Arisugacins A and B, Novel and Selective Acetylcholinesterase Inhibitors from *Penicillium* sp. FO-4259

I. Screening, Taxonomy, Fermentation, Isolation and Biological Activity

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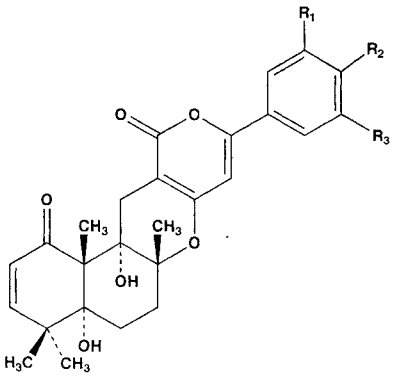
An *in vitro* screening method for selective acetylcholinesterase (AChE) inhibitors was established. Inhibitory activity of AChE and butyrylcholinesterase (BuChE) was measured and the culture broths of microorganisms that showed selective inhibition against AChE were characterized.

By using this method, a strain producing the novel and selective inhibitors of AChE, arisugacins A and B, was picked out among over seven thousand microorganisms tested. Arisugacins were obtained as white powders from the culture broth together with three known compounds, territrems B and C and cyclopenin that also showed selective inhibition against AChE. Arisugacins and territrems are members of the meroterpenoid compounds. They showed potent inhibitory activities against AChE with IC_{50} values in range of 1.0~25.8 nM. Furthermore, they showed greater than 2,000-fold more potent inhibition against AChE than BuChE.

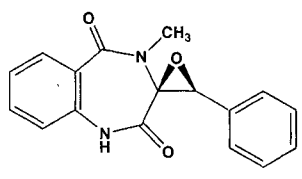
Alzheimer's Disease (AD) is a degenerative disorder of the central nervous system characterized clinically by the loss of memory, intellect and cognitive functions. The etiology and the pathophysiology of AD are still unknown. Currently, clinical study with an acetylcholinesterase (AChE) inhibitor, tacrine, has demonstrated a significant improvement of cognitive function in patients of AD[1]. Tacrine has been approved by the FDA as the first agent for the treatment of AD, although it suffers from dose-limiting side effects[2]. Clinical data of selective and nonselective AChE inhibitors suggested that the butyrylcholinesterase (BuChE) inhibition may be associated with peripheral side effects[3].

The standard assay for AChE was performed by the method of ELLMAN *et al.*[4]. Recently, a qualitative assay using silica gel TLC plates was reported by KIELY *et al.*[5]. DOCTOR *et al.* applied the method of ELLMAN *et al.* to a 96-well microtiter plate for the assay of large numbers of samples[6]. We established a rapid method to screen for selective AChE inhibitors from a large number of culture broths by comparing inhibitory activities against both AChE and BuChE. By using the method, we discovered the novel and selective AChE inhibitors, arisugacins A (previously reported as arisugacin[7]) and

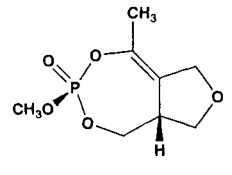
Fig. 1. Structures of arisugacins A and B, territrems B and C, cyclopenin and cyclophostin.



Arisugacin A $R_1=R_2=OCH_3$, $R_3=H$
Arisugacin B $R_1=R_3=H$, $R_2=OCH_3$
Territrem B $R_1=R_2=R_3=OCH_3$
Territrem C $R_1=R_3=OCH_3$, $R_2=OH$



Cyclopenin



Cyclophostin

B (Fig. 1) from the culture broth of fungal strain FO-4259.

In this paper, we describe the details of the discovery, taxonomy of the producing strain, fermentation, isolation and biological activities of arisugacins A and B. Structure elucidation including their relative configuration will be reported separately[8].

Materials and Methods

Chemicals

AChE (E.C. 3.1.1.7) from human erythrocytes, BuChE (E.C. 3.1.1.8) from horse serum, BW 284C51 and tetraisopropylprophosphoramide (*iso*-OMPA) were purchased from Sigma Chemicals Co. Ltd. Acetylcholine-HCl was purchased from Daiichi Pharmaceutical Co., Ltd. Tacrine and physostigmine were purchased from Research Biochemical Co., Ltd. Butyrylcholine-HCl was purchased from Nacalai Tesque Co., Ltd. Substrate-enzyme (Cholinesterase B test Kit) was purchased from Wako Pure Chemical Industries, Ltd. Diazinon and cyclophostin were kindly supplied by Nippon Kayaku Co., Ltd. Other chemicals were analytical grade.

