ES-242-1, A NOVEL COMPOUND FROM *Verticillium* sp., BINDS TO A SITE ON *N*-METHYL-D-ASPARTATE RECEPTOR THAT IS COUPLED TO THE CHANNEL DOMAIN

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A novel compound, ES-242-1, which binds to a site on *N*-methyl-D-aspartate (NMDA) receptor that is coupled to the channel domain, was isolated from the culture broth of a fungus, *Verticillium* sp. SPC-15898. ES-242-1 inhibited the [^{3}H]thienyl cyclohexylpiperidine ([^{3}H]TCP) binding to rat crude synaptic membrane fractions with an IC_{50} value of 116 nM, but did not inhibit the [^{3}H]kainate binding to its receptor, which is another subtype of the excitatory amino acid receptor.

The excitatory amino acids, L-glutamate and L-aspartate, are thought to play roles as excitatory neurotransmitters in the mammalian central nervous system. The receptors that mediate their actions are divided into the major three subtypes: *N*-methyl-D-aspartate (NMDA), quisqualate, and kainate.

The NMDA receptor is the best characterized, and is putatively involved in synaptic plasticity[1~3]. It is possibly of physiological importance, since excessive stimulation of the NMDA receptor is thought to be involved in pathogenesis of epilepsy, stroke, anoxia, hypoglycemia, and other neurodegenerative diseases[4]. Therefore, it has been proposed that blockade of NMDA receptor-mediated neurotoxicity may be an effective approach to the treatment of brain injury. Clinical studies, however, are limited by the small number of suitable NMDA antagonists that are available. Consequently, we have sought new NMDA antagonists with potential for this therapeutic application.

During the course of our screening studies designed to obtain a new NMDA antagonist of microbial origin, we isolated a novel compound, ES-242-1, which acts on the channel domain of NMDA receptor, from the culture broth of *Verticillium* sp. SPC-15898. In this paper, we report fermentation, isolation and characterization of ES-242-1. Structural elucidation studies will be reported in a separate paper.

Materials and Methods

Materials

[^{3}H]-1-[1-(2-thienyl)cyclohexyl]piperidine (TCP) and [^{3}H]kainate were purchased from New England Nuclear. MK-801 was chemically synthesized by Dr. H. OBASE and Mr. H. KATO in Pharmaceutical Research Laboratories of our company. Wistar rat brain was obtained from Nihon Seibutsu Zairyou Center, Tokyo, Japan. L-Glutamate was from Wako Pure Chemical Industries Ltd., Osaka, Japan. All other chemicals were analytical grade.

Microorganism

The producing organism, *Verticillium* sp. SPC-15898 (FERM BP-2604) was isolated from a soil

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collected in Kanagawa Prefecture in Japan.

Culture and Medium Conditions

A loopful of spores of microorganism, grown on an agar slant, was inoculated into 10 ml of seed medium composed of V8 vegetable juice (Campbell) 20% and $CaCO_3$ 0.3% (pH 6.4 before sterilization) in a test tube (21 i.d. × 200 mm). The agar slant medium consisted of malt extract 2%, glucose 2%, peptone (Kyokuto) 0.1% and agar 2.0% (pH 6.5 before sterilization). The inoculated tube was incubated at 25°C. A 10%-inoculation from the above vegetative medium was added to a 300-ml Erlenmeyer flask containing 50 ml of the same medium. After cultivation for 2 days on a rotary shaker (200 rpm) at 25°C, 50 ml of the second seed culture was transferred to 2-liter Erlenmeyer flask containing 500 ml of the fermentation medium composed of glucose 2%, dried mashed potato (Yukijirushi) 2%, peptone (Kyokuto) 0.5%, K_2HPO_4 0.05% and $Mg_3(PO_4)_2 \cdot 8H_2O$ 0.05% (pH 6.0 before sterilization) and incubated for 5 days at 25°C on a rotary shaker (200 rpm). The growth was monitored by packed cell volume (PCV) measurement. The ES-242-1 was produced mainly in mycelia, and its production was traced by measuring an inhibitory activity of [^{3}H]TCP binding. For this measurement, 2 ml of the culture broth was sampled and centrifuged. The precipitated mycelium was extracted with 2 ml of methanol. The supernatant mixed with an equal volume of 2-propanol was treated with 4 ml of saturated ammonium sulfate solution, stirred vigorously, and centrifuged. One ml of the upper organic layer was concentrated *in vacuo* to dryness. The dried materials were dissolved in a same volume of methanol. In either case, 10 μl of the methanol solution was provided for the assay.

