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SPF-5506-A_4, a New Peptaibol Inhibitor of Amyloid β-Peptide Formation Produced by *Trichoderma* sp.

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Abstract A new peptaibol compound, SPF-5506-A_4, was isolated from the fermentation broth of *Trichoderma* sp. SPF-5506. The chemical structure of the 14-residue peptide was determined by MS, NMR and amino acid sequence analyses. The absolute configuration of amino acid residues in the acid hydrolysate was determined by Marfey's method. The structure of SPF-5506-A_4 was established as Ac-Aib-L-Asn-L-Ile-Aib-L-Pro-L-Ser-L-Ile-Aib-L-Pro-L-Leu-L-Leu-Aib-L-Pro-L-leucinol. The compound inhibited amyloid β-peptide formation in primary guinea pig cerebral cortex neuron cell culture dose-dependently with an IC_{50} of 0.1 μg/ml. Cytotoxicity was not observed at concentrations of <3 μg/ml.

Keywords SPF-5506-A_4, peptaibol, amyloid β-peptide, inhibition, *Trichoderma*

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Introduction

Peptaibols are linear peptides composed of up to about 20 amino acids and characterized by an acylated *N*-terminus, an amino alcohol at the *C*-terminus and the presence of several α-dialkylated amino acids, mainly α-aminoisobutyric acid (Aib). A number of natural peptaibols have been reported from strains of *Trichoderma* and other fungal genera [1, 2]. Peptaibols are known to have various biological activities such as antimicrobial, antimycoplasmic, antiviral and antitumor activities [3~6].

Alzheimer's disease (AD) is a progressive neurodegenerative dementia, characterized by cerebral deposition of neurofibrillary tangles and β-amyloid plaques that are comprised predominantly of an aggregate of a 4 kDa peptide, amyloid β-peptide (Aβ) [7, 8]. Increased Aβ accumulation, aggregation, and deposition in the brain are key events in the pathogenesis of AD [9].

In the course of our screening for biologically active compounds from microbial sources, a fungal strain SPF-5506 was found to produce a new peptide compound named SPF-5506-A_4 (Fig. 1). The compound inhibited Aβ formation in cultured neuron cells at sub-micromolar concentrations that did not exhibit cytotoxicity. In this paper we report the taxonomy of the producing strain, fermentation, isolation, structure elucidation, and biological activity of SPF-5506-A_4.

Materials and Methods

General

UV spectra were recorded on a Hitachi U-2000

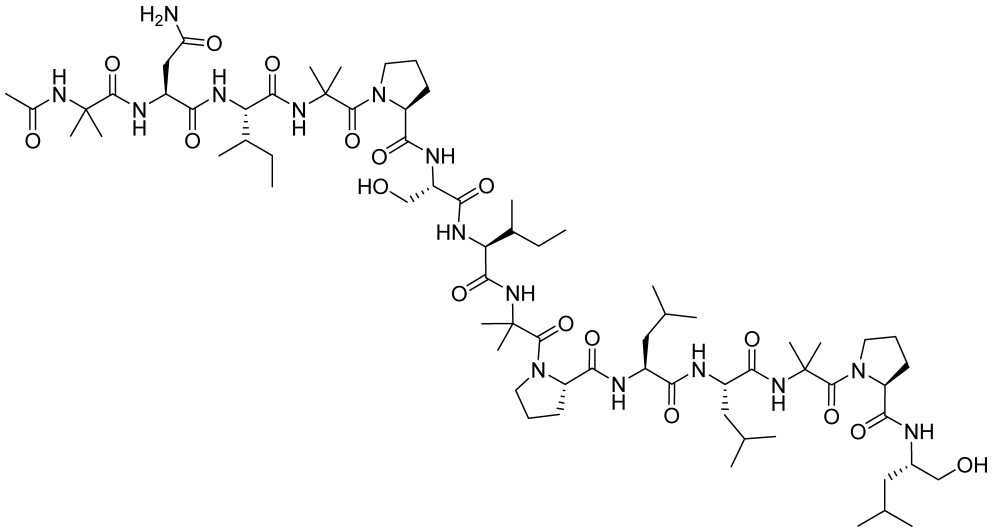

Fig. 1 Structure of SPF-5506-A_4.

spectrophotometer. FAB-MS spectra were obtained on a JEOL JMS-SX102A spectrometer. NMR spectra were recorded on a JEOL JNM α-500 spectrometer at 500 and 125 MHz for ^{1}H and ^{13}C, respectively. The chemical shifts are given in ppm referred to DMSO-d_6 as 2.49 ppm (^{1}H) and 39.50 ppm (^{13}C).