Assay methods of Cholinesterase

The inhibitory activities against AChE were measured by the method of OKABE *et al.*[9] with modifications as follows; 100 μl of AChE solution containing 0.65 unit activity/ml in 0.1 M potassium phosphate buffer (pH 7.6) and 10 μl of a cultured broth or 50% ethanol solution of purified material was added to each well of a 96-well plate. After incubation for 15 minutes at 37°C, 100 μl of substrate-enzyme solution (17.5 nmole of benzoylcholine, 14.75 nmole of 4-aminoantipilin, 0.0425 unit of cholineoxidase, 0.015 unit of peroxidase and 5.25 μmole of phenol in 0.1 M potassium phosphate buffer, pH 7.6) and 10 μl of acetylcholine solution (1 mg/ml) were added to each well. The reaction was carried out at 37°C for 30 minutes, and the formation of red quinone was measured with a Titertek microplate reader (Dainippon Pharmaceutical Co., Ltd.) at 492 nm. Values for percentages of inhibition were calculated relative to a control sample. The inhibitory activities against BuChE were measured as described above for AChE by substituting BuChE (0.125 units activity/ml) and butyrylcholine (1 mg/ml) for enzyme and substrate, respectively. The AChE and BuChE activities are inhibited by inhibitors of choline oxidase or peroxidase in these assays, because the enzymes are used for coupling reaction to form red quinone. These inhibitors were eliminated by testing the inhibitory activities against these enzymes using 10 μl of 10 mM choline chloride or 0.3% hydrogen peroxide instead of the substrate solutions.

Results and Discussion

Inhibition of AChE and BuChE Activities by Various Cholinesterase Inhibitors

Cholinesterases (ChEs) may be classified as AChE and BuChE on the basis of differential specificity of ACh and BuCh hydrolysis. The AChE is primarily associated with nerve and muscle, localizing typically at synaptic contacts, while BuChE is synthesized in liver with substantial amounts appearing in serum. Several ChE inhibitors have been shown to be selective for one of the two enzymes as reported by TAYLOR[10].

In our assay, ChE inhibitors were classified into 4 types by their IC_{50} values (concentrations required for inhibition of control enzyme activity by 50%) against AChE and BuChE as shown in Table 1. The type A ChE inhibitor, BW 284C51, exerted 920 times more potent effect on AChE than on BuChE. The type B ChE inhibitors, physostigmine and diazinon acted equally on both AChE and BuChE. Diazinon is a synthetic organophosphate and used as an insecticide. The type C ChE inhibitor, tacrine, exerted 17 times more potent effect on BuChE than on AChE. The type D ChE inhibitor, *iso*-OMPA, exerted 850 times more potent effect on BuChE than on AChE. Except for diazinon, the above results approximately coincided with the results of THOSEM *et al.*[3] obtained by using a radiometric assay against AChE from human erythrocytes and BuChE from human plasma, suggesting that the culture broths showing type A inhibition may contain the selective inhibitors of AChE.

Screening of the Selective Inhibitors of AChE and Discovery of Arisugacins

The culture broths showing over 50% inhibition of AChE were picked up in primary screening. In secondary screening, the broths that showed more potent inhibition against AChE compared with that against BuChE were picked out. Tertiary screening was done to choose lipophilic substances by extraction test using ethyl acetate. Lipophilic and low molecular substances

Table 1. Effects of various cholinesterase inhibitors.

Type	Inhibitor	IC_{50} (μM)	
		AChE	BuChE
A	BW 284C51	0.10	92.0
B	Physostigmine	0.03	0.03
	Diazinon	0.01	0.01
C	Tacrine	0.20	0.012
D	*iso*-OMPA	340	0.40

may have higher permeability to across the blood-brain barrier.

