ES-242-2, -3, -4, -5, -6, -7, AND -8, NOVEL BIOXANTHRACENES PRODUCED BY *Verticillium* sp., WHICH ACT ON THE *N*-METHYL-D-ASPARTATE RECEPTOR

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Verticillium sp. SPC-15898 was found to produce novel metabolites, designated ES-242-2~-8, which were structurally related to ES-242-1. These compounds were isolated from the culture broth and the physico-chemical and biochemical properties were examined. ES-242-2~-8 inhibited [^{3}H]thienyl cyclohexypiperidine ([^{3}H]TCP) binding to rat crude synaptic membranes (CSM) with IC_{50} values of 0.116, 2.9, *ca.* 2.9, 25.3, 1.0, 59, 24, and 13 μM, respectively. None of these compounds showed inhibitory effects against the binding of [^{3}H]kainate to its receptor, which is another subtype of the excitatory amino acid receptor.

In a previous paper[1], we reported that a novel bioxanthracene, ES-242-1, which binds to a site of the *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. In the present investigation, we have isolated seven novel compounds, designated ES-242-2, ES-242-3, ES-242-4, ES-242-5, ES-242-6, ES-242-7 and ES-242-8, from the same culture broth as ES-242-1, which are structurally related to ES-242-1. In this article, we describe the isolation, physico-chemical and biological properties of these compounds. Structural elucidation studies will be described in a separate paper.

Materials and Methods

Materials

All radioligands used were purchased from New England Nuclear. MK-801 was chemically synthesized by Dr. H. OBASE and Mr. H. KATO in the Pharmaceutical Research Laboratories of our company. Wistar rat brains were obtained from Nihon Seibutsu Zairyou Center, Tokyo, Japan. L-Glutamate was from Wako Pure Chemical Industries Ltd., Osaka, Japan. All other chemicals were of analytical grade.

Microorganism

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

Culture and Medium Condition

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% (v/v) and $CaCO_3$ 3% (pH 6.4 before sterilization) in a 250-ml Erlenmeyer flask. The agar slant medium consisted of glucose 0.4%, yeast extract 0.4%, meat

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extract 1%, and agar 2% (pH 6.0 before sterilization). The inoculated flask was incubated for 4 days at 25°C on a rotary shaker. A 30-ml aliquot of the seed culture was transferred into a 2-liter Erlenmeyer flask with baffle containing 300 ml of the seed medium, and the flask was incubated for 2 days at 25°C on a rotary shaker. The second seed culture was transferred into a 200-liter tank fermenter containing 100 liters of the seed medium. The tank was operated for 2 days at 25°C with agitation at 200 rpm and aeration of 60 liters per minute. Whole cultured broth of the tank fermenter was transferred into a 2,000-liter tank containing 1,000 liters of the fermentation medium composed of glucose 2%, potato starch 2%, peptone 2%, KH_2PO_4 0.05%, and $Mg_3(PO_4)_2 \cdot 8H_2O$. The fermentation was carried out for 4 days at 25°C with agitation at 120 rpm and aeration of 400 liters per minute.

Determination of Biological Activities

1-[1-(2-Thienyl)cyclohexyl]piperidine (TCP) binding assays were performed as described[2] with [^{3}H]TCP and rat brain membrane as a ligand and a source of receptors, respectively. Crude synaptic membranes were prepared from rat brain according to the method of MURPHY *et al.*[3], but without detergent treatment. L-Glutamate was added to the solution for the binding assay to maximally stimulate the TCP receptor binding. The reaction mixtures (1 ml) containing Tris-HCl (pH 7.4) 5 mM, [^{3}H]TCP (47.8 Ci/mmol) 2.6 nM, thawed crude synaptic membranes 150~250 μg, and L-glutamate 10 μM were incubated at room temperature. After 30 minutes, the reactions were 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 measured as that remaining in the presence of 50 μM of MK-801.

The assays of [^{3}H]kainate binding were carried out in the same way as [^{3}H]TCP binding assays. In brief, the reaction mixtures (1 ml) containing [^{3}H]kainate 5 nM, rat crude synaptic membranes 200 μg and Tris-HCl (pH 7.4) 50 mM were 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 reactions were stopped by filtration through the glassfilters, processed, and analyzed as described above.

