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Erinacine E as a Kappa Opioid Receptor Agonist and Its New Analogs from a Basidiomycete, *Hericium ramosum*

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A κ opioid receptor binding inhibitor was isolated from the fermentation broth of a basidiomycete, *Hericium ramosum* CL24240 and identified as erinacine E (**1**). Three analogs of **1** were produced by fermentation in other media and by microbial biotransformation. Of these compounds, **1** was shown to be the most potent binding inhibitor. Preliminary SAR studies of these compounds indicated that all functional groups and side chains were required for the activity. Compound **1** was a highly-selective binding inhibitor for the κ opioid receptor: 0.8 μM (IC_{50}) for κ, >200 μM for μ, and >200 μM for δ opioid receptor. Compound **1** suppressed electrically-stimulated twitch responses of rabbit vas deferens with an ED_{50} of 14 μM. The suppression was recovered by adding a selective κ opioid receptor antagonist nor-binaltorphimine, indicating that **1** is a κ opioid receptor agonist.

Opioid compounds have various physiological effects such as antinociception, neurotransmitter release, respiratory depression and addiction[1]. These compounds exert their effects by binding to specific membrane-bound receptors located throughout central and peripheral nervous systems[2]. The presence of at least three types of opioid receptors (μ, δ and κ) has been reported on the basis of their differences in apparent affinity for opioid ligands[3~6] and more recently of DNA sequences of all three receptors[7~15]. Because a major physiological effect attributed to the opioid system is analgesia[16,17], opioid drugs are important therapeutic agents in the clinical management of pain. Currently, all clinically important opioid drugs act through the μ receptor to induce analgesia. Morphine, for example, is a widely-used analgesic that also causes untoward side effects such as tolerance, dependence and respiratory depression. There is evidence to suggest that κ opioid receptors are present on the peripheral terminals of primary afferent neurons[18] and that activation of these receptors reduces hyperalgesia in a rat model of inflammation. Thus, κ agonists may exhibit antinociceptive activity without adverse side effects observed with μ receptor agonists.

In a screening program designed to discover κ receptor agonists from microbial secondary metabolites, the basidiomycete *Hericium ramosum* CL24240 was found to produce a κ receptor binding inhibitor which was identified as erinacine E (**1**)[19~21]. In order to generate new analogs of **1**, we have applied various media and biotransformation. In this paper, we describe κ opioid receptor agonistic activity of **1** in addition to isolation, structure elucidation and the binding inhibitory activities of new analogs.

Results

Generation of New Erinacine E analogs

A basidiomycete *Hericium ramosum* CL24240 in Medium-2 was found to produce a κ receptor binding

