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CJ-15,208, a Novel Kappa Opioid Receptor Antagonist from a Fungus, *Ctenomyces serratus* ATCC15502

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A novel κ opioid receptor binding inhibitor CJ-15,208 (**I**) was isolated from the fermentation broth of a fungus, *Ctenomyces serratus* ATCC15502. The structure of **I** was determined to be a cyclic tetrapeptide consisting of one tryptophan, one D-proline, and two L-phenylalanine. Compound **I** was a selective binding inhibitor for the κ opioid receptor: 47 nM (IC_{50}) for κ, 260 nM for μ, and 2,600 nM for δ. In the electrically-stimulated twitch response assay of rabbit vas deferens **I** recovered the suppression by a κ agonist asimadoline with an ED_{50} of 1.3 μM, indicating that it is a κ antagonist.

Opioid peptides act in the central and peripheral nervous system to exert numerous physiological and pharmacological effects. They bind opioid receptors on cell membranes to produce those effects. Drugs with opioid receptor binding activity are therapeutically useful for the treatment of pain and intoxication. For example, morphine (μ receptor agonist) and naloxone (nonselective opioid receptor antagonist) are widely used as an analgesic and an antidote, respectively[1~4]. However, morphine has unwanted side effects such as tolerance, dependency or respiratory depression, and its usage is strictly limited. Naloxone is reported to have a relatively-short plasma half-life of 60~90 minutes[5]. In the circumstances there is a clear medical need for more efficacious drugs with fewer side effects. Considerable pharmacological, biochemical[6~9] and molecular biological[10~20] studies of opioid receptors have suggested that there are at least four major classes designated μ, δ, κ and opioid receptor-like 1 (ORL1). While morphine has a binding activity for μ receptor, drugs with selective binding activity for δ, κ or ORL1 receptors may be more efficacious and have fewer side effects.

In a screening program designed to discover κ receptor agonists/antagonists from microbial secondary metabolites, a fungus *Ctenomyces serratus*[21] ATCC15502 was found to produce a novel κ antagonist CJ-15,208 (**I**). In this paper, we describe the fermentation, isolation, structure elucidation and biological activities of **I**.

Results

Isolation

Compound **I** was detected by HPLC using an ODS column described in the experimental section, and the fractions that include compound **I** were collected in each step. The fermentation broth (10 liters) was filtered after the addition of EtOH (15 liters) (Fig. 1). The filtrate was concentrated to aqueous solution (2 liters) and extracted twice with the same volume of EtOAc. The extract was dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue (7.2 g) was applied to a Sephadex LH-20 column (25×500 mm, Amersham Pharmacia Biotech) with MeOH at a flow rate of 1 ml/minute (9 ml/fraction), and active fractions (#35 to 58) were combined and evaporated

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Fig. 1. Isolation scheme of CJ-15,208 (**I**).

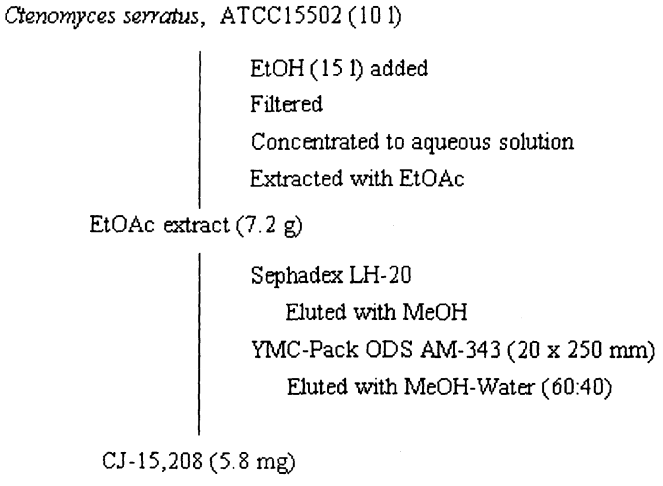


Table 1. Physico-chemical properties of CJ-15,208 (**I**).