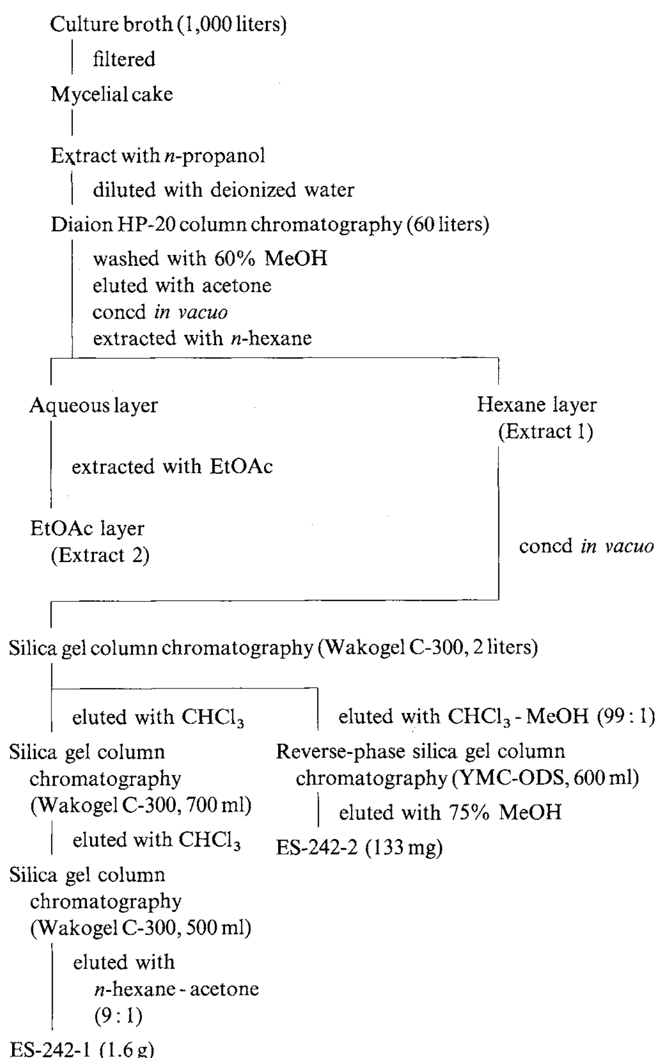
Results

We found the fungus, *Verticillium* sp. SPC-15898, which produces seven ES-242-1-related compounds having inhibitory potencies against [^{3}H]TCP binding to crude synaptic membrane. They gave the distinct Rf values from that of ES-242-1 on TLC developed with various solvent systems. The physico-chemical properties of these compounds designated ES-242-2, ES-242-3, ES-242-4, ES-242-5, ES-242-6, ES-242-7, and ES-242-8 are different from those of ES-242-1.

Purification and Isolation

The isolation procedure of the ES-242s from the culture broth of *Verticillium* sp. SPC-15898 is schematically shown in Figs. 1, 2, and 3. The compounds were extracted from the mycelial cake with *n*-propanol. The propanol extract was diluted with water and passed through a Diaion HP-20 column (Mitsubishi Chemical Industries Ltd., 60 liters). The column was washed with 60% methanol and eluted with acetone. Fractions containing the ES-242s were concentrated *in vacuo* to yield aqueous solution. The resultant aqueous solution was extracted with *n*-hexane (Extract 1) and then, with ethyl acetate (Extract 2). Extract 1 was concentrated *in vacuo* to yield oily brown material. This oily material was subjected to silica gel column chromatography (Wakogel C-300, 2 liters). The adsorbed material was eluted with chloroform and chloroform - methanol (99 : 1). Fractions eluted with chloroform were combined and concentrated to yield crude ES-242-1 (1.6 g). Fractions eluted with chloroform - methanol (99 : 1) was concentrated to dryness and the residue was fractionated on reverse-phase silica gel column chromatography

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



(YMC-ODS, 600 ml). The adsorbed material was eluted with 75% methanol solution. Fractions containing ES-242-2 were combined and evaporated to yield yellowish powder (133 mg).