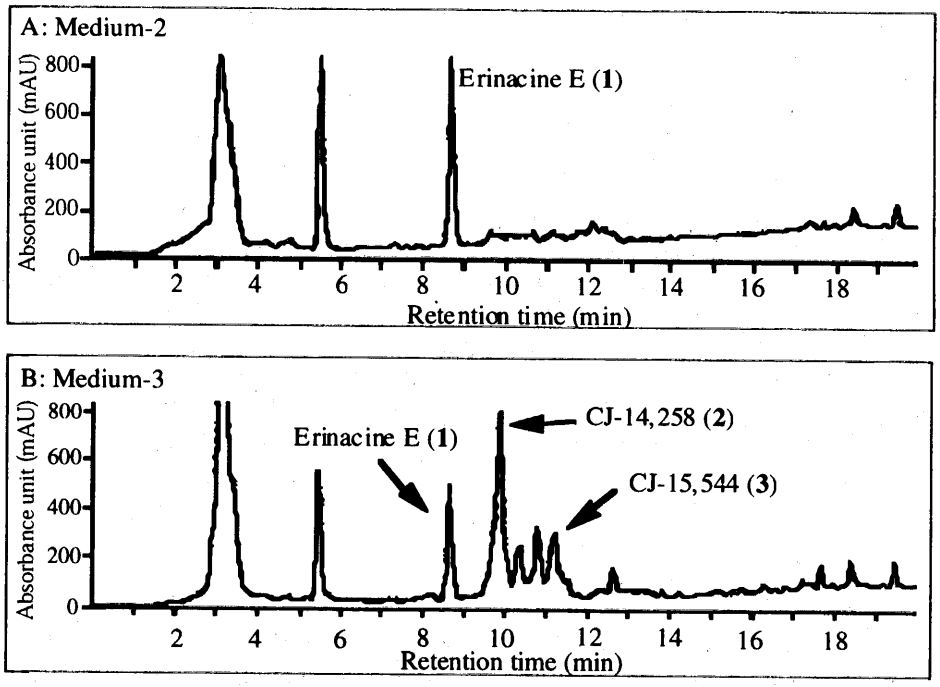
inhibitor which was identified as erinacine E (**1**) based on data from EI-MS, ^{1}H NMR, ^{13}C NMR, COSY, NOESY, C-H COSY, COLOC, Selective INEPT, NOE Difference and single crystal X-ray diffraction[19]. In order to generate new analogs, *Hericium ramosum* CL24240 was fermented in 104 different media. Out of them, when fermented in Medium-3, this strain was found to produce two new analogs, CJ-14,258 (**2**) and CJ-15,544 (**3**) (Fig. 1), with similar UV spectrum as **1**.

Biotransformation of **1** was examined by using 81 microorganisms. One of them, *Caldariomyces fumago* ATCC 16373 was found to transform **1** to a new analog, CP-412,065 (**4**) at the conversion rate of 29% (Fig. 2).

Physico-chemical Properties

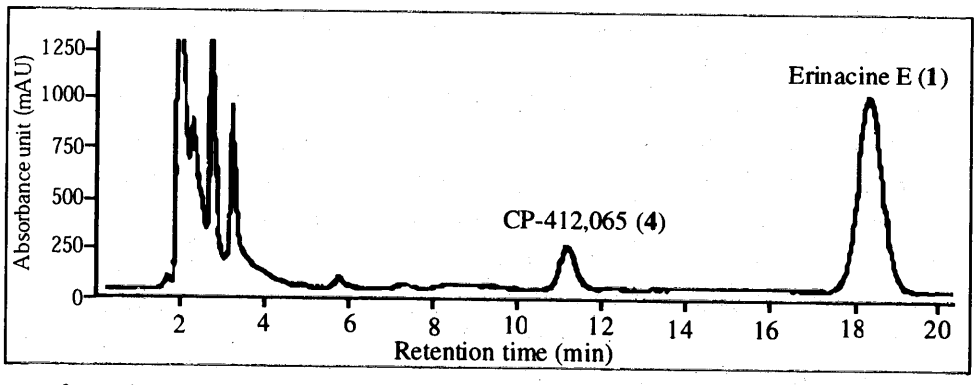
The physico-chemical properties of **2**~**4** are summarized in Table 1. They were obtained as white powders and were soluble in MeOH and acetone, but insoluble in H_2O and *n*-hexane. The UV spectra of **2** and **3** showed end absorption, whereas the spectrum of **4** showed

Fig. 1. HPLC profiles of ethanol extracts from fermentation broths.



Hericium ramosum CL24240 was fermented in each medium, and ethanol extracts from each fermentation broth were analyzed using a C18 reverse phase HPLC column as described in the Experimental section. Erinacine E (**1**) and its analogs were monitored by absorbance at 210 nm.

Fig. 2. HPLC profile of a biotransformation product generated by *Caldariomyces fumago* ATCC 16373.



Biotransformation was carried out in the presence of erinacine E (**1**), and the product was analyzed on a C18 reverse phase HPLC column as described in the Experimental section. Erinacine E (**1**) and its analog were monitored by absorbance at 215 nm.