Determination of Biological Activities

TCP binding assays were performed as described[5] with [^{3}H]TCP and rat brain membrane as a ligand and a source of receptors, respectively. Crude synaptic membrane was prepared from rat brain according to the method of MURPHY *et al.*[6], but without detergent treatment. L-Glutamate was added to the solution for the binding assay to maximally stimulate the TCP receptor binding. The reaction mixture (1 ml) containing Tris-HCl (pH 7.4) 5 mM, [^{3}H]TCP (47.8 Ci/mmol) 2.6 nM, thawed crude synaptic membrane 150 ~ 250 μg, and L-glutamate 10 μM was incubated for 30 minutes at room temperature. After which, the reaction was stopped by rapid filtration through Whatman GF/B glassfilters presoaked with 0.05% polyethylene-imine. The glassfilters were washed with five 3.5-ml portions of ice cold 5 mM Tris-HCl buffer (pH 7.4) by using a Brandel M-24R cell harvester. The washed filters were dried and the trapped radioactivity on the filters was determined by liquid scintillation counting in vials with 3 ml of cocktail (Omuniflour, Dupont). Non-specific binding was defined as that remaining in the presence of 50 μM of MK-801.

The inhibition of [^{3}H]TCP binding (%) was calculated as follows;

$$1-\frac{\begin{matrix}\text{Total } [^3\text{H}]\text{TCP binding} \\ \text{in presence of assay sample}\end{matrix} - \begin{matrix}\text{Non-specific} \\ \text{binding}\end{matrix}}{\begin{matrix}\text{Total } [^3\text{H}]\text{TCP binding} \\ \text{in absence of assay sample}\end{matrix} - \begin{matrix}\text{Non-specific} \\ \text{binding}\end{matrix}} \times 100$$

[^{3}H]Kainate binding experiment was carried out in the same way as [^{3}H]TCP binding assay. In brief, the reaction mixture (1 ml) containing [^{3}H]kainate 5 nM, rat crude synaptic membrane 200 μg and Tris-HCl (pH 7.4) 50 mM was incubated for 1 hour on ice. L-Glutamate (1 mM) was added to the reaction mixture for the calculation of non-specific binding of [^{3}H]kainate, after which, the reaction was stopped by filtration through the glassfilter, processed, and analyzed as described above.

HPLC Analysis of ES-242-1

The purity of ES-242-1 was determined by an HPLC system equipped with a Gilson Model 303 pump, Gilson Model 1001 UV detector and Shimadzu C-R6A Chromatopac. ES-242-1 dissolved in methanol (10 μl) was injected onto an octadecylated silica gel column (ODS-H-1151, 4.6 i.d. × 150 mm, Senshu Kagaku) and developed with 70% methanol solution at a flow rate of 1 ml/minute at room temperature, monitoring absorbance at 242 nm.

Results

Production of ES-242-1 by Fermentation

Numerous attempts to increase the yield of ES-242-1 were made; the resultant defined medium and optimum conditions for production are described under Materials and Methods. The time course of ES-242-1 production in 2-liter flask is shown in Fig. 1. The inhibitory potency against [^{3}H]TCP binding of the culture broth initiated on day 2 and was reached maximum on day 3. The color of the broth turned red during cultivation. PCV did not change drastically. The amount of ES-242-1 produced in mycelia is approximately 10-fold higher than that in broth filtrate.

