6O_9S·H_2O$: C, 63.18; H, 7.84; N, 8.83%.

(Boc–Tyr–D-Ala–Gly–Phe–Leu–)$_2$[–NHCH(CH_2SMBzl)NH–] (8). Compound **7** (0.65 g, 0.70 mmol) was dissolved in TFA (5 ml) at 0 °C. The reaction mixture was treated as described for compound **5**, yielding (H–Phe–Leu–)$_2$[–NHCH(CH_2SMBzl)–NH–]·2TFA, 0.67 g (100%). This TFA salt (0.67 g, 0.70 mmol), Et_3N (0.20 ml, 1.40 mmol) and Boc–Tyr–D-Ala–Gly–OH (0.57 g, 1.40 mmol) were dissolved in DMF (8 ml). HOBt (0.23 g, 1.53 mmol) and EDC·HCl (0.29 g, 1.53 mmol) were added to this reaction mixture at −10 °C. The solution was allowed to warm up to the room temperature. After 2 d stirring, the solution was evaporated in vacuo, and the residue was solidified with ice-water. The solid collected was washed as described for compound **5**. The crude product was purified by gel filtration on a Sephadex LH-20 column (3.6×110 cm) eluted with DMF; yield, 0.67 g (63%); mp 218—219 °C; $[\alpha]_D^{20}$ −6.9° (*c* 1.0, NMP); $R_f{}^1$, 0.60; $R_f{}^5$, 0.35. Found: C, 59.76; H, 7.04; N, 10.93%. Calcd for $C_{79}H_{106}N_{12}O_{17}S·3H_2O$: C, 59.68; H, 7.19; N, 10.71%.

(H–Tyr–D-Ala–Gly–Phe–Leu–)$_2$[–NHCH(CH_2SMBzl)NH–] (3). Compound **8** (0.31 g, 0.20 mmol) was treated with TFA (5 ml) at 0 °C for 30 min. After evaporation, the residual oil was dissolved in 30% AcOH (2 ml) and subjected to gel filtration on a Sephadex G-25 column (2.0×110 cm) eluted with 30% AcOH. The fractions containing a pure product monitored by TLC were pooled and repeatedly lyophilized from aqueous AcOH; yield, 0.22 g (76%); mp 171—173 °C; $[\alpha]_D^{20}$ +15.5° (*c* 0.5, AcOH); $R_f{}^7$, 0.48; $R_f{}^9$, 0.89; R_{Lys}, 0.61. Amino acid ratios: Tyr, 0.97; Ala, 1.00; Gly, 1.03; Phe, 1.02; Leu, 0.98. Found: C, 57.36; H, 6.80; N, 11.25%. Calcd for $C_{68}H_{90}N_{12}O_{13}S·2AcOH·4H_2O$: C, 57.36; H, 7.09; N, 11.15%.

[(H–Tyr–D-Ala–Gly–Phe–Leu–)$_2$[–NHCH(CH_2S–)NH–]]$_2$ (1). Compound **3** (0.13 g, 0.09 mmol) was treated with anhydrous liquid HF (9 ml) in the presence of (Methylthio)benzene (0.5 ml), *p*-cresol (0.5 ml) and dimethyl sulfide (0.5 ml) for 1 h at 0 °C. After HF and dimethyl sulfide were evaporated, the residue was dissolved in 10% AcOH and the solution was washed with diethyl ether in order to completely remove any scavengers, such as (Methylthio)benzene and *p*-cresol. For oxidation, the aqueous solution was bubbled for 3 d at room temperature, using a water pump to pass through the air into the solution. After evaporation, oily residue dissolved in 30% AcOH was purified on a Sephadex G-25 column (2.0×110 cm) eluted with 30% AcOH. The fractions containing a pure product were pooled and lyophilized repeatedly from water; yield, 40 mg (34%); mp 223 °C (decomp); $[\alpha]_D^{20}$ −13.4° (*c* 0.5, AcOH); $R_f{}^7$, 0.33; $R_f{}^8$, 0.76; $R_f{}^9$, 0.82; R_{Lys}, 0.71; R.T., 19.7 min. Amino acid ratios: Tyr, 1.01; Ala, 1.05; Gly, 1.07; Phe, 1.01; Leu, 1.00. Found: C, 56.76; H, 6.71; N, 12.31%. Calcd for $C_{120}H_{162}N_{24}S_2·4AcOH·H_2O$: C, 56.92; H, 6.94; N, 12.45%.