	CJ-15,208
Appearance	White amorphous powder
$[\alpha]_D$ (24°C)	-64° (*c* 0.05, DMSO)
Molecular formula	$C_{34}H_{35}N_5O_4$
Molecular weight	577
HRFAB-MS (*m/z*)	
Found :	576.2658 $(M-H)^-$
Calcd. :	576.2613 (for $C_{34}H_{34}N_5O_4$)
UV λ_{max} (nm, MeOH)	End, 280
IR ν_{max} (cm^{-1}, KBr)	3290, 1694, 1665, 1601, 1516, 1451, 1230, 1105, 741, 696
Solubility	
Soluble:	MeOH, DMSO
Insoluble:	Hexane

in vacuo.

The active fractions (1.3 g) were applied to an ODS column (250 g) and eluted with 75% aqueous MeOH. Evaporated material (213 mg) was applied to preparative HPLC on an ODS column (YMC-pack ODS AM-343, 20×250 mm, YMC Co. Ltd.) with MeOH - H_2O (60 : 40) at a flow rate of 6 ml/minute (6 ml/fraction). The eluted peak (retention time: 45~55 minutes) was collected and concentrated to yield **1** (5.8 mg) as white amorphous powder.

Physico-chemical Properties

The physico-chemical properties of **I** are summarized in Table 1. Compound **I** was soluble in MeOH and DMSO, but insoluble in *n*-hexane. The IR spectrum showed intense N-H (3290 cm^{-1}) and amide carbonyl (1694 cm^{-1}) signals, which are characteristic of peptides. The UV spectrum showed the absorption maxima at end and 280 nm, suggesting the presence of the indole chromophore.

Structure Elucidation

The molecular formula of **I** was determined to be $C_{34}H_{35}N_5O_4$ [*m/z* found: 576.2658 $(M-H)^-$, calcd. 576.2613 for $C_{34}H_{34}N_5O_4$] by HRFAB-MS. The ^1H and ^{13}C NMR spectra showed 35 protons and 34 carbons, supporting the molecular formula. The carbon signals were classified into six $-CH_2-$, four –CH–, fifteen –CH=, five –C= and four carbonyl carbons by analysis of the DEPT spectra. The ^{13}C-^1H COSY experiment established the connectivity of the proton and carbon atoms (Table 2). The degree of unsaturation from the molecular formula was 20: ten sites of unsaturation were assigned to double bonds (twenty sp^2 carbons), four were assigned to four carbonyl groups and thus the remainder were designated to be due to the six rings of **I**. The extensive connectivity of **I** was established through the ^1H-^1H COSY and selective INEPT[22] experiments, which indicated the presence of four amino acids (proline, tryptophan and two phenylalanine) as shown in Fig. 2. The results of ^1H-^1H COSY and selective INEPT studies of each amino acid are as follows. (1) Proline: the presence of the pyrrolidine ring was derived from the proton sequence, H-2 (δ 5.49)–H-3 (δ 2.23)–H-4 (δ 2.02)–H-5 (δ 3.49), in the ^1H-^1H COSY spectrum and the long-range coupling from H-2 to C-5 (δ 48.86) in the selective INEPT spectrum. The H-2 also showed the long-range coupling to the amide carbonyl carbon, C-1 (δ 170.59). (2) Tryptophan: The ^1H-^1H COSY spectrum showed three proton sequences, H-29 (δ 8.89)–H-18 (δ 4.42)–H-19 (δ 3.06 and δ 2.66), H-21 (δ 7.03)–H-22 (δ 10.64) and H-24 (δ 7.39)–H-25 (δ 7.11)–H-26 (δ 7.01)–H-27 (δ 7.72). An allylic coupling between H-19 and H-21 indicated the attachment of C-19 (δ 28.24) to C-20 (δ 112.18). This connection is supported by the long-range coupling from H-18 to C-20 in the selective INEPT. The long-range couplings to the amide carbon, C-17 (δ 174.02), were observed from H-29, H-18 and H-19. (3) Phenylalanine: The presence of two phenylalanines was suggested by the ^1H-^1H COSY spectrum, which showed

Table 2. ^{1}H and ^{13}C NMR chemical shifts of CJ-15,208 (**I**).