Microorganism

The producing strain SPF-5506 was isolated from a soil sample collected in Kyoto Prefecture, Japan. The strain has been deposited at the Patent Microorganisms Depositary, National Institute of Technology and Evaluation, Japan under the accession number FERM P-19571.

Taxonomy

Taxonomic study of strain SPF-5506 was performed according to the method of Rifai [10]. The media used were malt extract agar (MEA, malt extract 2.0%, agar 2.0%) and potato dextrose agar (PDA, Nihon Pharmaceutical). Morphological characteristics were examined by light microscope using Amma's lactophenol (phenol 10 g, lactic acid 10 g, glycerol 20 g, water 10 g). Color names were determined by using the Color Tone Manual [11].

Fermentation

A slant culture of the strain SPF-5506 was inoculated into 500-ml Sakaguchi flasks, each containing 75 ml of liquid medium composed of glucose 2.0%, sucrose 5.0%, cotton seed flour 2.0%, $NaNO_3$ 0.1%, L-His 0.1%, K_2HPO_4 0.05%, KCl 0.07%, $MgSO_4 \cdot 7H_2O$ 0.0014%, pH 7.0, and cultured for 6 days at 27°C with reciprocal shaking at 130 rpm.

Isolation

The fermentation broth (1.5 liters) of strain SPF-5506 cultured at 27°C for 6 days was extracted with 1.5 liters of 1-BuOH and the extract was concentrated under reduced pressure to yield 2.8 g of oily material. The material was dissolved in 50 ml of MeOH and applied to a column of Toyopearl HW-40F (Tosoh, 30×500 mm) and eluted with MeOH. Active fractions (653 mg) were pooled and injected into preparative HPLC equipped with Wakopak Wakosil-II5C18HG-Prep columns (30×100+30×250 mm, Wako Pure Chemical Industries, Ltd.). The elution was performed with 1.0% aqueous formic acid - acetonitrile (MeCN) (50 : 50 to 10 : 90 linear gradient in 40 minutes) at a flow rate of 20 ml/minute and detection of UV absorption at 225 nm. Active fractions (Rt: 35.7 minutes) were collected and evaporated under reduced pressure to afford SPF-5506-A_4 (5.0 mg) as a white powder.

Analysis of Amino Acid Composition

SPF-5506-A_4 (1 mg) was hydrolyzed in 0.5 ml of 6 N HCl for 20 hours at 110°C. The solution was evaporated under reduced pressure and dried under high vacuum. The residue was dissolved in 0.5 ml of water. Amino acid composition analysis was performed using a Hitachi L-8500 amino acid analyzer with the ninhydrin method. The concentration of each amino acid was calculated based on the peak area in comparison with an authentic amino acid mixture solution.

Determination of D/L Configuration of Amino Acids

The D/L configuration of the amino acids in the acid hydrolysate of SPF-5506-A_4 was determined according to Marfey's method [12]. Briefly, 25 μl of the aqueous

solution of the hydrolysate was combined with 50 μl of 1.0% 1-fluoro-2,4-dinitrophenyl-5-L-alanineamide (FDAA) in Me_2CO and 20 μl of 1 M $NaHCO_3$, and heated at 40°C for 1 hour. After cooling to room temperature, the reaction mixture was combined with 40 μl of 2 N HCl and 200 μl of MeCN. The samples (10 μl) were analyzed by reversed-phase HPLC using a Wakopak Wakosil-II5C18HG column (4.6×150 mm, Wako Pure Chemical Industries) with 0.05% aqueous TFA - MeCN (90 : 10 to 50 : 50 linear gradient in 60 minutes) at a flow rate of 1.0 ml/minute and detection of UV absorption at 340 nm. The retention times of the derivatized hydrolysate were compared with standards and confirmed by co-injection. Retention times (minute) of FDAA-derivatized standards were as follows: L-Ser (21.50), D-Ser (21.75), L-Asp (23.54), D-Asp (24.97), L-Pro (29.48), D-Pro (31.20), L-leucinol (40.44), D-leucinol (48.11), L-Ile (42.02), D-Ile (48.36), L-Leu (43.39), D-Leu (49.20).