By using the above method, culture broths of actinomycetes and fungi isolated mainly from soil samples were subjected to the screening. Table 2 shows the results. Among over seven thousand microorganisms, only two culture broths were picked out as candidates with active principles showing selective inhibition to AChE and lipophilic properties. An actinomycete WK-4164 was found to produce a known organophosphate, cyclophostin (Fig. 1), which has been isolated from *Streptomyces lavendulae* and reported as an inhibitor of AChE in screening for insecticides[11]. On the other hand, a fungus FO-4259 was found to produce two novel compounds, arisugacins A[7] and B and three known compounds, territrems B and C[7] and cyclopenin (Fig. 1). Territrems B and C were reported as tremorigenic metabolites of *Aspergillus terreus*[12,13]. Cyclopenin was reported as an alkaloid of *Penicillium cyclopium* and *Penicillium viridicatum*[14].

Though only 7% of actinomycetes strains passed the secondary screening, 35% of fungus strains passed. This may be due to physostigmine like substances produced by actinomycetes. Physostigmine and its related compounds are type B ChE inhibitors and are produced by plants and actinomycetes[15,16].

Taxonomy of the Arisugacins-producing Organism

Strain FO-4259 was originally isolated from a soil sample collected in Arisugawa-no-miya park at Minato-ku, Tokyo, Japan. From the characters described below, the fungus was identified as *Penicillium* sp.[17]

For identification of the fungus, Czapek yeast extract agar (CYA), malt extract agar, 25% glycerol nitrate agar and yeast extract-soluble starch agar were used. This strain grew rapidly to form moss green to dark green colonies with a diameter of 50~70 mm after incubation for 14 days at 25°C. At 37°C, growth was nil. The colonies' surface was velvety. Reverse color of the colonies were pale yellow brown to dusty yellow. No soluble pigment was produced. The conidial structures were abundantly produced on various agar media. Morphological observation was done under a microscope (Olympus Vanox-S AH-2). When strain FO-4259 was grown on CYA at 25°C for 7 days, the conidiophores were born from substrate hyphae, and penicillia were mainly biverticillate as shown in Fig. 2. Phialides were ampulliform and $7.5\sim10.0\times2.5\sim3.0\,\mu m$. The conidia were globe to subglobe in shape and $2.5\sim3.0\,\mu m$ in diameter, and its surface was rough.

From the above characteristics, strain FO-4259 was identified as *Penicillium* sp. and named *Penicillium* sp. FO-4259. This strain was deposited at the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology Japan, as *Penicillium* sp. FO-4259 with the accession number FERM P-14680.

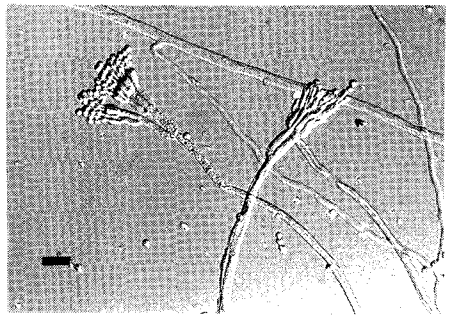
Fermentation

The production of arisugacins A and B were carried out as follows. A loopful of mycelia from a slant culture of *Penicillium* sp. FO-4259 was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a seed medium consisting of glucose 2.0%, Polypepton 0.5%, yeast extract 0.2%, agar 0.1%, KH_2PO_4 0.1% and $MgSO_4\cdot7H_2O$ 0.05% (adjusted to pH 6.0 before sterilization). After incubation at 27°C for 72 hours on a rotary shaker, one per cent of the seed culture was inoculated into each of fifty 1000-ml Roux flasks containing 200 ml of a producing medium consisting of soluble starch 3.0%, glycerol 1.0%, soybean meal 2.0%, dry yeast 0.3%, KCl 0.3%, $CaCO_3$ 0.2%, $MgSO_4\cdot7H_2O$ 0.05% and KH_2PO_4 0.05% (adjusted to pH 6.5 before sterilization). The fermentation was carried out at 27°C for 14 days under static condition. Fig. 3 shows a typical time course of arisugacins A and B production by *Penicillium* sp. FO-4259. The production of arisugacins A and B reached 2.46 μg/ml and 40 μg/ml after day-23 and day-19, respectively. The production was analyzed by HPLC

Table 2. Results of screening for acetylcholinesterase inhibitors from microorganisms.