No.	^{13}C (ppm)	^{1}H (ppm)
Pro		
1	170.59 s	
2	59.82 d	5.49 (1H, m)
3	33.63 t	2.23 (2H, m)
4	21.52 t	2.02 (2H, m)
5	48.86 t	3.49 (2H, dd, J = 3.52, 8.61 Hz)
Phe-1		
7	175.15 s	
8	60.97 d	4.10 (1H, dd, J = 8.64, 10.50 Hz)
9	36.92 t	3.07 (2H, m)
10	138.19 s	
11/15	130.25 d	7.08 (1H, d, J = 7.85 Hz)
12/14	129.23 d	7.22 (1H, t, J = 7.85 Hz)
13	127.45 d	7.12 (1H, m)
16		9.05 (1H, broad s)
Trp		
17	174.02 s	
18	58.20 d	4.42 (1H, dt, J = 3.50, 10.3 Hz)
19	28.24 t	3.06 (1H, m), 2.66 (1H, dd, J = 3.50, 10.30 Hz)
20	112.18 s	
21	124.32 d	7.03 (1H, broad s)
22		10.64 (1H, broad s)
23	137.65 s	
24	112.36 d	7.39 (1H, d, J = 7.83 Hz)
25	121.93 d	7.11 (1H, m)
26	119.44 d	7.01 (1H, m)
27	119.70 d	7.72 (1H, d, J = 7.83 Hz)
28	128.80 s	
29		8.89 (1H, d, J = 10.30 Hz)
Phe-2		
30	174.20 s	
31	59.46 d	4.84 (1H, m)
32	38.62 t	3.30 (1H, m), 3.03 (1H, m)
33	139.35 s	
34/38	130.83 d	7.66 (1H, d, J = 7.29 Hz)
35/37	129.10 d	7.29 (1H, t, J = 7.29 Hz)
36	127.45 d	7.25 (1H, m)
39		8.40 (1H, d, J = 9.50 Hz)

Solvent: a 1:1 mixture of acetone-d_6 and DMSO-d_6.
Internal standard: DMSO-d_6, δ 39.5 for ^{13}C and δ 2.50 for ^{1}H.

Fig. 2. ^{1}H-^{1}H COSY and selective INEPT experiments of CJ-15,208 (**I**).

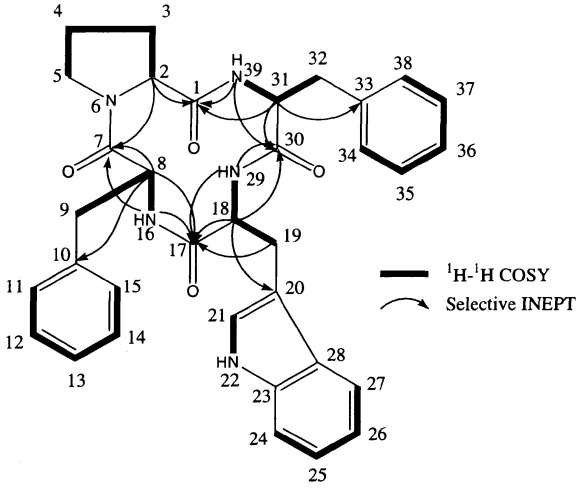
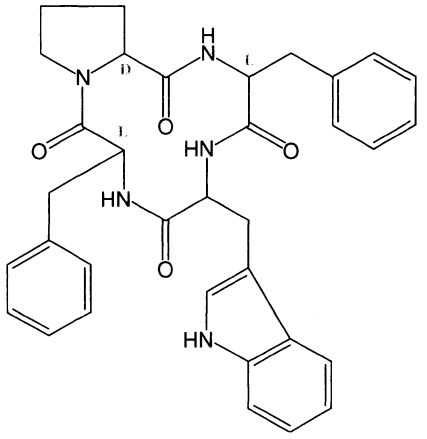