Aβ Formation in Primary Neuron Cell Culture

The cerebral cortices from E28-35 fetal SLC-Hartley guinea pig embryos were dissected and enzymatically dissociated with papain in Dulbecco's phosphate-buffered saline (DPBS), then triturated and allowed to settle to produce a single cell suspension. The cell suspension was diluted to 6×10^5 cells/ml with Minimum Essential Medium (Invitrogen) supplemented with 2.0% fetal bovine serum, 5 mM glucose, 24 mM $NaHCO_3$ and 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid (HEPES). The cells were distributed at 6×10^4 cells/well in poly-D-lysine-coated 96-well culture plates and incubated at 37°C for 2 hours in a CO_2 incubator (5.0% CO_2, 95% air, saturated humidity). After incubation, the medium was replaced by Neurobasal Medium (Invitrogen) supplemented with 0.5 mM L-Gln and 2% B-27 Supplement (50×) (Invitrogen), and incubated at 37°C for 8 days in a CO_2 incubator. Media were changed every 3 days. At day 9, media were discarded, and fresh media containing test samples were added, and incubated for 2 days in a CO_2 incubator. After incubation, the media were taken and frozen at −80°C for subsequent Aβ ELISA analysis and cytotoxicity assay.

Determination of Aβ by ELISA

Test samples were diluted with blocking buffer (1.0% Block Ace (Dainippon Sumitomo Pharma) in DPBS) and 50 μl aliquots were placed on 96-well ELISA plates precoated with 4G8 anti-Aβ monoclonal antibody (Senetek), and incubated at 4°C for 2 days. Wells were washed 4 times with PBS-T (0.02% Tween 20 in DPBS) and combined with 50 μl of 2.5 μg/ml 6E10 anti-Aβ monoclonal antibody (Senetek) in blocking buffer. After incubation overnight at 4°C, wells were washed 4 times with PBS-T, mixed with 50 μl of streptavidin-horseradish peroxidase conjugate (Amersham Biosciences, 1/5000 dilution in blocking buffer), and incubated for 1 hour at room temperature. After washing 4 times with PBS-T, 100 μl of substrate solution (Immunopure TMB Substrate, Pierce) were added and incubated at room temperature for 10 minutes. The reaction was stopped by addition of 100 μl of 2 N H_2SO_4, and absorbance was measured on a plate reader at 450 nm. The concentration of Aβ was calculated by comparison with a standard sample (Aβ 1-40, Sigma-Aldrich).

Determination of Cytotoxicity

Cytotoxicity of test compound toward the cultured primary guinea pig cerebral cortex neuron cells was determined by measuring lactate dehydrogenase (LDH) activity in the conditioned media of the cells using MTX-LDH Assay kit (Kyokuto Pharmaceutical). The conditioned media of the cells fully lysed by addition of 0.2% Tween 20 were used as 100% cytotoxicity control.

Results

Taxonomy

The growth of strain SPF-5506 on agar media was rapid. Colonies grown on MEA and PDA at 25°C for 4 days were 9 cm in diameter. The colony surface was floccose and white at first, but turning yellowish green after sporulation. The reverse side of colony was colorless or pale yellow. Soluble pigment was not observed. The hyphae were 3～5 μm in diameter with smooth walls. Conidiophores were relatively long and sparingly branched. Phialides were in whorl of 2 or 3 and ampulliform. Conidia were yellowish green, smooth walled, globose or subglobose and 3.0～3.5×3.5～4.0 μm in size. Based on these characteristics, the strain SPF-5506 was identified as a member of the genus *Trichoderma*, and named *Trichoderma* sp. SPF-5506.