Table 1. Physico-chemical properties of CJ-14,258 (**2**), CJ-15,544 (**3**) and CP-412,065 (**4**).

	CJ-14,258 (**2**)	CJ-15,544 (**3**)	CP-412,065 (**4**)
Appearance	White powder	White powder	White powder
Formula	$C_{25}H_{38}O_6$	$C_{25}H_{34}O_6$	$C_{25}H_{34}O_6$
HRFAB-MS	(negative)	(negative)	(positive)
Calcd.	433.2590 (for $C_{25}H_{37}O_6$)	429.2278 (for $C_{25}H_{33}O_6$)	431.2430 (for $C_{25}H_{35}O_6$)
Found:	433.2587	429.2286	431.2390
$[\alpha]_D$ (25°C, MeOH)	−73.4 (*c* 0.74)	−78.6 (*c* 0.056)	Not measured
UV λ_{max} (MeOH, nm)	End	End	256 (ε 5714)
IR ν_{max} (KBr) cm^{-1}	3420, 2930, 1458, 1086	3396, 2930, 1727, 1450, 1059	Not measured

Table 2. ^1H and ^{13}C NMR assignments of CJ-14,258 (**2**), CJ-15,544 (**3**) and CP-412,065 (**4**) in CD_3OD.

Position	**2**		**3**		**4**
	^{13}C (ppm)[a]	^1H (ppm)[b]	^{13}C (ppm)[a]	^1H (ppm)[b]	^1H (ppm)[b]
1	40.2 t	1.08 (1H, m)	40.9 t	1.55 (1H, m)	6.28 (1H, d, *J*=5.5 Hz)
		1.60 (1H, m)		1.65 (1H, m)	
2	29.8 t	2.30 (2H, m)	30.1 t	2.31 (2H, m)	6.32 (1H, d, *J*=5.5 Hz)
3	140.2 s		141.3 s		
4	141.2 s		139.1 s		
5	49.3 d	2.35 (1H, m)	45.2 d	2.84 (1H, m)	2.95 (1H, d, *J*=12.0 Hz)
6	42.5 s		43.0 s		
7	30.1 t	1.50 (1H, m)	29.2 t	1.40 (1H, m)	1.19 (1H, m)
		1.60 (1H, m)		1.70 (1H, m)	1.73 (1H, m)
8	38.6 t	1.50 (2H, m)	38.8 t	1.55 (2H, m)	1.33 (2H, m)
9	51.5 s		51.6 s		
10	27.3 t	1.80 (1H, m)	32.6 t	2.65 (1H, m)	2.63 (1H, m)
		1.90 (1H, m)		2.75 (1H, m)	2.78 (1H, m)
11	37.8 t	1.00 (1H, m)	126.9 d	5.79 (1H, m)	5.67 (1H, m)
		2.20 (1H, m)			
12	46.5 d	1.70 (1H, m)	140.3 s		
13	48.0 d	1.97 (1H, dd, *J*=12.7, 8.6 Hz)	52.1 d	3.29 (1H, m)	3.24 (1H, bs)
14	95.5 d	3.99 (1H, d, *J*=8.4 Hz)	99.7 d	4.42 (1H, d, *J*=6.2 Hz)	3.93 (1H, s)
15	98.8 d	4.81 (1H, d, *J*=8.1 Hz)	80.8 d	4.39 (1H, bs)	4.72 (1H, bs)
16	20.4 q	0.99 (3H, s)	18.2 q	0.97 (3H, s)	1.04 (3H, s)
17	26.3 q	1.08 (3H, s)	25.8 q	1.06 (3H, s)	0.77 (3H, s)
18	29.2 d	2.80 (1H, heptet, *J*=6.8 Hz)	29.0 d	2.95 (1H, heptet, *J*=6.8 Hz)	2.98 (1H, heptet, *J*=7.0 Hz)
19	22.5 q	1.00 (3H, d, *J*=6.8 Hz)	22.9 q	1.00 (3H, d, *J*=6.8 Hz)	1.09 (3H, d, *J*=7.0 Hz)
20	23.1 q	1.00 (3H, d, *J*=6.8 Hz)	22.9 q	1.02 (3H, d, *J*=6.8 Hz)	1.10 (3H, d, *J*=7.0 Hz)
1′	109.3 d	4.90 (3H, s)	111.9 d	5.16 (1H, s)	4.96 (1H, s)
2′	82.2 s		86.3 s		
3′	87.0 d	3.80 (1H, d, *J*=9.2 Hz)	208.5 s		4.29 (1H, d, *J*=6.3 Hz)
4′	70.3 d	4.15 (1H, ddd, *J*=10.5, 9.2, 5.5 Hz)	81.3 s		
5′	66.4 t	3.12 (1H, dd, *J*=11.5, 10.5 Hz)	68.7 t	3.41 (1H, d, *J*=11.6 Hz)	3.26 (1H, d, *J*=12.0 Hz)
		3.79 (1H, dd, *J*=11.5, 5.5 Hz)		4.27 (1H, d, *J*=11.6 Hz)	3.94 (1H, d, *J*=12.0 Hz)