Microorganism	Number of strains	Screening		
		1st.	2nd.	3rd.
Actinomycetes	4935	173	12	1 (WK-4164)
Fungi	2214	126	44	1 (FO-4259)
Total	7149	299	56	2

Fig. 2. Photo micrograph of penicillia of strain FO-4259 on Czapeck yeast extract. (scale: 20 μm)

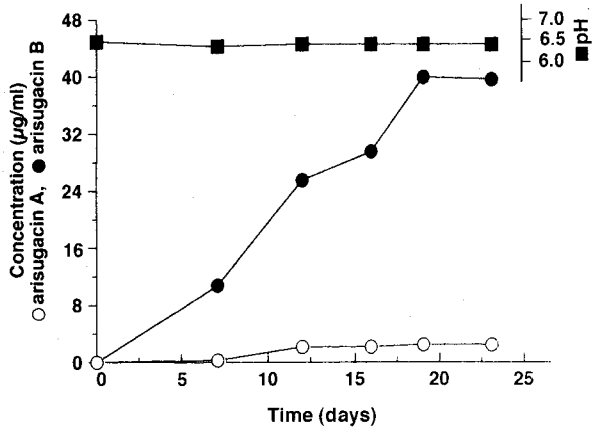


after extraction of whole culture broth with the same volume of ethanol.

Isolation

The isolation procedures for arisugacins A and B and co-produced compounds are summarized in Fig. 4. The whole culture broth (10 liters) was subjected to organic solvent extraction, column chromatography using silica gel and preparative HPLC using ODS to give a white powder of arisugacin A (4.0 mg) and arisugacin B (96 mg), in addition to the known compounds, territrem B (100 mg), territrem C (140 mg) and cyclopenin (220 mg). The NMR studies of arisugacins A and B revealed their structures as 5′-demethoxy-territrem B and 3′,5′-demethoxy-territrem B, respectively (Fig. 1). They are members of the meroterpenoid compounds that have mixed polyketide-terpenoid structure†. Physico-chemical properties and structure elucidation of arisugacins A and B will be reported separately[8]. The other compounds, territrems B and C and cyclopenin were identified from MS and ^{1}H and ^{13}C NMR data.

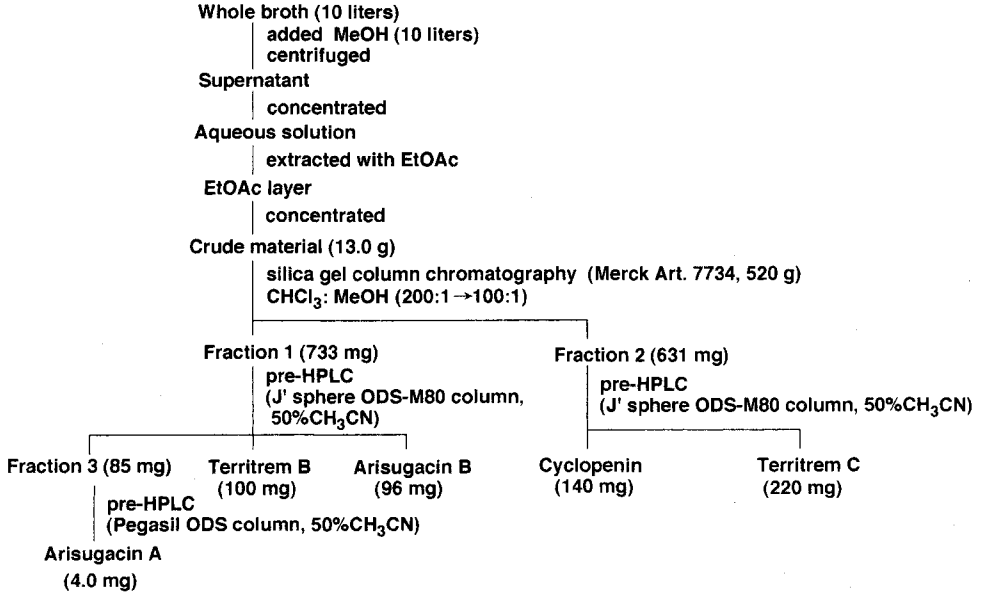
Fig. 3. Typical time course of arisugacins A and B production by *Penicillium* sp. FO-4259.