Fig. 3. Structure of CJ-15,208 (**I**).



two NH–α-methine–β-methylene sequences, H-16 (δ 9.05)–H-8 (δ 4.10)–H-9 (δ 3.07) and H-39 (δ 8.40)–H-31 (δ 4.84)–H-32 (δ 3.30/3.03), and two phenyl proton sequences, H-11/15 (δ 7.08)–H-12/14 (δ 7.22)–H-13 (δ 7.12) and H-34/38 (δ 7.66)–H-35/37 (δ 7.29)–H-36 (δ 7.25). The long-range couplings from H-16 and H-8 to the amide carbonyl carbon, C-7 (δ 175.15), were observed in the selective INEPT spectrum. The other long-range couplings from H-39 and H-31 to the amide carbonyl carbon, C-30 (δ 174.20), were also observed in the selective INEPT spectrum. Although six rings were required from the molecular formula and the number of sp^2 carbons, the confirmed four amino acids have five rings. Thus, the structure of **I** was determined to be *cyclic*. The cyclic amino acid sequence of **I** was determined by a selective INEPT study. The carbonyl carbon of tryptophan was determined to be C-17 by the long-range coupling from H-19 to C-17 in the selective INEPT spectrum. The long-range couplings from H-16 and H-8 to C-17 revealed the attachment of N-16 to C-17. The long-range couplings from H-2 to C-7, from H-39 and H-31 to C-1, and from H-29 and H-18 to C-30 were observed, respectively. These spectral data revealed the cyclic amino acid sequence, -Pro-Phe-Trp-Phe-. The absolute stereochemistry of the Pro and Phe of **I** was determined to be D and L by HPLC analysis of the hydrolysates of **1**, respectively. The absolute

Table 3. Binding inhibition of CJ-15,208 (**I**) for κ, μ and δ receptors.

Compound	IC_{50} (nM)		
	κ [^{3}H] CI-977	μ [^{3}H] DAMGO	δ [^{3}H] DPDPE
CJ-15,208 (**I**)	47	260	2,600
U-69,593 (κ agonist)	20	5,700	>10,000
SNC 80 (δ agonist)	>100	>100	6.4
Naloxone (opioid antagonist)	9.0	2.0	110

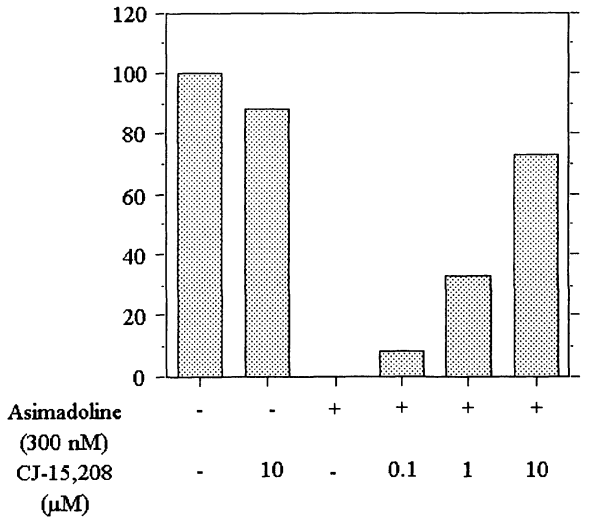
Bindings of [^{3}H] CI-977, [^{3}H] DAMGO and [^{3}H] DPDPE (1 nM) to Guinea pig brain membrane were performed in the presence of CJ-15,208 (**I**), U-69,593, SNC80 or naloxone. The binding inhibitory activity was measured as described in Experimental section.

stereochemistry of the Trp was not determined, because the Trp was not detected in the standard reaction conditions. Finally, the structure of **1** was determined to be *cyclo* (-D-Pro-L-Phe-Trp-L-Phe-) as shown in Fig. 3.

