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# Terreulactones A, B, C, and D: Novel Acetylcholinesterase Inhibitors Produced by *Aspergillus terreus*

## I. Taxonomy, Fermentation, Isolation and Biological Activities

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In the course of screening for selective inhibitors of acetylcholinesterase from the microbial metabolites, four new meroterpenoid compounds, terreulactones A, B, C and D were isolated from solid state fermentation of *Aspergillus terreus* Fb000501. They showed potent inhibitory activities against acetylcholinesterase with $IC_{50}$ values in range of 0.06~0.42 $\mu$M. In addition, they exhibited more than 500~3000 times selectivity for acetylcholinesterase compared with butyrylcholinesterase.

Alzheimer's disease is a neurodegenerative disorder with the neuropathological characteristics that cholinergic functions declined in the basal forebrain and cortex[1,2]. Accordingly, enhancement of cholinergic neurotransmission have been considered as one potential therapeutic approach against Alzheimer's disease. One treatment strategy to enhance cholinergic function is the use of acetylcholinesterase (AChE, EC 3.1.1.7) inhibitors to increase the amount of acetylcholine present in the synapses between cholinergic neurons[3,4]. Acetylcholinesterase inhibitors like tacrine, one of the most extensively evaluated acetylcholinesterase inhibitors, have been shown to significantly improve cognitive function in Alzheimer's disease[5,6]. Tacrine, however, has been known to cause hepatotoxic side effects by also inhibiting butyrylcholinesterase (BuChE, EC 3.1.1.8) which is found in plasma[7]. In this respect, an inhibitor selective for acetylcholinesterase has attracted particular attention for treatment of the Alzheimer's-type dementia. Arisugacins A~H[8~11], territrems A~C[12], and quinolactacins A1 and A2[13] have been isolated as selective inhibitors of acetylcholinesterase from microbial metabolites. In the course of our screening for selective inhibitors of acetylcholinesterase from microbial metabolites, we isolated four new meroterpenoid compounds named terreulactones A, B, C and D from solid state fermentation of *Aspergillus terreus* Fb000501 (Fig. 1). Terreulactones A, B, C and D are meroterpenoid type compounds that have mixed polyketide-terpenoid structures, which are not common in microbial metabolites. Especially, terreulactone A is a sesquiterpene lactone type meroterpenoid incorporating an uniquely fused lactone skeleton in its sesquiterpene moiety[14]. We report here the taxonomy of the producing strain, fermentation, isolation, and biological activity of terreulactones A, B, C and D. The structure determination will be described in the following paper[15].

### Materials and Methods

#### Chemicals

Acetylcholinesterase (E.C. 3.1.1.7) from electric eel, butyrylcholinesterase (E.C. 3.1.1.8) from horse serum, acetylthiocholine iodide (ATCh), butyrylthiocholine iodide

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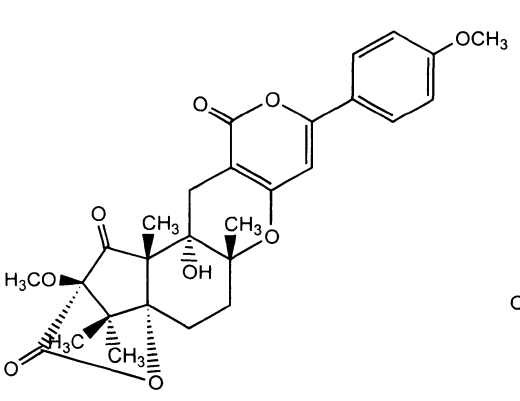
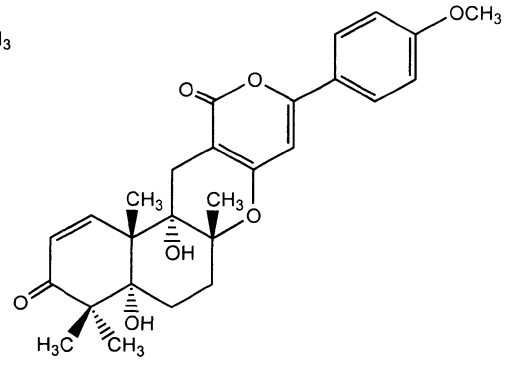
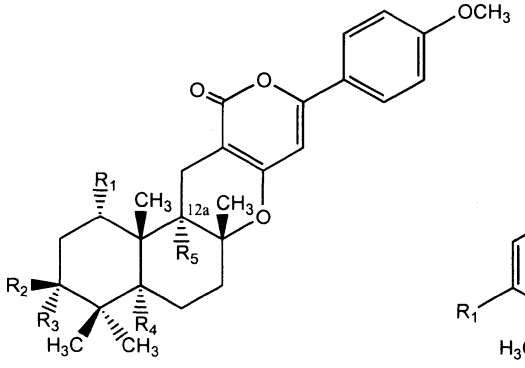
Fig. 1. Relative structures of terreulactones A, B, C and D.