Biological Activities

The inhibitory activities of arisugacins A and B, territrems B and C, cyclopenin and cyclophostin against AChE and BuChE are shown in Table 3. The inhibitory activity of arisugacin A against AChE was 1.3, 7.6, 6.8, 25.8, 200 and 2040 times stronger than those of cyclophostin, territrems B, C, arisugacin B, tacrine and cyclopenin, respectively. Arisugacins A and B, territrem B and C, cyclopenin and cyclophostin showed more than 21000, 20000, 2632, 3824, 2000 and 34.6-fold potent inhibition, respectively, against AChE than BuChE. Arisugacins A and B, territrems B and C and cyclopenin were highly selective inhibitors against AChE. But, cyclophostin is only moderately selective to AChE. Territrems B and C have been reported as inhibitors of AChE in molluscan neurons[23] and insect head[24]. Cyclophostin has been reported as an inhibitor of AChE in head of housefly and brown planthopper[11]. The selectivity of these compounds against AChE and the inhibitory activity of cyclopenin against AChE are reported for the first time in this paper.

Fig. 4. Isolation procedure for arisugacins A and B and related compounds.



† Structurally related, bioactive natural products include pyripyropenes[18~21], oxalicine A[22].

Table 3. Inhibitory activities of arisugacins, territrems, cyclopenin, cyclophostin and tacrine against acetylcholinesterase and butyrylcholinesterase.

Compound	IC_{50} (nM)	
	AChE	BuChE
Arisugacin A	1.0	>21,000
Arisugacin B	25.8	>516,000
Territrem B	7.6	>20,000
Territrem C	6.8	>26,000
Cyclopenin	2040.0	>4,080,000
Cyclophostin	1.3	45.0
Tacrine	200.0	12.0

Furthermore, we are interested in structure-activity relationships of arisugacins A and B and related compounds (territrems B and C) and their binding to AChE. The structures and the inhibitory activities against AChE of these compounds differ only in the substituents on their aromatic moieties (Fig. 1 and Table 3). In a recent paper, it has been suggested by PENG[25] that the enone and the pyrone groups present in territrem B play an important biological role. Detailed studies on biological activities of arisugacins are in progress.

Further investigations are necessary to evaluate the activities of arisugacins on experimental animal model of AD.

Arisugacins A and B were discovered in the culture of a territrem-producing strain (FO-4259) which was picked out by using this method. Both of arisugacins and territrems were selective AChE inhibitors. Therefore, this screening method was proven to be useful for the rapid screening of selective AChE inhibitors from a large number of culture broths.