Structure Determination

The physico-chemical properties of SPF-5506-A_4 are summarized in Table 1. The molecular formula was determined to be $C_{70}H_{121}N_{15}O_{17}$ by HRFAB-MS and ^1H and ^{13}C NMR. Amino acid analysis of the total acidic hydrolysate revealed the presence of Aib, Asx (Asn or Asp), Ile, Leu, Pro and Ser in a molar ratio of 4 : 1 : 2 : 2 : 3 : 1. The absence of reactivity with ninhydrin and the presence of sharp singlet peak at 1.9 ppm in ^1H

Table 1 Physico-chemical properties of SPF-5506-A_4

Appearance	White powder
Molecular formula	$C_{70}H_{121}N_{15}O_{17}$
FAB-MS (*m*/*z*, pos)	1445 $(M+H)^+$, 1329, 1231, 1146, 1033, 920, 822, 737, 624, 537, 440, 355, 242, 128
FAB-MS (*m*/*z*, neg)	1443 $(M-H)^-$
HRFAB-MS (*m*/*z*, pos)	
Found:	1444.9158 $(M+H)^+$
Calcd.:	1444.9143
UV λ_{max} (MeOH)	End absorption
Solubility	
Soluble:	MeOH, DMSO
Insoluble:	H_2O, *n*-Hexane
Color reaction	
Ninhydrin	Negative
Amino acid analysis (molar ratio)	Aib (4), Asx (1), Ile (2), Leu (2), Pro (3), Ser (1)
Configuration of amino acids in acid hydrolysate	L-Asp, L-Ile, L-Leu, L-Pro, L-Ser, L-leucinol

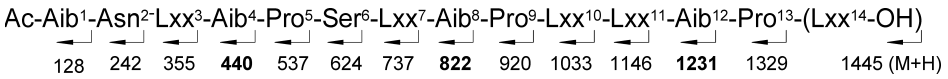

Fig. 2 Positive ion FAB-MS fragmentation of SPF-5506-A_4.

Fragments with strong ion peaks are indicated in bold. Lxx represents Leu or Ile.

NMR suggested the presence of an acetylated *N*-terminal residue. Low resolution FAB-MS data contained a set of fragment peaks and suggested that the *N*-terminal residue had mass unit of 128 Da (=acetyl-Aib). A possible peptide sequence was deduced by the FAB-MS data as shown in Fig. 2. Three major fragment ions observed at *m*/*z* 440, 822 and 1231 were in agreement with the preferential cleavage at the Aib-Pro bond, which is typical of peptaibols as in the case of harzianins [13], trichorozins [14] and trichorovins [15].

Detailed analysis of COSY, HMBC and HOHAHA spectra enabled the assignment of all proton and carbon NMR resonances (Table 2). The *C*-terminal residue with a mass unit of 116 Da was determined to be leucinol. The Asx was determined to be Asn. The *N*-terminal residue was confirmed to be acetyl-Aib. The presence of NOE correlations among the amide protons of Aib-1 (δ_H 8.62), Asn-2 (δ_H 8.32), Ile-3 (δ_H 7.62) and Aib-4 (δ_H 7.93) revealed the sequence Aib-Asn-Ile-Aib (Fig. 3). Similarly, the sequences Ser-Ile-Aib and Leu-Leu-Aib were established by NOESY. The NOE correlations of the δ-methylene protons of the three Pro (Pro-5, 9 and 13) with the amide and methyl protons of three Aib (Aib-4, 8 and 12) extended the three partial sequences as Aib-Asn-Ile-Aib-Pro, Ser-Ile-Aib-Pro and Leu-Leu-Aib-Pro. The entire sequence, Ac-Aib-Asn-Ile-Aib-Pro-Ser-Ile-Aib-Pro-Leu-Leu-Aib-Pro-leucinol, was completely established by the following HMBC correlations: 1) the carbonyl carbon of Pro-5 (δ_C 173.13) with the amide (δ_H 7.52) and α methine (δ_H 4.10) protons of Ser-6; 2) the carbonyl carbon (δ_C 172.81) of Pro-9 with the amide proton (δ_H 7.52) of Leu-10; and 3) the carbonyl carbon (δ_C 170.86) of Pro-13 with the amide proton (δ_H 7.04) of leucinol-14 (Fig. 3). This was consistent with the deduced sequence shown in Fig. 2.

The configuration of each amino acid in the total acid hydrolysate was analyzed by Marfey's method. All the amino acid residues from SPF-5506-A_4 showed the L configuration. Thus the structure of SPF-5506-A_4 was determined to be Ac-Aib-L-Asn-L-Ile-Aib-L-Pro-L-Ser-L-Ile-Aib-L-Pro-L-Leu-L-Leu-Aib-L-Pro-L-leucinol.