[a] Chemical shifts are shown with reference to CD_3OD as 49.8 ppm.
[b] Chemical shifts are shown with reference to CD_3OD as 3.30 ppm.

maximum absorption at 256 nm, suggesting the presence of a *cis*-diene moiety in the structure. The IR absorption bands at about 3400 cm^{-1} of **2** and **3** implied the presence of a hydroxy group. The presence of a ketone in **3** was assumed by the IR absorption at 1727 cm^{-1}.

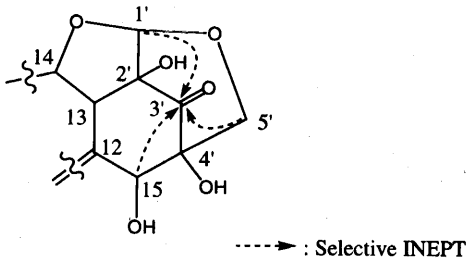
Structure Elucidation

Structure Elucidation of **3** and **4**

The structures of **3** and **4** were determined by comparison of their spectral properties with those of **1**.

The HRFAB-MS of **3** gave a parent ion peak at *m/z* 429.2286 [(M−H)$^-$; calcd. for $C_{25}H_{33}O_6$, 429.2278], indicating the loss of one mole of hydrogen from **1**. The ^{1}H and ^{13}C NMR spectra (Table 2) showed that loss of the oxymethine proton and carbon signals for the 3′ position of **1** occurred together with addition of a carbonyl carbon signal (δ 208.5). These results indicated that **3** was the keto derivative at 3′ position of **1**. The position of the ketone moiety was also suggested by the observation of the long range couplings from H-15 (δ 4.39), H-1′ (δ 5.16) and H-5′ (δ 4.27) to C-3′ in the Selective INEPT spectra (Fig. 3). Thus, the structure of **3** was determined as shown in Fig. 4. The ^{1}H NMR spectrum of **4** was similar to that of **1** except for the presence of signals for two protons H-1 (δ 6.28) and H-2 (δ 6.32) in place of two methylene proton signals of H-1 and H-2 for **1**. The LRFAB-MS of 4 showed a molecular ion peak at *m/z* 431, indicating the dehydrogenation of **1**. The UV spectrum (λ_{max} 256 nm) of **4** indicated the presence of conjugated *cis*-diene. Taken together, the structure of **4** was determined as the 1,2-dehydrogenated derivative of **1** as shown in Fig. 4.

Fig. 3. Selective INEPT experiment of CJ-15,544 (**3**).