Acknowledgment

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References

1) SUMMERS, W. K.; L. V. MAJOVSKI, G. M. MARSH, K. TACHIKI & A. KLING: Oral tetrahydroaminoacridine in long term treatment of senile dementia, Alzheimer type. N. Engl. J. Med. 315: 1241~1245, 1986
2) RELMAN, A. S.: Tacrine as a treatment for Alzheimer's dementia. N. Engl. J. Med. 324: 349~352, 1991
3) THOMSEN, T.; B. ZENDEH, J. P. FISHER & H. KEWITZ: *In vitro* effects of various cholinesterase inhibitors on acetyl- and butyrylcholinesterase of healthy volunteers. Biochem. Pharmacol. 41: 139~141, 1991
4) ELLMAN, G. L.; K. D. COURTNEY, V. ANDRES, Jr. & R. M. FEATHERSTONE: A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Parmacol. 7: 88~95, 1961
5) KIELY, J. S.; W. H. MOOS, M. R. PAVIA, R. D. SCHWARZ & G. L. WOODARD: A silica gel plate-based qualitative assay for acetylcholinesterase activity: A mass method to screen for potential inhibitors. Anal. Biochem. 196: 439~442, 1991
6) DOCTOR, B. P.; L. TOKER, E. ROTH & I. SILMAN: Microtiter assay for acetylcholinesterase. Anal. Biochem. 166: 399~403, 1987
7) ŌMURA, S.; F. KUNO, K. OTOGURO, T. SUNAZUKA, K. SHIOMI, R, MASUMA & Y. IWAI: Arisugacin, a novel and selective inhibitor of acetylcholinesterase from *Penicillium* sp. FO-4259. J. Antibiotics 48: 745~746, 1995
8) KUNO, F.; K. SHIOMI, K. OTOGURO, T. SUNAZUKA & S. ŌMURA: Arisugacins A and B, novel and selective acetylcholinesterase inhibitors from *Penicillium* sp. FO-4259. II. Structure elucidation. J. Antibiotics 49: 748~751, 1996
9) OKABE, H.; K. SAGESAKA, A. NOMA & N. NAKAJIMA: Enzymatic assay of cholinesterase activity. II. Clinical application. Jap. J. Clin. Path. 25: 755~758, 1977
10) TAYLOR, P.: The cholinesterases. J. Biol. Chem. 266: 4025~4028, 1991
11) KUROKAWA, T.; K. SUZUKI, T. HAYAOKA & T. NAKAGAWA: Cyclophostin, acetylcholinesterase inhibitor from *Streptomyces lavendulae*. J. Antibiotics 46: 1315~1318, 1993
12) LING, K. H.; C.-K. YANG & F.-T. PENG: Territrems, tremorgenic mycotoxins of *Aspergillus terreus*. Appl. Environ. Microbiol. 37: 355~357, 1979
13) LING, K. H.; H.-H. LIOU, C.-M. YANG & C.-K. YANG: Isolation, chemical structure, acute toxicity, and some physicochemical properties of territrem C from *Aspergillus terreus*. Appl. Environ. Microbiol. 47: 98 ~100, 1984
14) MOHAMMED, Y. S. & M. LUCKNER: The structure of cyclopenin and cyclopenol, metabolic products from *Penicillium cyclopium* Westling and *Penicillium viridicatum* Westling. Tetrahedron Lett. 28: 1953~1958, 1963
15) IWASA, T.; S. HARADA & Y. SATO: Miticidal antibiotics C-8030 B, C, D and physostigmine produced by *Streptomyces pseudogriseolus* subsp. *iriomotensis* subsp. nov. J. Takeda Res. Lab. 40: 12~26, 1981
16) MURAO, S. & H. HAYASHI: Physostigmine and N^8-norphysostigmine, insecticidal compounds, from *Streptomyces* sp. Agric. Biol. Chem. 50: 523~524, 1986
17) PITT, J. I.: The Genus *Penicillium* and its Teleomorphic States, *Eupenicillium* and *Talaromyces*. Academic press, New York, 1979
18) ŌMURA, S.; H. TOMODA, Y. K. KIM & H. NISHIDA: Pyripyropenes, highly potent inhibitors of acyl-CoA cholesterol acyltransferase produced by *Aspergillus fumigatus*. J. Antibiotics 46: 1168 ~1169, 1993
19) TOMODA, H.; Y. K. KIM, H. NISHIDA, R. MASUMA & S. ŌMURA: Pyripyropenes, novel inhibitor of acyl-CoA: cholesterol acyltransferase produced by *Aspergillus fumigatus*. I. Production, isolation, and biological properties. J. Antibiotics 47: 148 ~153, 1994

20) KIM, Y. K.; H. TOMODA, H. NISHIDA, T. SUNAZUKA, R. OBATA & S. ŌMURA: Pyripyropenes, novel inhibitors of acyl-CoA: cholesterol acyltransferase produced by *Aspergillus fumigatus*. II. Structure elucidation of pyripyropenes A, B, C and D. J. Antibiotics 47: 154 ~ 162, 1994

21) TOMODA, H.; N. TABATA, D.-J. YANG, H. TAKAYANAGI, H. NISHIDA, S. ŌMURA & T. KANEKO: Pyripyropenes, novel ACAT inhibitors produced by *Aspergillus fumigatus*. III. Structure elucidation of pyripyropenes E to L. J. Antibiotics 48: 495~503, 1995

22) UBILLAS, R.; C. L. BARNES, H. GRACZ, G. E. ROTTINGHAUS & M. S. TEMPESTA: X-Ray crystal structure of oxalicine A, a novel alkaloid from *Penicillium cyclopium*. J. Chem. Soc. Chem. Commun. 1618~1619, 1989

23) ARVANOV, V. L.; K. H. LING, R.-C. CHEN & M.-C. TSAI: Effect of territrem-B on cholinergic responses of snail neuron. Neurosci. Lett. 152: 69~71, 1993

24) DOWD, P. F.; F. C. PENG, J. W. CHEN & K. H. LING: Toxicity and anticholinesterase activity of the fungal metabolites territrems to the corn earworm, *Helicoverpa zea*. Entomol. Exp. Appl. 65: 57~64, 1992

25) PENG, F. C.: Acetylcholinesterase inhibition by territrem B derivatives. J. Natural Products 58: 857~862, 1995