(H–Tyr–D-Ala–Gly–Phe–Leu–)$_2$[–NHCH(CH_2SH)NH–] (2). Compound **1** (20 mg, 0.01 mmol) in 50% AcOH was added zinc dust (60 mg, 0.88 mmol); the mixture was then stirred for 10 h at room temperature. Zinc dust was removed by filtration through a bet of Hyflo Super-Cell and the filtrate was evaporated. The oily residue dissolved in 30% AcOH (1.5 ml) was applied on a column (2.0×110 cm) of Sephadex G-25 eluted with 30% AcOH. The fractions containing a pure product were pooled and lyophilized repeatedly from water; yield, 16 mg (63%); mp 183—186 °C; $[\alpha]_D^{20}$ +13.4° (*c* 0.5, AcOH); $R_f{}^7$, 0.53; $R_f{}^9$, 0.85; R_{Lys}, 0.62; R.T., 19.2 min. Amino acid ratios: Tyr, 1.04; Ala, 1.07; Gly, 1.09; Phe, 1.02; Leu, 1.00. Found: C, 56.58; H, 6.91; N, 12.49%. Calcd for $C_{60}H_{82}N_{12}O_{12}S·2AcOH·2.5H_2O$: C, 56.50; H, 7.04; N, 12.35%.

[Boc–Cys(S–)–NH_2]$_2$ (9). To a solution of (Boc–Cys–OH)$_2$ (1.45 g, 3.30 mmol) and NMM (0.72 ml, 6.56 mmol) dissolved in THF (8 ml), IBCF (0.85 ml, 6.56 mmol) was added at −10 °C. After stirring for 10 min, a mixture of aqueous ammonium and DMF (1:5 by volume, 20 ml) was added, and the stirring was continued for 3 h at room temperature. The solution was evaporated in vacuo, and ice-water was added to precipitate a solid. The solid collected was successively washed with 0.1 M $NaHCO_3$, 10% citric acid and H_2O and then recrystallized from DMF–$CHCl_3$–diethyl ether to afford compound

9; yield, 1.20 g (83%); mp 216—217 °C; $[\alpha]_D^{20}$ −134.3° (*c* 1.0, NMP); $R_f{}^1$, 0.75; $R_f{}^2$, 0.35. Found: C, 43.91; H, 6.98; N, 13.08%. Calcd for $C_{16}H_{30}N_4O_6S_2$: C, 43.82; H, 6.90; N, 12.78%.

[Boc–Phe–Leu–Cys(S–)–NH$_2$]$_2$ (10). Compound **9** (0.84 g, 1.93 mmol) was dissolved in TFA (10 ml) at 0 °C. After 30 min, the solution was evaporated to leave an oil, which was solidified by the addition of dry diethyl ether to yield (H–Cys–NH$_2$)$_2$·2TFA (0.79 g, 88%); $R_f{}^9$, 0.90; $R_f{}^{10}$, 0.95. To a solution of this TFA salt (0.79 g, 1.70 mmol), Et$_3$N (0.48 ml, 3.40 mmol) and Boc–Phe–Leu–OH (1.28 g, 3.40 mmol) in DMF (15 ml) were added HOBt (0.55 g, 4.07 mmol) and EDC·HCl (0.72 g, 3.73 mmol) at −10 °C. The reaction mixture was gradually allowed to warm up to the room temperature and stirred for 2 d. The solution was evaporated in vacuo, and ice-water was added to precipitate a solid. The collected solid was successively washed, as described for compound **5**. It was recrystallized from DMF–CHCl$_3$–diethyl ether to give compound **10**; yield, 1.31 g (81%); mp 214—215 °C; $[\alpha]_D^{20}$ −50.0° (*c* 1.0, NMP); $R_f{}^1$, 0.39; $R_f{}^3$, 0.50. Found: C, 56.90; H, 7.23; N, 11.61%. Calcd for $C_{46}H_{70}N_8O_{10}S_2$·0.5H$_2$O: C, 57.06; H, 7.39; N, 11.57%.

[Boc–Phe–Leu–NHCH(CH$_2$S–)NH$_2$]$_2$·2TFA (11). To a stirred solution of compound **10** (1.05 g, 1.10 mmol) in DMF–H$_2$O (1 : 1 by volume, 12 ml) was added BTIB (1.65 g, 4.39 mmol) at 0 °C under a nitrogen atmosphere. After 10 min, pyridine (0.71 ml, 8.76 mmol) was added, and the stirring was continued for 4 h. The solution was evaporated in vacuo, and the residue was solidified by addition of ice-water. The collected solid was washed with water and diethyl ether; yield, 1.18 g (95%); $R_f{}^3$, 0.37. This solid was used for the next reaction without further purification.