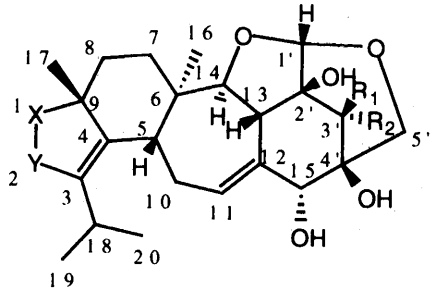
Structure Elucidation of **2**

The spectral data of **2** obtained from HRFAB-MS ($C_{25}H_{38}O_6$) and NMR (Table 2) experiments suggested that its structure was similar to that of striatin A (**5**)[22]. The C∼F rings of **2** was established through the interpretation of ^{1}H-^{1}H COSY, COLOC and Selective INEPT spectra. ^{1}H-^{1}H COSY showed the two partial structures, I [–CH–CH$_2$–CH$_2$–CH(–CH–O–)–CH–CH(–O–)–] and II [–CH(–O–)–CH(–O–)–CH$_2$–O–] as shown in Fig. 5. In the partial structure I, the long range couplings were observed from H-10 (δ 1.80 and 1.90) and H-13 (δ 1.97) to C-6 (δ 42.5) in Selective INEPT. Therefore, the seven membered ring C was suggested. The D ring was established by the long range couplings from H-1′ (δ 4.90) to C-13 (δ 48.0) and from H-14 (δ 3.99) to C-2′ (δ 82.2) in the COLOC and from H-13 (δ 1.97) to C-2′ and from H-1′ to C-14 (δ 95.5) and C-2′ in the Selective INEPT. The long range couplings from H-3′ (δ 3.80) to C-13 (δ 48.0), C-15 (δ 98.8) and C-2′ (δ 82.2) in Selective INEPT indicated the six membered ring E. Finally, the long range couplings from H-3′ and H-5′ (δ 3.12 and 3.79) to C-1′ (δ 109.3) in the COLOC and from H-3′ to C-2′ in the Selective INEPT revealed the F ring. The NOE experiments of **2** suggested the same relative configuration as striatin A except for the hydroxy group at 15 position (Fig. 6).

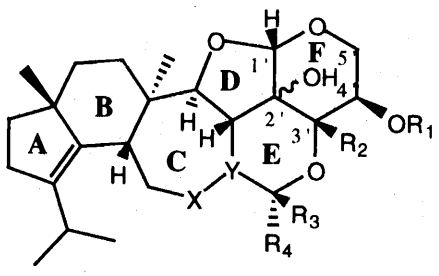
Biological Properties

Table 3 shows inhibitory effects of **1** and its new analogs **2**, **3**, **4** on [^{3}H] opioid agonists binding to Guinea pig brain membrane. Naloxone, a nonselective opiate antagonist, inhibited bindings of all three different ligands as reported previously[2,5]. Compound **1** was the most potent and selective binding inhibitor for κ receptor.

Fig. 4. Structures of erinacines **1**∼**4** and striatin A (**5**).



	R_1	R_2	X–Y
Erinacine E (**1**)	OH	H	CH_2–CH_2
CJ-15,544 (**3**)	=O		CH_2–CH_2
CP-412,065 (**4**)	OH	H	CH=CH



	R_1	R_2	R_3	R_4	X–Y
Striatin A (**5**)	Ac	OH	OMe	H	CH=C
CJ-14,258 (**2**)	H	H	H	OH	CH_2–CH

Fig. 5. ^{1}H-^{1}H COSY, COLOC and selective INEPT experiments of CJ-14,258 (**2**).

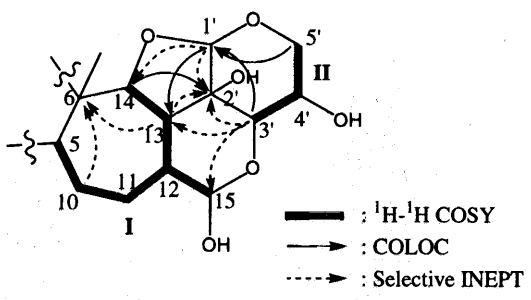


Fig. 6. NOE experiment of CJ-14,258 (**2**).