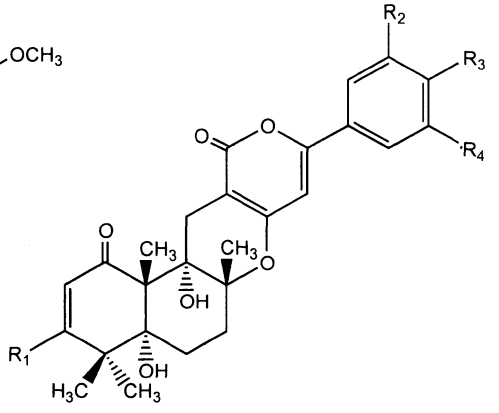
Terreulactone A (**1**)



Terreulactone B (**2**)



| | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ |
|---|---|---|---|---|---|
| Terreulactone C (**3**) | H | =O | | OH | OH |
| Arisugacin C | H | =O | | OH | H |
| Arisugacin D | H | H | $OCOCH_3$ | OH | OH |
| Arisugacin E | H | H | OH | OH | H |
| Arisugacin F | H | OH | H | H | H |
| Arisugacin G | H | =O | | H | H |
| Arisugacin H | OH | H | $OCOCH_3$ | OH | OH |



| | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| Terreulactone D (**4**) | $OCH_3$ | $OCH_3$ | $OCH_3$ | $OCH_3$ |
| Arisugacin A | H | $OCH_3$ | $OCH_3$ | H |
| Arisugacin B | H | H | $OCH_3$ | H |
| Territrem A | H | $-OCH_2-$ | | $OCH_3$ |
| Territrem B | H | $OCH_3$ | $OCH_3$ | $OCH_3$ |
| Territrem C | H | $OCH_3$ | OH | $OCH_3$ |

(BuTCh), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) and tacrine were purchased from Sigma Chemicals Co., Ltd.

## Taxonomy

The producing fungal strain Fb000501 was originally isolated from a soil sample collected in Chile. Taxonomic studies of strain Fb000501 were conducted according to the method of KLICH and PITT[16], and RAPER and FENNELL[17]. The color names used in this study were taken from the color standard of Munsell. For the identification of the fungus, Czapek's solution agar (CZA, Difco), Czapek yeast extract agar (CYA, CZA supplemented with 0.5% yeast extract), and malt extract agar (MEA; malt extract 2.0%, peptone 0.1%, dextrose 2.0%, and agar 2.0%) were used.

## Assay of Acetylcholinesterase and Butyrylcholinesterase

The inhibitory activities against acetylcholinesterase were evaluated according to Ellman's coupled enzyme assay[18] with modification as follows; 0.08 units AChE dissolved in 0.1 M potassium phosphate buffer (pH 7.4) and 5 $\mu$l of a 50% acetone extract of cultured microbes or methanol solution of purified compounds were added to each well of a 96-well plate. After incubation for 3 minutes, ATCh and DTNB dissolved in 0.1 M potassium phosphate buffer (pH 7.4), respectively, were added to final 20 $\mu$M and 30 $\mu$M, respectively, to each well. The reaction was carried

Table 1. Cultural characteristics of strain Fb000501.