[(Boc–Phe–Leu–)$_2$[–NHCH(CH$_2$S–)NH–]]$_2$ (12). Compound **11** (1.18 g, 1.05 mmol), Et$_3$N (0.29 ml, 2.09 mmol) and Boc–Phe–Leu–OH (0.79 g, 2.09 mmol) were dissolved in DMF (10 ml). To this solution, HOBt (0.34 g, 2.51 mmol) and EDC·HCl (0.44 g, 2.30 mmol) were added at −10 °C and stirred. After 1 h, the reaction mixture was gradually allowed to warm up to the room temperature, and the stirring was continued for 3 d. The solution was evaporated in vacuo and the residue was solidified by the addition of ice-water. The solid collected was successively washed as described for compound **5**. The purification was carried out by gel filtration on a column (3.6×110 cm) of Sephadex LH-20 eluted with DMF. The fractions containing a pure product were collected to crystallize; yield, 0.68 g (40%); mp 244—245 °C; $[\alpha]_D^{20}$ −5.6° (*c* 1.0, NMP); $R_f{}^1$, 0.50; $R_f{}^4$, 0.32. Found: C, 61.36; H, 7.68; N, 10.42%. Calcd for $C_{84}H_{126}N_{12}O_{16}S_2$·H$_2$O: C, 61.44; H, 7.86; N, 10.24%.

[(Boc–Tyr–D-Ala–Gly–Phe–Leu–)$_2$[–NHCH(CH$_2$S–)NH–]]$_2$ (13). Compound **12** (0.56 g, 0.35 mmol) was dissolved in TFA (5 ml) at 0 °C. The reaction mixture was treated as described above, yielding [(H–Phe–Leu–)$_2$[–NHCH(CH$_2$S–)NH–]]$_2$·4TFA (0.58 g, 99%). This TFA salt (0.58 g, 0.35 mmol), Et$_3$N (0.21 ml, 1.39 mmol) and Boc–Tyr–D-Ala–Gly–OH (0.62 g, 1.39 mmol) were dissolved in DMF (8 ml), and then HOBt (0.23 g, 1.81 mmol) and EDC·HCl (0.32 g, 1.66 mmol) were added at −10 °C. The reaction mixture was treated as described for compound **5**. Purification was carried out of on a column (3.6×110 cm) of Sephadex LH-20 eluted with DMF; yield, 0.41 g (68%); mp 220—222 °C; $[\alpha]_D^{20}$ −2.8° (*c* 1.0, NMP); $R_f{}^1$, 0.60; $R_f{}^5$, 0.35; $R_f{}^6$, 0.51. Found: C, 59.76; H, 7.04; N, 10.93%. Calcd for $C_{79}H_{106}N_{12}O_{17}S_2$·5H$_2$O: C, 59.68; H, 7.19; N, 10.71%.

[(H–Tyr–D- Ala–Gly–Phe–Leu–)$_2$[–NHCH(CH$_2$S–)NH–]]$_2$ (1). Compound **13** (57 mg, 0.02 mmol) was treated as for compound **1**. Purification was performed by gel filtration on a column (2.0×110 cm) of Sephadex G-25 eluted with 30% AcOH; yield, 31 mg (58%); mp 220 °C (decomp); $[\alpha]_D^{20}$ −12.6° (*c*0.5, AcOH); $R_f{}^7$, 0.33; $R_f{}^8$, 0.76; $R_f{}^9$, 0.82; R_{Lys}, 0.71; R.T., 19.7 min.

Receptor Binding Assays. Receptor binding assays using rat brain membrane preparations and NG108-15 cells were carried out essentially as described previously.[16] [^{3}H]DADLE (40 Ci/mmol, New England Nuclear) and [^{3}H]-DAGO (38 Ci/mmol, New England Nuclear) were used as tracers specific for the δ and μ receptors, respectively, at a final concentration of 0.25 nM. Incubations were carried out for 60 min at 25 °C in 50 mM Tris-HCl buffer (pH 7.5) containing bestain (1 μM) and bacitracin (100 μg ml^{-1}) as enzyme inhibitors.

Dose-response curves were constructed utilizing seven to ten dose levels in duplicate. The results were analyzed by the computer program ALLFIT[17] and the data were used to construct least-squares estimates of the logistic curves relating the binding labeled ligand to concentrations of unlabeled ligand.

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11) The abbreviation according to biochemical nomenclature by IUPAC-IUB Joint Commission, *Eur. J. Biochem.*, **138**, 9 (1984), are used through out. Unless otherwise specified, the amino acids are L-compounds. Additional abbreviations are as follows: BTIB, bis[(trifluoroacetoxy)iodo]-benzene; DADLE, [D-Ala^2, D-Leu^5]enkephalin; DAGO, [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin; DMF, *N*,*N*-dimethylformamide; EDC·HCl, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; *gem*-Cys, geminal cysteine in which the carboxyl group of cysteine was converted into the amino group; HOBt, 1-hydroxybenzotriazole; IBCF, isobutyl chloroformate; MBzl, *p*-methoxybenzyl; NG108-15, neuroblastoma × glioma hybrid cells of specified cultured cell line denoted as 108-15; NMM, *N*-methylmorpholine; NMP, *N*-methylpyrrolidone; TFA, trifluoroacetic acid; and THF, tetrahydrofuran.

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