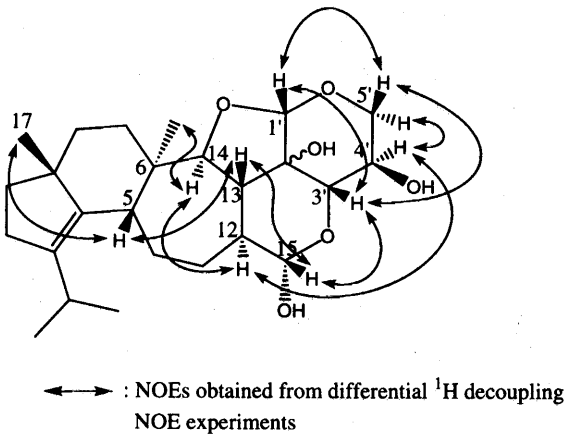


Table 3. Binding inhibitory activities of erinacine E (**1**), its related compounds and naloxone.

	IC_{50} (μM)		
	Kappa [^{3}H] CI-977	Mu [^{3}H] DAGO	Delta [^{3}H] DPDPE
Erinacine E (**1**)	0.80	>200	>200
CJ-14,258 (**2**)	32	>200	>200
CJ-15,544 (**3**)	4.5	>200	>200
CP-412,065 (**4**)	>200	>200	>200
Naloxone	0.0090	0.0020	0.11

Bindings of [^{3}H] CI-977, [^{3}H] DAGO, [^{3}H] DPDPE (1 nM) to Guinea pig brain membrane were performed in the presence of erinacine E (**1**), its related compounds (**2**~**4**) or naloxone. The binding inhibitory activity was measured as described in Experimental section.

Compound **1** also suppressed the electrically-stimulated contraction of rabbit vas deferens in a dose-dependent manner with an ED_{50} of 14 μM[23] (Fig. 7). Norbinaltorphimine, a known selective κ antagonist[24~26] (30 nM), recovered the suppression caused by **1** (Fig. 8), indicating that **1** is a selective agonist for the κ receptor. The rabbit vas deferens exhibited contraction similar to the initial response after washing out all the compounds, meaning that **1** doesn't have significant toxicity to the tissue.

Discussion

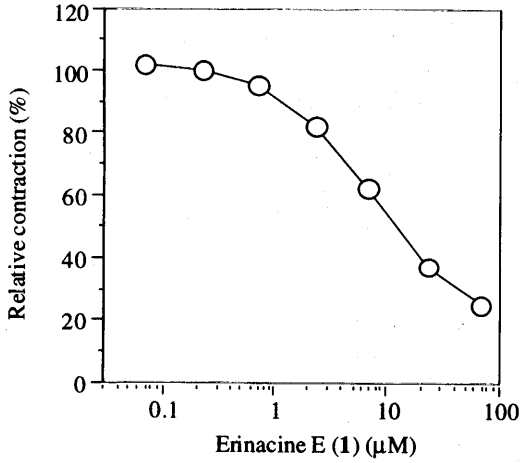
Erinacine E produced by *Hericium erinaceum* has been reported to be a stimulator of nerve growth factor (NGF) synthesis[19]. In this study, erinacine E (**1**) from a *Hericium ramosum* CL24240 was found to be also a κ receptor agonist. Preliminary SAR of the isolated analogs indicated that disruption of any moieties of erinacine E (**1**) results in remarkable loss of the original activity. Especially, A-ring moiety seems to be most important, because the analog modified in this moiety, CP-412,065, showed complete loss of the activity.