| Media* | Diameter of colony (cm) | Conidia | Mycelium | Reverse | Exudate | Pigment |
|---|---|---|---|---|---|---|
| CZA | 25°C 5.0 - 5.4<br>37°C 9.0 - 9.4 | moderate,<br>brownish orange | white, velutinous,<br>with shallow radial furrow<br>with margins thin and irregular | brown | none | amber |
| CYA | 25°C 3.4 - 3.9<br>37°C - | heavy,<br>yellowish brown | white, floccose,<br>radially sulcate | wood brown | hyaline to yellowish | amber |
| MEA | 25°C 2.8 - 3.0<br>37°C 8.0 - 9.0 | moderate,<br>brownish yellow | inconspicuous, white,<br>velutinous | grayish yellow | none | yellowish |

*Strain Fb000501 was cultured for 9 days.

out at room temperature for 5 minutes and the initial rate of the enzyme was analyzed by measuring the formation of 5-thio-2-nitrobenzoate, yellow anion, at 412 nm of UV wavelength with microplate reader (Molecular Devices Co., Ltd.). 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 using 0.16 unit BuChE and 20 $\mu$M BuTCh instead of AchE and ATCh for enzyme and substrate, respectively.

## Result and Discussion

### Screening of the Selective Inhibitors of AChE

The 50% acetone extracts of liquid-culture or solid-culture of microorganisms such as actinomycetes and fungi isolated mainly from soil samples were screened and microorganisms showing over 50% inhibition of AChE were selected. Then, among them, microorganisms that showed more potent inhibitory activity against AChE compared with that against BuChE were picked out. By screening around five thousand microorganisms with the above method, five fungi were selected as candidates with active principles showing selective inhibition of AChE. Among them, four strains turned out to produce known territrem compounds. The fungus Fb000501, however, was found to produce four novel compounds, terreulactones A, B, C and D, but not to produce territems and arisugacins.

### Taxonomy of Producing Strain Fb000501

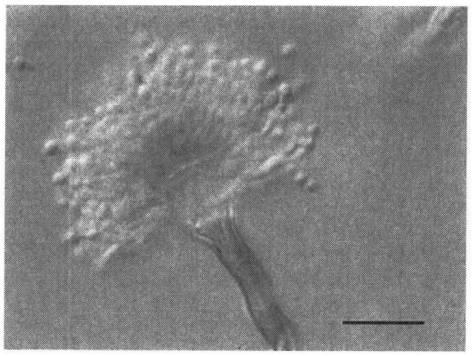
Cultural characteristics of the producing strain Fb000501 were examined after incubation at 25°C and 37°C for 9 days on CYA, CZA, and MEA (Table 1). This fungal strain grew moderately to form brownish orange colonies with a diameter of 5.0~5.4 cm and 9.0~9.4 cm at 25°C and 37°C, respectively, on CZA. The mycelia were white, velutinous and form shallow radial furrow with thin and irregular margins. The reverse color of the colonies was brown and the soluble pigment was amber. The conidial structures were abundantly produced on various agar media. Morphological observation was carried out under a microscope after incubation on CZA at 25°C for 9 days (Fig. 2). The conidiophores were strictly biseriate and borne from submerged hyphae. Conidial heads were in compact column and 120~200 $\mu$m long at maturity. Stipes were smooth-walled and sometimes blackened, 90~170 $\mu$m in length and 43~70 $\mu$m in width. Vesicles were spherical or dome-like, 11~16 $\mu$m in diameter and formed metulae covering the upper half to two thirds of the vesicle tightly packed. The conidia were hyaline, smooth-walled, globose to subglobose and 2.4~2.9×2.2~2.5 $\mu$m in size.

From the above characteristics, strain Fb000501 was identified as *Aspergillus terreus* Thom 1918[17] and named *Aspergillus terreus* Fb000501.

### Fermentation

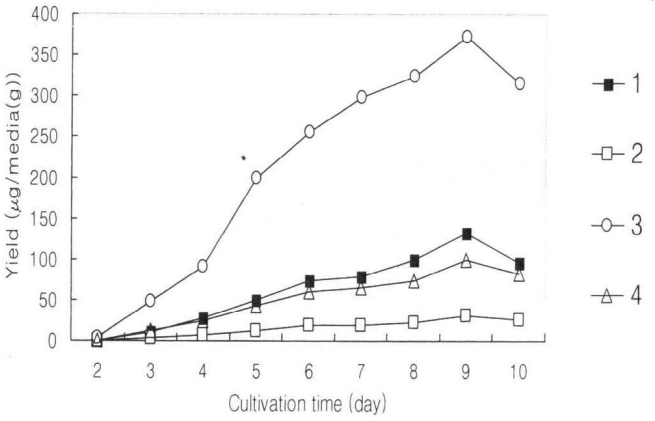
Fermentation was carried out in solid state of moistured wheat-bran because terreulactones A~D were not produced

Fig. 2. Photomicrograph of aspergilla of strain Fb000501.