Biological Activity

SPF-5506-A_4 inhibited Aβ formation in cultured guinea pig cerebral cortex neuron cells dose-dependently with an IC_{50} of 0.1 μg/ml (Fig. 4). The compound did not show cytotoxicity toward the cells at concentrations of <3 μg/ml.

Table 2 ^{1}H and ^{13}C NMR data for SPF-5506-A_4 in DMSO-d_6

Position		δ_C	δ_H	Position		δ_C	δ_H
Acetyl	CO	170.99		Aib-8	α	56.07	
	CH_3	22.87	1.90 (3H, s)		β	23.04	1.44 (3H, s)
Aib-1	α	55.82			β	25.56	1.37[c] (3H, s)
	β	23.86	1.35 (3H, s)		CO	172.94	
	β	26.06	1.31[a] (3H, s)		NH		7.94 (s)
	CO	174.87		Pro-9	α	63.07	4.24 (m)
	NH		8.62 (s)		β	28.21	1.71 (m), 2.21 (m)
Asn-2	α	51.53	4.31 (m)		γ	25.50	1.86 (2H, m)
	β	35.40	2.03 (m), 2.07 (m)		δ	48.30	3.28 (m), 3.69 (m)
	γ-CO	172.15			CO	172.81	
	δ-NH_2		7.05 (s), 7.59 (br s)	Leu-10	α	53.10	3.95 (m)
	CO	171.54			β	38.66	1.57 (m), 1.83 (m)
	NH		8.32 (d, 6.4)		γ	24.51	1.65 (m)
Ile-3	α	57.22[b]	4.32 (m)		δ	20.57	0.82 (3H, t, 6.7)
	β	35.91	1.88 (m)		δ	22.96	0.91 (3H, t, 6.4)
	γ	24.48	1.25 (m), 1.69 (m)		CO	172.54	
	δ	10.31	0.71 (3H, t, 6.3)		NH		7.52 (d, 4.4)
	β-CH_3	15.14	0.82 (3H, t, 6.7)	Leu-11	α	50.72	4.28 (m)
	CO	172.22			β	39.59	1.37 (m), 1.56 (m)
	NH		7.62 (d, 9.1)		γ	24.12	1.65 (m)
Aib-4	α	56.07			δ	20.25	0.73 (3H, t, 5.8)
	β	23.29	1.44 (3H, s)		δ	22.74[e]	0.78 (3H, t, 6.8)
	β	25.56	1.38[c] (3H, s)		CO	172.34	
	CO	172.83			NH		7.09 (d, 9.1)
	NH		7.93 (s)	Aib-12	α	55.84	
Pro-5	α	63.25	4.18 (m)		β	23.79	1.35 (3H, s)
	β	28.62[d]	1.61 (m), 2.25 (m)		β	25.50	1.32[a] (3H, s)
	γ	25.67	1.86 (2H, m)		CO	171.34	
	δ	48.54	3.43 (m), 3.69 (m)		NH		7.48 (s)
	CO	173.13		Pro-13	α	61.95	4.24 (m)
Ser-6	α	58.04	4.10 (m)		β	28.60[d]	1.61 (m), 2.06 (m)
	β	60.90	3.71 (m), 3.76 (m)		γ	25.29	1.72 (2H, m)
	OH		4.92 (t, 5.5)		δ	47.90	3.17 (m), 3.62 (m)
	CO	170.33			CO	170.86	
	NH		7.52 (d, 4.0)	Leucinol-14	α	48.72	3.72 (m)
Ile-7	α	57.27[b]	4.19 (m)		β	39.21	1.37 (m), 1.56 (m)
	β	35.63	1.88 (m)		γ	24.44	1.65 (m)
	γ	24.48	1.18 (m), 1.69 (m)		δ	21.54[e]	0.78 (3H, d, 6.7)
	δ	11.34	0.78 (3H, t, 6.3)		δ	23.64	0.87 (3H, d, 6.7)
	β-CH_3	15.40	0.82 (3H, t, 6.7)		CH_2-OH	63.91	3.10 (m), 3.29 (m)
	CO	172.32			NH		7.04 (d, 7.6)
	NH		7.18 (d, 9.2)				

[a~e] Assignments may be interchanged. Chemical shifts are given in ppm and coupling constants (J) in Hz.