Two-fifth amounts of Extract 2 were concentrated to dryness to yield a brown oily material. The oily material was dissolved in a small amount of chloroform and applied to a silica gel column (Merck Art. 7734, 2 liters). ES-242-3, -4, -5, -6, and -7 were eluted at elution volumes from 4 to 6 liters (Fraction A) and ES-242-8 was eluted from 6 to 7 liters (Fraction B). Fraction A was evaporated to dryness and applied to a silica gel column (Wakogel C-300, 500 ml) and the column was washed with *n*-hexane - acetone (9:1). ES-242-6, -1, and -5 were eluted with *n*-hexane - acetone (8:2) at elution volumes from 850 to 1,100 ml, from 1,500 to 2,300 ml, and from 2,300 to 3,000 ml, respectively. Fractions containing each compound were combined separately and concentrated *in vacuo*. ES-242-1 was crystallized in *n*-hexane - acetone to yield slightly yellowish crystals (1.2 g). Final purification of ES-242-5 and -6 were achieved by silica gel column chromatography. ES-242-5 and -6 were obtained as yellowish powder (76.6 and 26.3 mg,

Fig. 2. Purification procedure of ES-242-3, -4, -5, -6, and -7.

2/5 amount of Extract 2 in Fig. 1
concd *in vacuo*
Silica gel column chromatography (Merck Art. 7734, 2 liters)
eluted with $CHCl_3$

Fraction A
Silica gel column chromatography (Wakogel C-300, 500 ml)
washed with *n*-hexane - acetone (9 : 1)
eluted with *n*-hexane - acetone (8 : 2)

Silica gel column chromatography (Wakogel C-300, 20 ml)
eluted with *n*-hexane - acetone (8 : 2)
ES-242-6 (26.3 mg)

Crystallization
ES-242-1 (1.2 g)

Silica gel column chromatography (Merck Art. 9385, 200 ml)
eluted with *n*-hexane - acetone (8 : 2)
ES-242-5 (76.6 mg)

eluted with EtOAc
Fraction C
crystallized in EtOAc

Mother liquor
Silica gel column chromatography (Merck Art. 9385, 300 ml)
eluted with *n*-hexane - acetone (7 : 3)

Silica gel column chromatography (Merck Art. 9385, 300 ml)
eluted with *n*-hexane - acetone (7 : 3)

Reverse-phase silica gel column chromatography (YMC-ODS, 150 ml)
eluted with 75% MeOH
ES-242-7 (6.3 mg)

Reverse-phase silica gel column chromatography (YMC-ODS, 20 ml)
eluted with 75% MeOH
Crude ES-242-3 (2.6 mg)

Crystallization
n-hexane - acetone
ES-242-4 (102 mg)

Crystals of ES-242-1 (483 mg)

Fraction B
[Fraction B is processed in Fig. 3]

respectively).

Fractions eluted with ethyl acetate (Fraction C) were concentrated to dryness and dissolved in a small amount of ethyl acetate. After the solution was stored at 4°C for several days, ES-242-1 was crystallized. The crystalline ES-242-1 (483 mg) was removed by filtration, and the mother liquor was evaporated to dryness. The residue was fractionated on silica gel column chromatography

Fig. 3. Purification procedure of ES-242-8.

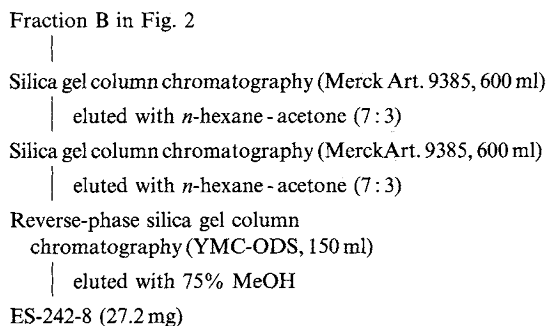


Table 1. Physico-chemical properties of ES-242s.

	ES-242-2	ES-242-3	ES-242-4	ES-242-5	ES-242-6	ES-242-7	ES-242-8
Appearance	Pale yellow powder	Pale yellow powder	Colorless needles	Pale yellow powder	Pale yellow powder	Pale yellow powder	Pale yellow powder
MP	161 ~ 162°C	137 ~ 139°C	184 ~ 185°C	157 ~ 158°C	n.d.	158 ~ 159°C	162 ~ 163°C
Specific rotation	$[\alpha]_D^{21} = +44°$ (*c* 0.15, $CHCl_3$)	$[\alpha]_{546\,nm}^{22} = +50°$ (*c* 0.16, $CHCl_3$)	$[\alpha]_D^{21} = -54°$ (*c* 0.18, $CHCl_3$)	$[\alpha]_D^{20} = +21°$ (*c* 0.12, $CHCl_3$)	n.d.	$[\alpha]_D^{25} = +108°$ (*c* 0.16, $CHCl_3$)	$[\alpha]_D^{25} = +5°$ (*c* 0.16, $CHCl_3$)
Color test							
I_2 vapor	Positive	Positive	Positive	Positive	Positive	Positive	Positive
$FeCl_3$	Positive	Positive	Positive	Positive	n.d.	n.d.	n.d.
H_2SO_4	Positive	Positive	Positive	Positive	n.d.	n.d.	n.d.
Solubility							
H_2O	Insoluble	Insoluble	Insoluble	Insoluble	Insoluble	Insoluble	Insoluble
MeOH	Soluble	Soluble	Soluble	Soluble	Slightly soluble	Soluble	Soluble
DMSO	Soluble	Soluble	Soluble	Soluble	Soluble	Soluble	Soluble
TLC, Rf value							
System I[a]	0.30	0.26	0.24	0.32	0.42	0.24	0.24
System II[b]	0.76	0.78	0.80	0.72	0.45	0.55	0.63