Isolation and Purification

The isolation procedure for ES-242-1 is outlined in Fig. 2. ES-242-1 was mainly purified from mycelia obtained by centrifugation (8,000 rpm, 10 minutes) of the fermentation broth. The mycelial cake was extracted with methanol. The extract was concentrated *in vacuo* to give aqueous solution which was then extracted with *n*-hexane. The hexane layer was concentrated *in vacuo*, to yield an oily material, which was dissolved in a small volume of chloroform and applied to a silica gel column (Wakogel C-300, 24 × 250 mm). Adsorbed material was eluted with chloroform. Fractions containing ES-242-1 were pooled and concentrated *in vacuo* to yield crude ES-242-1. The crude ES-242-1 thus obtained was dissolved in methanol and allowed to stand for 1 day at 4°C to yield light yellow crystalline ES-242-1 (10 mg). The mother liquor

Fig. 1. Time course of ES-242-1 production in a 2-liter Elrenmeyer flask.

○ pH, △ PCV. Inhibition of [^{3}H]TCP binding; ● in culture supernatant or ▲ in mycelium.

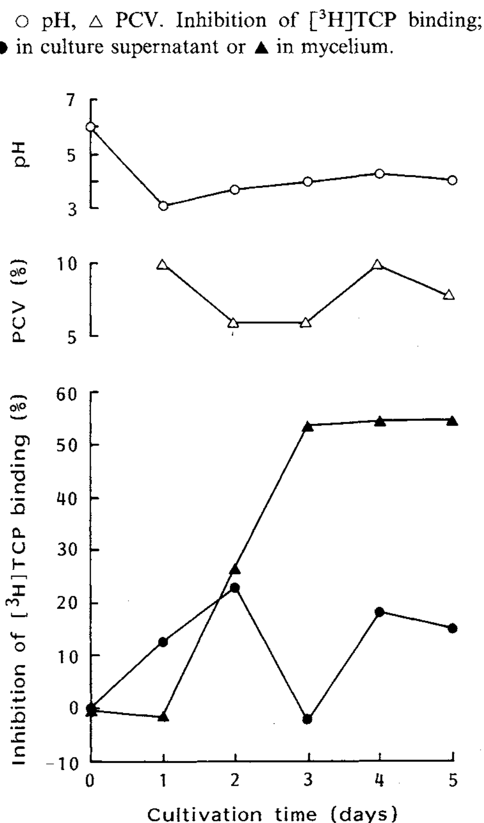


Fig. 2. Purification procedure of ES-242-1.

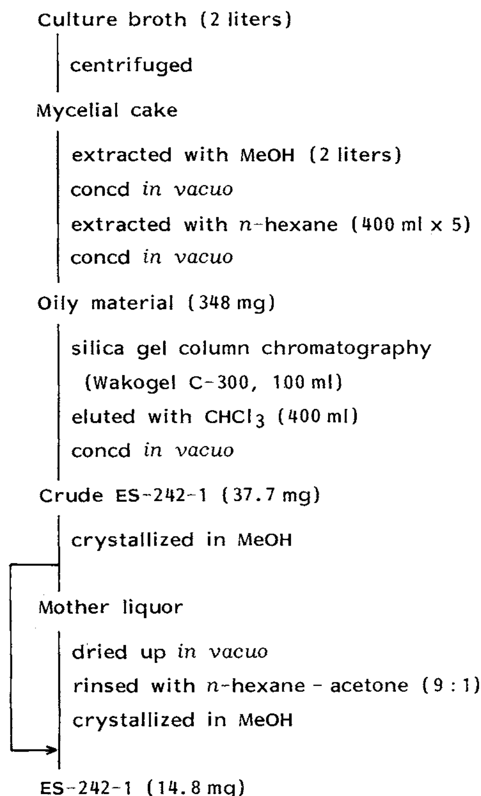
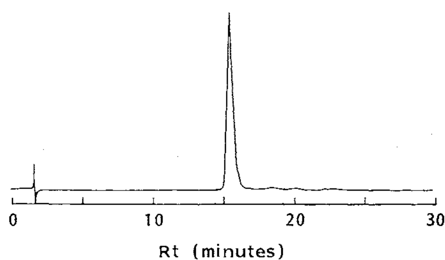


Fig. 3. HPLC analysis of purified ES-242-1.



Conditions were described in Materials and Methods. ES-242-1 (200 ng) was injected and eluted at a Rt of 15.3 minutes.