Experimental

General

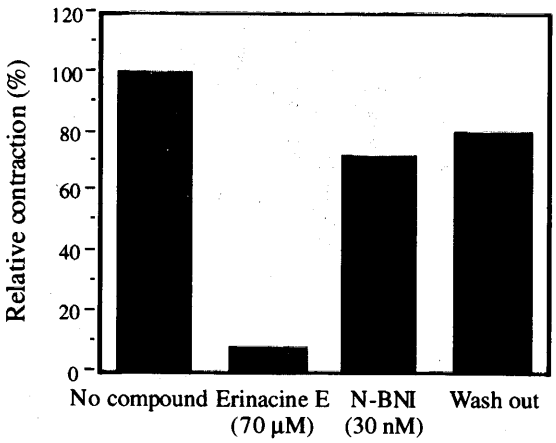
Spectral and physico-chemical data were obtained on the following instruments. For **2** and **3**: UV, JASCO Ubest-30; IR, Shimadzu IR-470; NMR, JEOL JNM-GX270 updated with an LSI-11/73 host computer, TH-5 tunable probe and version 1.6 software; HRFAB-MS, KRATOS model IS; Optical rotations, JASCO DIP-370 with a 10 cm cell. For **4**: HRFAB-MS, VG Analytical ZAB 2 SE high field mass spectrometer; NMR, Bruker 300 mHz spectrometer.

Fig. 7. Effect of erinacine E (**1**) 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, erinacine E (**1**) was cumulatively added up to 70 μM, and twitch response evoked by electric field stimulation was monitored. The detail was described in the text.

Fig. 8. Effect of nor-binaltorphimine (N-BNI) on suppression of the contraction caused by erinacine E (**1**).



N-BNI (30 nM) was added after complete suppression of twitch response caused by erinacine E (**1**). To check the damage on vas deferens, twitch response was also monitored after washing out all the compounds. The detail was described in the text.

Producing Strain

The producing strain, the basidiomycete *Hericium ramosum* CL24240, was obtained from Virginia Polytech, U.S.A. The taxonomical properties of the basidiomycete have been reported by D. HALL and D. E. STUNTZ[27].

Isolation of Erinacine E (1)

Hericium ramosum CL24240 was maintained on plates of malt agar medium (malt extract 2.5% and agar 1.5%) for 10~21 days. The cell suspension from the plates (in 2 ml sterile H_2O) was used to inoculate two 500-ml flasks containing 100 ml of Medium-1 (glucose 2%, malt extract 2%, yeast extract 0.18%, maltose 0.24% and agar 0.1%, pH 5.4~5.6). After incubation at 28°C for 7 days, 5 ml aliquots were inoculated into twenty 500-ml flasks containing 100 ml of Medium-2 (glucose 2%, malt extract 2%, yeast extract 0.18%, maltose 0.24% and wheat bran 2%, pH 5.4~5.6). Incubation was carried out at 28°C for 14 days. Inhibitory activity of κ opioid receptor binding was monitored by the inhibition of [^{3}H] CI-977 binding to Guinea pig brain membranes. Fermentation broth (1 liter) was treated with 1 volume of 70% aqueous EtOH for 4~6 hours and filtered with the aid of celite. This filtrate was concentrated to aqueous solution and then extracted with EtOAc (2 liters × 2). The extract was concentrated *in vacuo* to give a crude residue (190 mg). This residue was applied to a silica gel column (20 ml) packed in CH_2Cl_2. The activity was recovered in the CH_2Cl_2-MeOH (9:1) fractions. The fractions showing the activity were pooled and chromatographed on a Sephadex LH-20 (Pharmacia) column (25 × 500 mm) equilibrated and eluted with MeOH. The fractions showing the inhibitory activity were collected to yield **1** (30 mg).