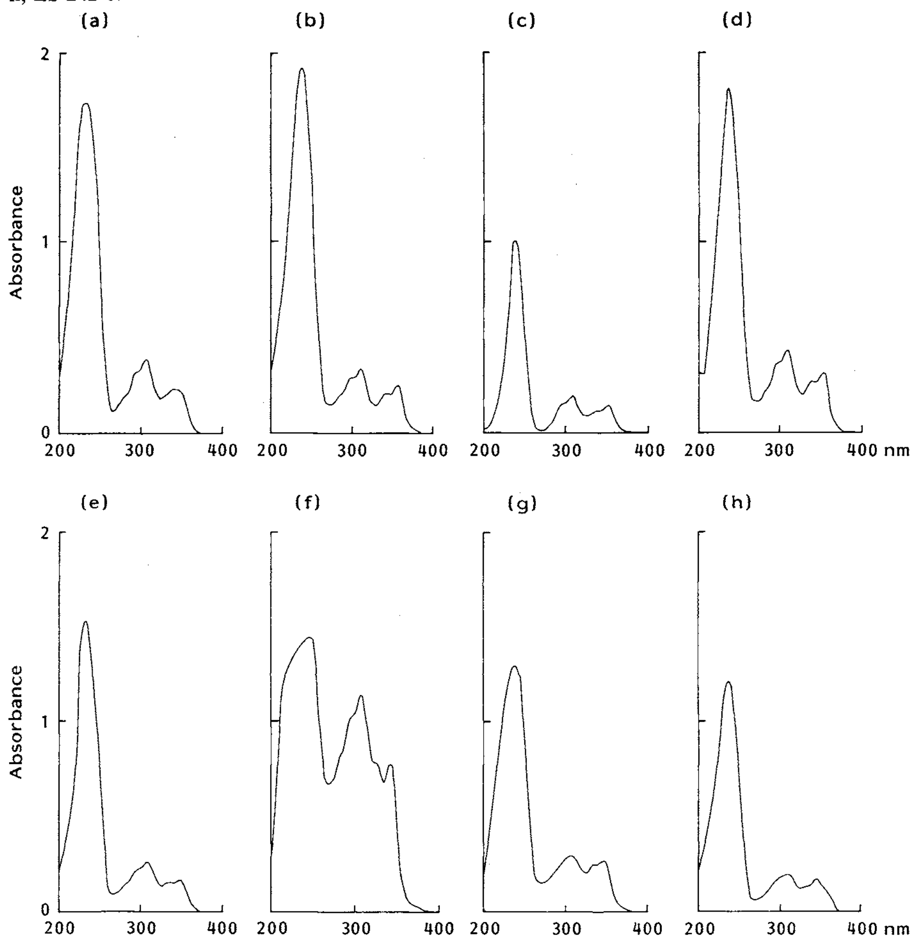
[a] Plate: Silica gel 60 (Merck, Art. 5628), solvent; *n*-hexane - acetone (3 : 2).

[b] Plate: RP-18 (Merck, Art. 13724), solvent; MeOH.

n.d.: Not determined.

Fig. 4. UV spectra of ES-242s.

a; ES-242-1, b; ES-242-2, c; ES-242-3, d; ES-242-4, e; ES-242-5, f; ES-242-6, g; ES-242-7, h; ES-242-8.