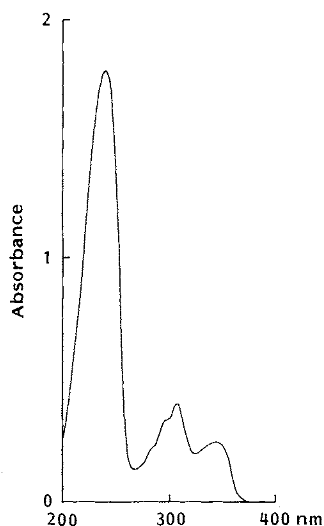
Table 1. Physico-chemical properties of ES-242-1.

Appearance	Slightly yellowish crystal
Molecular formula	$C_{34}H_{36}O_{10}$
EI-MS	604.2268 (calcd 604.2306)
MP (°C)	233~237
$[\alpha]_D$	+11° (*c* 0.46, MeOH)
IR (KBr) cm^{-1}	3380, 1735, 1623, 1576, 1457, 1381, 1361, 1157, 1097, 1050
UV λ_{max}^{MeOH} (nm)	239, 297, 309, 347
TLC, Rf value	
System I[a]	0.72
System II[b]	0.53
Solubility	
Soluble:	MeOH, $CHCl_3$, DMSO, $(CH_3)_2CO$, EtOAc
Insoluble:	H_2O, hexane

[a] Silica gel $60F_{254}$ (Merck, Art. No. 5628), solvent; *n*-hexane - acetone (1 : 1).

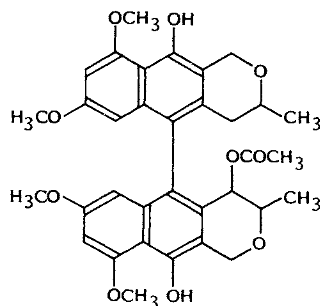
[b] Silica gel $60F_{254}$ (Merck, Art. No. 5628), solvent; $CHCl_3$ - EtOAc (1 : 1).

Fig. 4. The UV spectrum of ES-242-1.



The UV spectrum of ES-242-1 was obtained by using Hitachi spectrophotometer 220A. ES-242-1 was dissolved in MeOH (10 μg/ml) and the spectrum was scanned through 1 cm of light path.

Fig. 5. The structure of ES-242-1.



was concentrated *in vacuo* to dryness. The material was rapidly rinsed with a mixture of *n*-hexane - acetone (9 : 1) to remove impurities and dissolved in methanol. The solution was kept standing at 4°C to crystallize ES-242-1 (4.8 mg). Finally, 14.8 mg of ES-242-1 was purified from 2 liters of the culture broth. Purity of ES-242-1 was determined by HPLC analysis. ES-242-1 was eluted as a single peak at a retention time of 15.3 minutes (Fig. 3), when 70% methanol solution was used as a mobile phase.

Physico-chemical Properties of ES-242-1

Physico-chemical properties of ES-242-1 are summarized in Table 1. ES-242-1 is readily soluble in methanol, acetone, dimethyl sulfoxide, ethyl acetate and chloroform, and virtually insoluble in water and *n*-hexane. The molecular formula of ES-242-1 was determined to be $C_{34}H_{36}O_{10}$ on the basis of HREI-MS. The UV spectrum of ES-242-1 is shown in Fig. 4. The structure of ES-242-1 was determined as shown in Fig. 5, on the basis of ^1H and ^{13}C NMR spectrum data. Details of the structural elucidation studies will be reported in a separate paper.

Table 2. Inhibitory activity of ES-242-1 against [^{3}H]TCP binding.

Ligands	IC_{50} (nM)		
	ES-242-1	MK-801	Ketamine
[^{3}H]TCP	116	5	5,900
[^{3}H]Kainate	>10,000	>10,000	>10,000

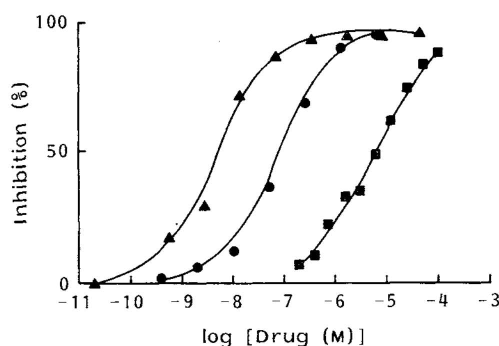


Fig. 6. Displacement curve of ES-242-1.