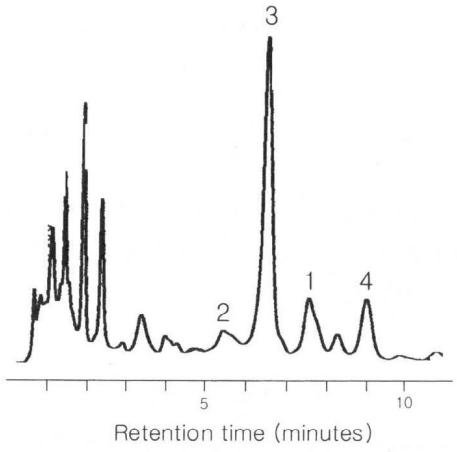
Bar presents 20 $\mu$m.

Fig. 3. Fermentation profiles for the production of terreulactones A (**1**), B (**2**), C (**3**) and D (**4**).




in liquid culture media containing glucose 2%, yeast extract 0.2%, polypeptone 0.5%, $MgSO_4$ 0.05%, and $KH_2PO_4$ 0.1% (pH 5.7 before sterilization). A piece of strain Fb000501 was inoculated from a mature plate culture into 500 ml baffled Erlenmeyer flasks each containing 100 ml of a sterile seed medium with the above composition. After incubation at 28°C for 3 days on a rotary shaker (150 rpm), 7 ml of the seed cultrue was transferred to 500 ml Erlenmeyer flasks containing 90 g of moistured wheat-bran. The fermentation was carried out at 28°C for 10 days under a stationary condition. The typical time course production of terreulactones A, B, C and D in 500 ml Erlenmeyer flask is shown in Fig. 3. The production of terreulactones A, B, C and D began at day 3 and the maximal production reached at day 9 with the yields of 131, 30, 373 and 100 $\mu$g/g, respectively. Beyond day 9, the production of terreulactones A, B, C and D started to decrease. Fig. 4 shows the HPLC chromatogram for a chloroform extract of 80% acetone extract of solid culture after 9 days of cultivation.

Fig. 4. HPLC chromatogram of a solid culture after the cultivation for 9 days.