The UV spectra of the ES-242s were obtained by using Hitachi spectrophotometer 220A. ES-242-1, -5, -7, and -8 were dissolved in methanol at a concentration of 10 μg/ml. ES-242-2, -3, and -4 were dissolved at a concentration of 20 μg/ml. ES-242-6 was dissolved in methanol at a concentration of 100 μg/ml. The spectra were scanned through 1 cm light path.

(Merck Art. 9385, 300 ml). Fractions containing ES-242-4 were combined and evaporated to dryness. ES-242-4 was crystallized in *n*-hexane - acetone (102 mg). Fractions containing both ES-242-3 and -7 were combined, concentrated *in vacuo*, and subjected to silica gel column chromatography developed with *n*-hexane - acetone (7 : 3). Fractions containing each compound were separately combined and concentrated *in vacuo*. Final purification of both compounds were achieved by reverse-phase silica gel column chromatography (YMC-ODS). ES-242-3 and -7 were obtained as yellowish powder (2.6 and 6.3 mg, respectively).

Fraction B was concentrated *in vacuo* to yield crude ES-242-8. Further purification of ES-242-8 was achieved twice by silica gel column chromatography and once by reverse-phase silica gel column chromatography. ES-242-8 was obtained as yellowish powder (27.2 mg).

Physico-chemical Properties

Physico-chemical properties of the ES-242 compounds are summarized in Table 1. Based on their

Fig. 5. Chemical structures of the ES-242s.

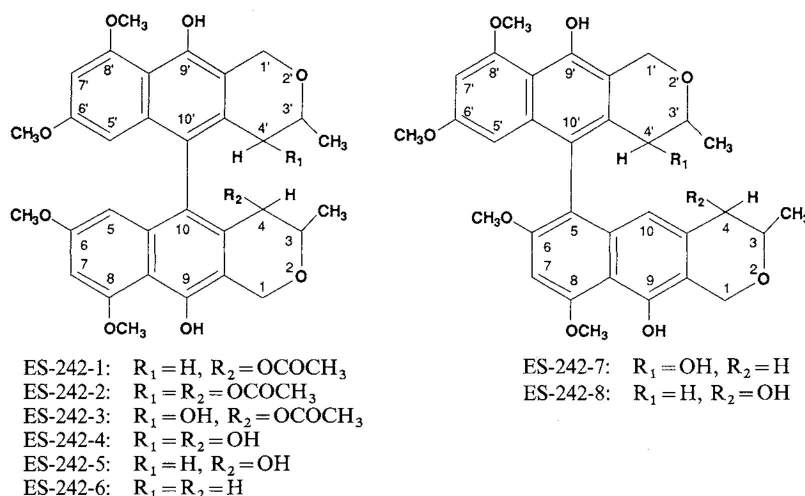
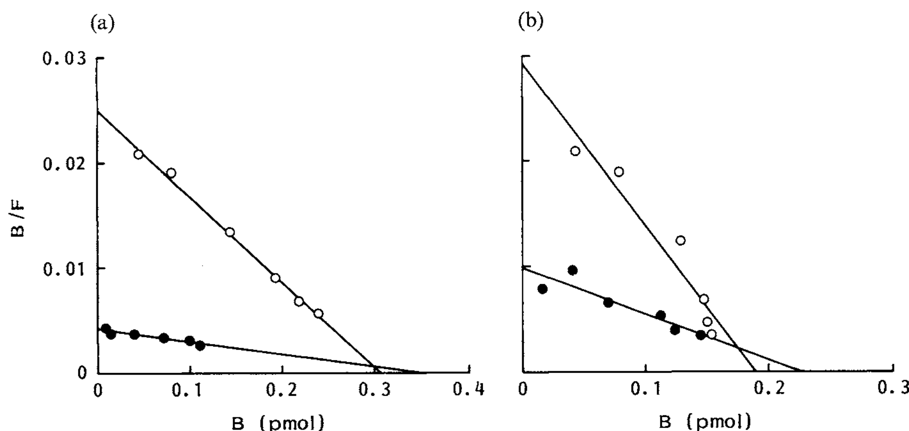


Fig. 6. Scatchard analyses of the inhibition of ES-242-1 or -2 against [^{3}H]TCP binding to rat crude synaptic membrane.

(a) Scatchard analysis in the absence (○) or presence of 332 nM ES-242-1 (●). (b) Scatchard analysis in the absence (○) or presence of 3 μM ES-242-2 (●).



Binding studies were carried out as described in Materials and Methods. Each compound was dissolved in methanol at various concentrations and 10 μl was added to a reaction mixture.