▲ MK-801, ● ES-242-1, ■ ketamine. Inhibition (%) was defined as described in Materials and Methods.

Biochemical Properties

The inhibitory activity of ES-242-1 against [^{3}H]TCP binding is shown in Table 2 and Fig. 6. ES-242-1 displaced the [^{3}H]TCP binding to the synaptic membrane in a dose-dependent manner with an IC_{50} value of 116 nM. The inhibitory potency of ES-242-1 was about 20-fold lower than that of MK-801 (IC_{50} 5 nM) and 40-fold higher than that of ketamine (IC_{50} 5.9 μM), both of which are non-competitive NMDA antagonists acting on the ion channel domain of NMDA receptor[7]. ES-242-1 was not effective in the inhibition of the binding of [^{3}H]kainate, a ligand for non-NMDA type of the glutamic acid receptor, at concentrations up to 10 μM.

ES-242-1 exhibited no antimicrobial activity against *Staphylococcus aureus* KY 4779, *Enterococcus faecalis* KY 4280, *Bacillus subtilis* KY 4773, *Escherichia coli* KY 4271, *Klebsiella pneumoniae* KY 4275, *Proteus vulgaris* KY 4277, *Shigella sonnei* KY 4281, *Salmonella typhosa* KY 4278, *Pseudomonas aeruginosa* KY 4276 or *Candida albicans* KY 5011.

No inhibition of protein kinase C by ES-242-1 was observed at concentrations up to 10 μM.

Discussion

The NMDA type of excitatory amino acid receptor has been shown to possess a number of distinct sites through which its function may be regulated or pharmacologically modified[1,8]. These include:

(1) The agonist recognition site, at which glutamate, aspartate, and NMDA bind to open the NMDA receptor channel[8]. The glutamate analogues 2-amino-5-phosphonovaleric acid (APV), γ-D-glutamylglycine (γ-DGG), CGS19755 (*cis*-4-phosphonomethyl-2-piperidine carboxylic acid)[9], and 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonoic acid (CPP) are competitive antagonists acting on the same site;

(2) an allosteric site, at which glycine regulates agonist-induced channel opening, and at which 7-chlorokynurenic acid[10], and HA-966 (3-amino-1-hydroxypyrrolidine-2)[11] displace glycine binding competitively, and producing noncompetitive antagonism of receptors to NMDA; and

(3) sites with the receptor-associated ion channel for Mg^{2+} and for drugs[7] such as MK-801, ketamine, TCP, and diarylguanidine derivatives[12]. Recent observations suggest that there may be additional site on the NMDA receptor where Zn^{2+} [13,14], polyamine[15], and tricyclic antidepressants[16,17] act to modulate the properties of the NMDA receptor.

In this work we have isolated a novel compound, ES-242-1, from the culture broth of *Verticillium* sp., which belongs to category (3) and acts at a site of the channel domain on the NMDA receptor. Indeed, ES-242-1 displaced [^{3}H]TCP binding to crude synaptic membranes at a low concentration (IC_{50} 116 nM) without affecting the [^{3}H]kainate binding. This is the first report describing the discovery of a compound acting on a site with NMDA receptor-associated ion channel from a microbial source. ES-242-1 is a new chemical entity, binds to the NMDA receptor, and as such may provide a new tool with which to understand the molecular pharmacology of this receptor. It may possess neuroprotective properties useful in the treatment of diseases involving glutamate toxicity. Further investigations concerning the biochemical and

pharmacological properties of ES-242-1 are being undertaken.