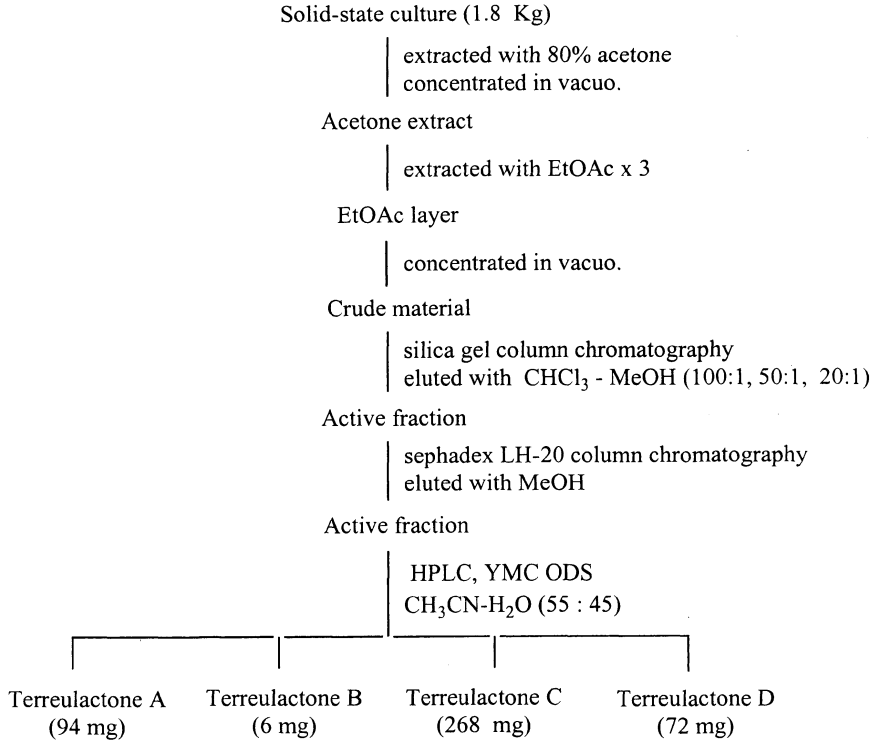



The $CHCl_3$ extract of 80% acetone extract of solid culture was dissolved in MeOH and analyzed by a Cosmosil $C_{18}$ column (4.6×150 nm, 0.9 ml/minute, UV at 300 nm) eluted with $CH_3CN$ - $H_2O$ (50 : 50).
**1**=Terreulactone A, **2**=Terreulactone B,
**3**=Terreulactone C, **4**=Terreulactone D

## Isolation

The isolation procedure of terreulactones A, B, C and D is schematically shown in Fig. 5. The fermented whole medium (1.8 kg) was extracted with 80% acetone and the extract was concentrated *in vacuo* to an aqueous solution, which was then extracted with an equal volume of EtOAc three times. EtOAc extract was concentrated *in vacuo* to dryness. The crude extract was subjected to $SiO_2$ (Merck Art No. 7734.9025) column chromatography followed by stepwise elution with $CHCl_3$ - MeOH (100 : 1, 50 : 1, 20 : 1). The active fractions eluted with $CHCl_3$ - MeOH (100 : 1 and 50 : 1) were pooled and concentrated *in vacuo* to give an oily residue. The residue was applied again to a Sephadex LH-20 and then eluted with MeOH. The active fraction dissolved in MeOH was further purified by reverse phase HPLC column (20×250 mm, YMC $C_{18}$) chromatography with a photodiode array detector. The column was eluted with $CH_3CN$ - $H_2O$ (55 : 45) at a flow rate of 8 ml/minutes

Fig. 5. Isolation procedures of terreulactones A (**1**), B (**2**), C (**3**) and D (**4**).




to afford terreulactones A (94 mg), B (6 mg), C (268 mg) and D (72 mg) at retention times of 22.4, 16.7,19.8 and 25.5 minutes, respectively, as white powders.

## Biological Activity

The inhibitory activities of terreulactones A, B, C and D against acetylcholinesterase are shown in Fig. 6. Terreulactones A, B, C and D inhibited acetylcholinesterase in a dose-dependent fashion with $IC_{50}$ ($\mu$M) values of 0.23, 0.09, 0.06 and 0.42 $\mu$M, respectively. The inhibitory activity of terreulactone C against acetylcholinesterase was most potent with 3.8, 1.5, 7 and 1.5 times stronger activity than those of terreulactones A, B, D and tacrine, respectively. Terreulactones A, B, C and D, however, did not inhibit butyrylcholinesterase even at 200 $\mu$M (Table 2). Therefore, terreulactones A, B, C and D showed more than 869, 2222, 3333, and 476 times, respectively, potent inhibitory activity against AChE compared with that against BuChE while tacrine, as a positive control, had a low selectivity with a stronger inhibitory activity on butyrylcholinestearse ($IC_{50}$ ($\mu$M); 0.01) rather than acetylcholinesterase ($IC_{50}$ ($\mu$M) ; 0.09) in this assay system. By Lineweaver-Burk plot analysis, terreulactone C exhibited noncompetitive inhibition with substrate as shown in Fig. 7 and its $K_i$ and $K_m$ values for acetylcholinesterase were $7.0\times10^{-8}$ and $2.0\times10^{-5}$ M, respectively. And by a plot of Vmax *versus* amount of enzyme added, terreulactone C exhibited irreversible inhibition (data not shown). Also other

Fig. 6. Inhibitory activity of terreulactones A (**1**), B (**2**), C (**3**) and D (**4**) on acetylcholinesterase.