HPLC Analysis

In order to generate new analogs, *Hericium ramosum* CL24240 was fermented in 104 media (10 ml in 25-ml tube) in the manner described above. Each fermentation broth was treated with 10 ml of EtOH followed by centrifugation at 3,000 rpm for 5 minutes. One ml aliquots of the supernatants were concentrated to dryness under nitrogen gas and then reconstituted in MeOH. HPLC analyses were performed on a Hewlett Packard HP1090 system. Each sample was loaded on a C18 reverse phase column (YMC Pack ODS AM 6 × 50 mm) and eluted with a linear gradient of MeOH–H_2O from 7:3 to 10:0 (v/v) at a flow rate of 0.8 ml/minute in 20 minutes. Erinacine E analogs were monitored by absorbance at 210 nm.

For analysis of biotransformed products, each sample was loaded on a Puresil C18 reverse phase column (Waters, 4.6 × 150) and eluted isocratically at 1.0 ml/minute with MeOH-H_2O (7:3). Biotransformation

products were monitored at 215 nm.

Isolation of CJ-14,258 (2) and CJ-15,544 (3)

The seed culture (5 ml) prepared in Medium-1 was transferred to five 500-ml flasks containing 100 ml of Medium-3 (glucose 1%, glycerol 3%, peptone 0.5%, NaCl 0.2%, agar 0.1% and wheat bran 2%, pH 7.0). After incubation at 28°C for 14 days, the fermentation broth was treated with 70% aqueous EtOH, filtered and extracted with EtOAc as described above. The crude extract (1 g) was applied to a reverse phase column (YMC Pack ODS AM 20×250 mm) and eluted with 82% aqueous MeOH at a flow rate of 8 ml/minute. Twenty mg of **2** and 3.7 mg of **3** were obtained.

Biotransformation

Cultures of *C. fumago* ATCC 16373 were maintained on slants of potato, dextrose, yeast extract agar (ATCC medium 337), and grown in Iowa medium (glucose 2%, NaCl 0.5%, K_2HPO_4 0.5%, yeast extract 0.5%, soybean meal 0.5%, pH 7.0)[28]. These cultures were inoculated with a loopful of inoculum from a slant culture and then grown in 300-ml flasks containing 25 ml of Iowa medium on a G-53 gyrotary shaker (New Brunswick Scientific, Edison, NJ) at 28°C and 220 rpm agitation for 24 hours. Biotransformation was carried out in steel-capped 16×125 mm glass tubes containing 2.5 ml of Iowa medium inoculated with 0.25 ml of the culture. Forty tubes containing the culture were incubated for 48 hours under the conditions described above and then treated with 0.25 mg of **1** per tube (25 μl of 10 mg/ml solution in dimethylsulfoxide). The tubes were incubated for 13 days under the same condition after addition of **1**.

Isolation of CP-412,065 (4)

Broths (100 ml) from the 40 tubes were pooled and extracted twice with 100 ml of EtOAc. The EtOAc extracts were concentrated by rotary evaporation under reduced pressure. The concentrated extract was applied on a 20×150 mm column of 40 μm octadecyl prep LC packing material (Bakerbond) and eluted with a stepwise gradient of MeOH and H_2O. Fractions containing a transformed product were concentrated by rotary evaporation and subjected to a C18 column at the same conditions as HPLC analysis. Fractions containing the biotransformed product were pooled and concentrated to yield 2.9 mg of **4**.

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'*-tetra-acetic 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 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 Japan) 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, reaction was terminated by harvesting the reaction mixtures onto a polyetheleneimine-soaked glassfilter (Wallac, Filtermat A). 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[23]. Briefly, vasa deferentia were isolated from rabbits and placed in organ baths filled with Mg^{2+}-free Krebs solution. The preparation was suspended through two platinum ring electrodes. After equilibration period (60 minutes) in order to check the tissue responses, twitch response was evoked by electric field stimulation. After stimulation, medium was changed, and 30 minutes later twitch response was reevoked by the same way. When the maximal tension of each response reached to plateau, the cumulative dosing of **1** was begun. To analyze data, the tension of effective threshold dosing was defined as 100% response. Each concentration altered plateau tone was expressed as a relative tension.

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