Biological Properties

Table 3 shows inhibitory effects of **I** on [^{3}H] opioid agonists binding to Guinea pig brain membrane. Known κ and δ agonists, U-69,593[23] and SNC 80[24,25], showed selective inhibition for κ and δ receptors, respectively. Naloxone, a nonselective opiate antagonist, inhibited binding of all three different ligands, as reported previously[2,8]. Compound **I** inhibited κ receptor binding preferably with an IC_{50} of 47 nM. Rabbit vas deferens is known to contain the κ receptor exclusively, and is useful to see if a κ selective binding inhibitor is a κ agonist or a κ antagonist[26]. Addition of 10 μM of **I** had no suppressive effect, indicating **I** is not an agonist (Fig. 4). On the other hand, **I** recovered the suppression with an ED_{50} of 1.3 μM when electrically-stimulated twitch responses of rabbit vas deferens was suppressed in advance by the κ agonist asimadoline[27]. The rabbit vas deferens exhibited contraction similar to the initial response after washing out all the compounds (data not shown). These data indicate that **I** is a selective κ antagonist without significant toxicity.

Fig. 4. Effect of CJ-15,208 (**I**) on twitch response of rabbit vas deferens evoked by electric field stimulation.



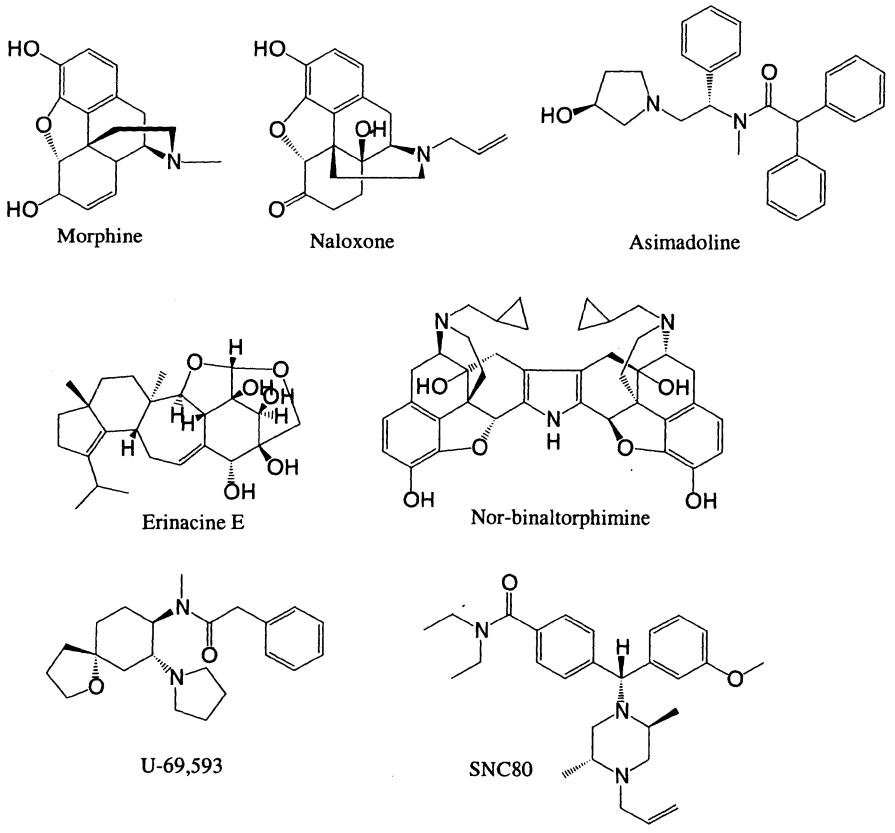
Vasa deferentia were isolated from Japanese white rabbits and placed in organ baths filled with Mg^{2+}-free Krebs solution. After equilibration, 10 μM of **I** was added and twitch response evoked by electric field stimulation was monitored. After complete suppression of contraction by asimadoline, **I** was cumulatively added up to 10 μM, The detail is described in the text.