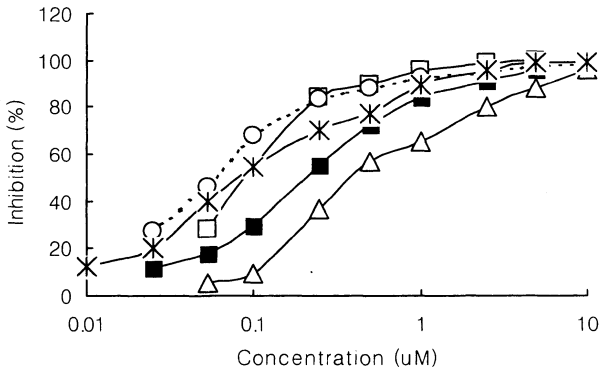
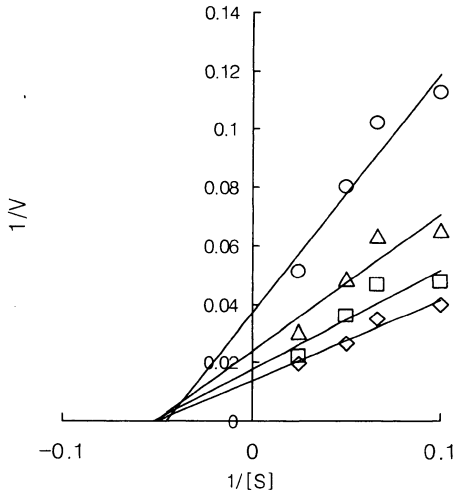


Table 2. Inhibitory activities of terreulactones A, B, C and D against acetylcholinesterase and butyrylcholinesterase.

| | $IC_{50}$ (μM) | | Selectivity (BuChE/AChE) |
|---|---|---|---|
| | AChE | BuChE | |
| Terreulactone A | 0.23 | > 200 | > 869 |
| Terreulactone B | 0.09 | > 200 | > 2222 |
| Terreulactone C | 0.06 | > 200 | > 3333 |
| Terreulactone D | 0.42 | > 200 | > 476 |
| Tacrine | 0.09 | 0.01 | 0.1 |

terreulactones showed noncompetitive and irreversible inhibition with substrate like terreulactone C.

Fig. 7. Lineweaver-Burk plot of inhibition of acetylcholinesterase by terreulactone C.




◇: 0 M, □: 0.0025 μM, △: 0.05 μM, ○: 0.1 μM

## Discussion

In the course of screening for selective inhibitors of acetylcholinesterase, we discovered four new compounds, terreulactones A, B, C and D, which were isolated from the solid-fermented culture of *Aspergillus terreus* Fb000501. Terreulactones A, B, C and D are meroterpenoid type compounds that have mixed polyketide-terpenoid structures, which are not common in microbial metabolites. Especially, terreulactone A is a new meroterpenoid incorporating an uniquely fused lactone skeleton in its sesquiterpene moiety. Terreulactone A is the first sesquiterpene lactone type meroterpenoid of microbial origin as far as I know. So far a few meroterpenoid such as arisugacins A~H[8~11], territrems A~C[12], pyripyropene[19] and oxalicine[20] were isolated from microbial metabolites. Arisugacins A and B, territrems A~C, pyripyropene and oxalicine were produced by *Penicillium* sp. FO-4259, *Aspergillus terreus*, *Aspergillus fumigatus*, and *Penicillium oxalicum*, respectively. Interestingly, arisugacins C~H were isolated only from the mutant strain of *Penicillium* sp. FO-4259, an arisugacins A and B-producing strain. Since the structures of terreulactones A~D including stereochemistry are related to those of arisugacins and territrems with acetylcholinesterase inhibitory activity, terreulactones seem to be biogenetically related to arisugacins. More interestingly, arisugacins and territrems were not detected in this study. ŌMURA *et al.* have reported the total synthesis of arisugacin A[21] and pyripyropene A[22] to make a variety of analogs to clarify the structure-activity relationships. It has been suggested by PENG that the enone and pyrone groups present in territrem B play an important biological role[23]. And CHEN revealed that territrem B inhibits acetylcholinesterase with a noncovalent yet irreversible binding mechanism[24]. But further investigations are necessary to evaluate the structure-activity relationships, binding mechanism to acetylcholinesterase, and activities on experimental animal model.

Acknowledgment

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