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References

1) COTMAN, C. W.; D. T. MONAGHAN & A. H. GANONG: Excitatory amino acid neurotransmission: NMDA receptors and Hebb-type synaptic plasticity. Annu. Rev. Neurosci. 11: 61~80, 1988
2) COLLINGRIDGE, G. L. & W. SINGER: Excitatory amino acid receptors and synaptic plasticity. Trends Pharmacol. Sci. 11: 290~296, 1990
3) COLLINGRIDGE, G. L. & T. V. P. BLISS: NMDA receptors—Their role in long-term potentiation. Trends Neurosci. 10: 288~293, 1987
4) ROTHMAN, S. M. & J. W. OLNEY: Excitotoxicity and the NMDA receptor. Trends Neurosci. 10: 299~302, 1987
5) VIGNON, J.; R. CHICHEPORTICHE, M. CHICHEPORTICHE, J.-M. KAMENKA, P. GENESTE & M. LAZDUNSKI: [^{3}H]TCP: A new tool with high affinity for the PCP receptor in rat brain. Brain Res. 280: 194~197, 1983
6) MURPHY, D. E.; E. W. SNOWHILL & M. WILLIAMS: Characterization of quisqualate recognition sites in rat brain tissue using DL-[^{3}H]α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and a filtration assay. Neurochem. Res. 12: 775~782, 1987
7) LODGE, D. & K. M. JOHNSON: Noncompetitive excitatory amino acid receptor antagonists. Trends Pharmacol. Sci. 11: 81~86, 1990
8) WATKINS, J. C. & H. J. OLVERMAN: Agonists and antagonists for excitatory amino acid receptors. Trends Neurosci. 10: 265~272, 1987
9) MURPHY, D. E.; A. J. HUTCHISON, S. D. HURT, M. WILLIAMS & M. A. SILLS: Characterization of the binding of [^{3}H]-CGS 19755: a novel *N*-methyl-D-aspartate antagonist with nanomolar affinity in rat brain. Br. J. Parmacol. 95: 932~938, 1988
10) KEMP, J. A.; A. C. FOSTER, P. D. LEESON, T. PRIESTLEY, R. TRIDGETT, L. L. IVERSEN & G. N. WOODRUFF: 7-Chlorokynurenic acid is a selective antagonist at the glycine modulatory site of the *N*-methyl-D-aspartate receptor complex. Proc. Natl. Acad. Sci. U.S.A. 85: 6547~6550, 1988
11) FOSTER, A. C. & J. A. KEMP: HS-966 antagonizes *N*-methyl-D-aspartate receptors through a selective interaction with the glycine modulatory site. J. Neurosci. 9: 2191~2196, 1989
12) KEANA, J. F. W.; R. N. MCBURNEY, M. W. SCHERZ, J. B. FISCHER, P. N. HAMILTON, S. M. SMITH, A. C. SERVER, S. FINKBEINER, C. F. STEVENS, C. JAHR & E. WEBER: Synthesis and characterization of a series of diarylguanidines that are noncompetitive *N*-methyl-D-aspartate receptor antagonists with neuroprotective properties. Proc. Natl. Acad. Sci. U.S.A. 86: 5631~5635, 1989
13) PETERS, S.; J. KOH & D. W. CHOI: Zinc selectively blocks the action of *N*-methyl-D-aspartate on cortical neurons. Science 236: 589~593, 1987
14) WESTBROOK, G. L. & M. L. MAYER: Micromolar concentrations of Zn^{2+} antagonize NMDA and GABA responses of hippocampal neurons. Nature 328: 640~643, 1987
15) RANSOM, R. W. & N. L. STEC: Cooperative modulation of [^{3}H]MK-801 binding to the *N*-methyl-D-aspartate receptor-ion channel complex by L-glutamate, glycine, and polyamines. J. Neurochem. 51: 830~836, 1988
16) SILLS, M. A. & P. S. LOO: Tricyclic antidepressants and dextromethorphan bind with higher affinity to the phencyclidine receptor in the absence of magnesium and L-glutamate. Mol. Pharmacol. 36: 160~165, 1989
17) SERNAGOR, E.; D. KUHN, L. VYKLICKY, Jr. & M. L. MAYER: Open channel block of NMDA receptor responses evoked by tricyclic antidepressants. Neuron 2: 1221~1227, 1989