Discussion

This is the first report on a cyclic peptide κ antagonist from microbial origin among several κ antagonists reported to date. Compound **I** consists of one tryptophan, one D-proline, and two L-phenylalanine residues, but there is no *cis-trans* isomerism observed in solution NMR studies in spite of the presence of the D-proline residue in the molecule.

These three kinds of amino acid are included in the endogenous κ ligand dynorphin A (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH),

Fig. 5. Structures of reference compounds.



but the significance of the configuration of proline is unclear. It is interesting to remember that tryptophan is not included in δ or μ ligands[1,2]. Structure-activity relationship (SAR) studies of dynorphin A and molecular simulation of its binding to κ receptor have been investigated[28,29], and basic residues like lysine-11 and arginine-7 were found to be important for the interaction. In the case of the well-known κ antagonist nor-binaltorphimine, a basic nitrogen is also reported to be indispensable for its antagonistic activity[30~33]. On the other hand, tryptophan-14 is reported to be necessary in the interaction between dynorphin and micelles[34]. In order to determine the exact binding requirements for the selectivity among opioid receptors, cyclic dynorphin analogs with selective κ agonistic activity have been synthesized[35]. However, they are still large peptides with 11 amino acid residues so that there is no evidence about the specific binding requirements at the κ receptor. Since **I** shows potent and selective activity to the κ receptor despite the small peptide (tetrapeptide) with no basic amino acid residue, further SAR studies of **I** may offer information for the minimum requirements of the interaction. In fact, *Ctenomyces serratus* ATCC15502 was found to produce several analogs with very low titers (data not shown) in addition to **I**, and these analogs may help understanding of the interaction.

In general, cyclic peptides seem to have desirable pharmacological profiles rather than linear peptides. For example, cyclic prodrugs of the opioid peptides [Leu^5]-enkephalin (Tyr-Gly-Gly-Phe-Leu-OH) and DADLE (Tyr-D-Ala-Gly-Phe-D-Leu-OH) using phenylpropionic acid, coumarinic acid or (acyloxy)alkoxy linkers have been shown to have better metabolic stability and transcellular permeation properties[36~38]. Compound **I** naturally has a cyclic skeleton, and it may be useful not only as a pharmacological tool but also in the treatment of detoxication of narcotics dependency.

We have previously reported that erinacine E isolated from a basidiomycete *Hericium ramosum* is a kappa agonist[39]. Discovery of both kappa agonist and antagonist implies that natural products are still fruitful sources for unprecedented chemical structures as morphine was discovered from a plant.

Experimental

General

Spectral and physico-chemical data were obtained by the following instruments: UV, JASCO Ubest-30; IR, Shimazu IR-470; NMR, JEOL JNM-GX270 updated with an LSI-11/73 host computer, TH-5 tunable probe and version 1.6 software; FAB-MS, JEOL JMS-700; optical rotations, JASCO DIP-370 with a 5 cm cell.

Producing Microorganism

The producing strain, the fungus *Ctenomyces serratus* ATCC15502 was obtained from the American Type Culture Collection (ATCC). It was deposited under the accession number FERM BP-5731 to National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology Tsukuba, Ibaraki, Japan. The taxonomical properties of this strain have been reported[21], describing that this strain is an ascomycete *Ctenomyces serratus*.

Fermentation

Ctenomyces serratus ATCC15502 was maintained on potato dextrose agar slant (Difco). A vegetative cell suspension from the slant was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of the seed medium (potato dextrose broth 2.4%, yeast extract 0.5% and agar 0.1%). The flask was shaken at 26°C for 4 days on a rotary shaker (7-cm throw at 210 rpm). Five-ml aliquots were inoculated into five 500-ml Erlenmeyer flasks containing 100 ml of the seed medium, and shaken at 26°C for 4 days. The second seed cultures were inoculated into one hundred 500-ml flasks containing the production medium (glucose 1%, glycerol 3%, peptone 0.5%, NaCl 0.2% and agar 0.1%, pH 7.0) and 20 g wheat bran. Incubation was carried out at 26°C for 10 days.