UV spectra (Fig. 4), these compounds were shown to possess the same chromophore as ES-242-1. The chemical structures of the ES-242s were elucidated on the basis of physico-chemical analysis and various spectral data (Fig. 5). Details of these studies will be described in a separate paper.

Biological Activities

The ES-242 compounds inhibited [^{3}H]TCP binding to rat crude synaptic membrane in a dose-dependent manner. IC_{50} values for [^{3}H]TCP

Table 2. Inhibitory effects of ES-242s on the binding of [^{3}H]TCP or [^{3}H]kainate.

Compounds	IC_{50} Values (μM)	
	[^{3}H]TCP	[^{3}H]Kainate
ES-242-1	0.12	>15
ES-242-2	2.9	>15
ES-242-3	*ca.* 2.9	n.t.
ES-242-4	25	>15
ES-242-5	1.0	>15
ES-242-6	59	n.t.
ES-242-7	24	>15
ES-242-8	13	>15

All of ES-242s were dissolved in MeOH.
n.t.: Not tested.

binding were listed in Table 2. The rank order of potency was ES-242-1 > -5 > -2 = -3 > -8 > -4 = -7 > -6. Kinetic analysis showed that ES-242-1 and ES-242-2 inhibited the [^{3}H]TCP binding in a competitive manner (Fig. 6). None of the ES-242s were effective on [^{3}H]kainate binding to its receptor at concentrations up to 10 μM (Table 2).

Discussion

In a previous paper[1], we reported a novel ligand for the NMDA receptor, ES-242-1, isolated from a culture broth of a fungus, *Verticillium* sp. SPC-15898. The present studies demonstrate the isolation of seven new compounds, which contain bioxanthracene structure as ES-242-1, produced by the same fungus.

The ES-242s do not contain nitrogen, unlike the other noncompetitive NMDA antagonists such as MK-801[4], TCP[2], ketamine[5], and other NMDA antagonists[6,7], suggesting that nitrogen atoms may not be essential for a compound to act on NMDA receptor.

The ES-242 compounds possess 10, 10′ or 5, 10′-bioxanthracene structure having two oxanthracene rings connected with a single C-C bond. ES-242-2 ~ -8 are inhibitors for [^{3}H]TCP binding to the NMDA receptor as ES-242-1, however, their inhibitory potencies are lower than that of ES-242-1. Minor structural changes of the ES-242s cause big differences in inhibitory potency against [^{3}H]TCP binding to the NMDA receptor. These results may show a strategy for synthesis of more strongly active compounds acting on the NMDA receptor.

Excitatory amino acid receptors are divided into three major subtypes: NMDA, kainate/quisqualate (non-NMDA), and metabotropic. The ES-242s inhibited the binding of [^{3}H]TCP, which is a noncompetitive NMDA antagonist, in a competitive manner without affecting the binding of [^{3}H]kainate, which is an agonist for non-NMDA receptor. These results suggest that the ES-242s may selectively interact with the NMDA type of excitatory amino acid receptor.

We have found that ES-242-1 and -2 prevented neuronal cell death induced by glutamate and under anoxic conditions *in vitro* (unpublished observation). Thus, the ES-242s, which are new chemical entities that may provide a new tool with which to understand the molecular pharmacology of this receptor. These compounds may possess neuroprotective properties useful in the treatment of diseases involving glutamate toxicity.

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References

1) TOKI, S.; K. ANDO, M. YOSHIDA, I. KAWAMOTO, H. SANO & Y. MATSUDA: 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. J. Antibiotics 45: 88~93, 1992
2) 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
3) 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
4) WONG, E. H. F.; J. A. KEMP, T. PRIESTLEY, A. R. KNIGHT, G. N. WOODRUFF & L. L. IVERSEN: The anticonvulsant MK-801 is a potent *N*-methyl-D-aspartate antagonist. Proc. Natl. Acad. Sci. U.S.A. 83: 7104~7108, 1986
5) HONEY, C. R.; Z. MILJKOVIC & J. F. MACDONALD: Ketamine and phencyclidine cause a voltage-dependent block of responses to L-aspartic acid. Neurosci. Lett. 61: 135~139, 1985
6) 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
7) LODGE, D. & K. M. JOHNSON: Noncompetitive excitatory amino acid réceptor antagonists. Trends Pharmacol. Sci. 11: 81~86, 1990