HPLC Analysis

HPLC analysis was performed on a Hewlett Packard HP1090 system. Samples were subjected to an ODS column (YMC-pack ODS AM-312, 6.0×150 mm, YMC Co. Ltd.) maintained at 42°C and eluted with MeOH - H_2O (60 : 40) at a flow rate of 0.8 ml/minute. Compound **I** was monitored by absorbance at 220 nm. Under these conditions, **I** was eluted at the retention time of 12.6 minutes.

Amino Acid Analysis

Acid hydrolysis of **I** was performed with 6 N-HCl aq at 110°C for 6 hours. The reaction mixture was dried, re-dissolved in fresh water and analyzed by Hitach amino acid analysis system, L-8500. The detected amino acids were 1.0 : 0.6 mol equivalent of Phe and Pro, respectively.

A part of the hydrolysis mixture was reacted with (+)-1-(9-fluorenyl)ethyl chloroformate to make diastereomeric amino acid and analyzed with the HPLC conditions as described in the literature[40] to determine stereochemistries of the amino acid. The analysis revealed that the hydrolysis mixture contains 1.0 : 1.0 mol equivalent of L-Phe and D-Pro, respectively.

Preparation of Brain Membranes

The Guinea pig whole brains obtained from Japan SLC, Inc. (Shizuoka, Japan) were rinsed in ice-cold phosphate-buffered saline and briefly homogenized in homogenization buffer [50 mM HEPES - 50 mM Tris-HCl (pH 7.5) containing 0.24 M sucrose, 5 mM $MgCl_2$, 2 mM ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 100 μM phenylmethylsulfonyl fluoride (PMSF) and 50 μM leupeptin]. The homogenate was centrifuged at 3,000×*g* at 4°C for 10 minutes. The supernatant was recentrifuged at 20,000×*g* at 4°C for 30 minutes. The pellet (membrane fraction) obtained was suspended in the homogenization buffer. The membrane fractions were stored at −80°C until use.

Opioid Receptor Binding Assay

Assays were performed in 96-well microtiter plates containing a 200 μl-aliquot of reaction mixture per well consisting of 40 mM HEPES - 40 mM Tris - HCl, pH 7.5, 0.192 M sucrose, 4 mM $MgCl_2$, 1.6 mM EGTA, 80 μM PMSF, 40 μM leupeptin, Guinea pig brain membrane fraction and 1 nM of radiolabeled ligand. [^{3}H]CI-977, [D-ala^2, *N*-methyl-phe^4, glyol^5][tyrosyl-3,5-^{3}H]enkephalin (DAGO, both from Amersham Pharmacia Biotech) and [D-pen^{2,5}][tyrosyl-2,6-^{3}H]enkephalin (DPDPE, from DUPONT) were used for κ, μ and δ opioid receptor binding assays, respectively. After incubation at room temperature with shaking for 30 minutes, the reaction was terminated by harvesting the reaction mixture onto a polyethyleneimine - soaked glassfilter (Filtermat A, Wallac). After drying, the radioactivity of the glassfilter was measured in a scintillation counter.

Rabbit Vas Deferens Twitch Response Assay

The antagonist activity was determined by the electric field stimulation-induced twitch response method using Japanese white rabbit vas deferens[26]. Briefly, vasa deferentia were isolated from rabbits sacrified by over-dosing of pentobarbital and they were placed in organ baths filled with Mg^{2+}-free Krebs solution. The preparation was

suspended through two platinum ring electrodes. After an equilibration period (60 minutes) in order to check the tissue responses, twitch response was evoked by electric field stimulation. After stimulation, the medium was changed, and 30 minutes later twitch response was reevoked by the same method. When the maximal tension of each response reached a plateau, 10 μM of **I** was added to see if it had an agonistic property. After that, a κ agonist asimadoline (10 μM) was added to suppress contraction, and the cumulative dosing of **I** was begun. To analyze the data in each preparation, the maximum tension was defined as 100%, and tension decreased by each concentration of compound was expressed as a relative tension. Altered tension was identified in the plateau phase after the treatment of test compound in a various concentration.

Acknowledgments

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