Discussion

We have isolated a new peptaibol SPF-5506-A_4 from the fermentation broth of *Trichoderma* sp. SPF-5506 as a potent inhibitor of Aβ formation in cultured neuron cells. To our knowledge this is the first report of peptaibols having inhibitory activity against Aβ formation. Aβ is known to be formed by intracellular proteolytic cleavage of amyloid precursor protein (APP) by β- and γ-secretases

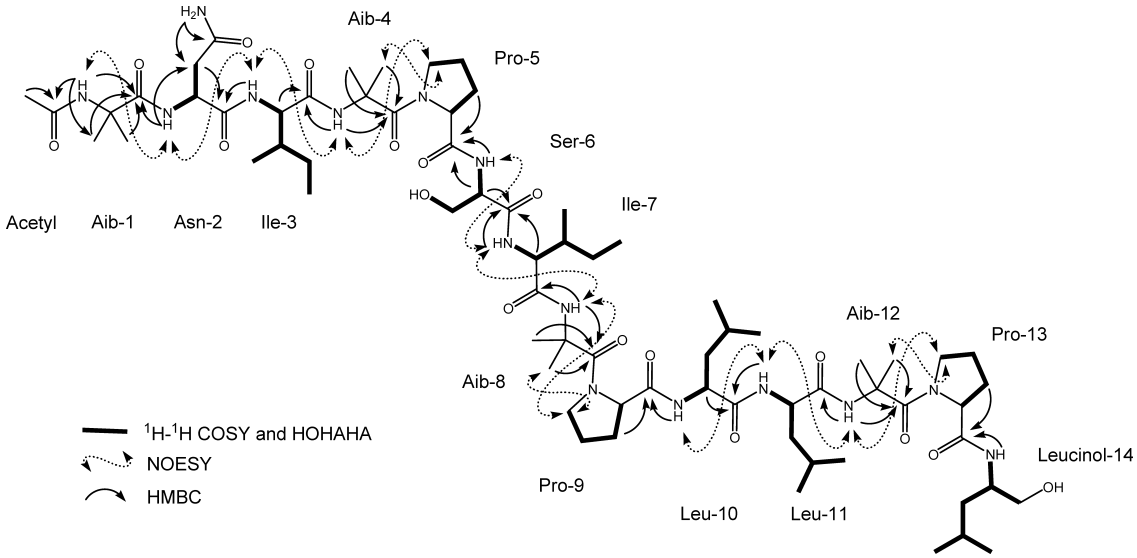

Fig. 3 ^{1}H-^{1}H COSY, HMBC, HOHAHA and NOE correlations observed in SPF-5506-A$_4$.

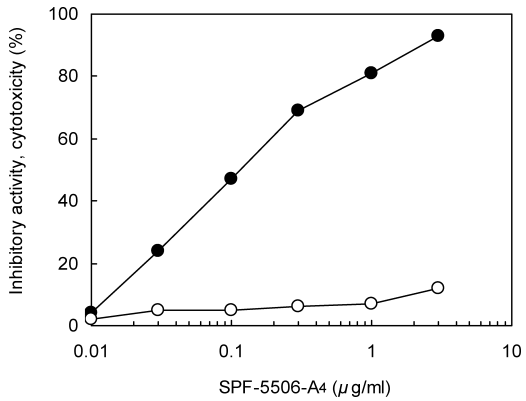

Fig. 4 Concentration-response of SPF-5506-A$_4$ against Aβ formation (●) and cytotoxicity (○) in primary guinea pig neuron cell culture assay.

and secreted from the cells [16]. In our study, the production of APP within the cell was not inhibited by SPF-5506-A$_4$ at a dose at which Aβ production was well inhibited (data not shown). Therefore, SPF-5506-A$_4$ may exert its activity by inhibiting enzymatic processing of APP or secretion pathway of Aβ. Peptaibols are known to interact with lipid bilayers and increase membrane permeability [17, 18]. SPF-5506-A$_4$ may affect membrane environment, leading to the inhibition of production or secretion of Aβ. The exact mechanism(s) of the inhibition remains to be elucidated.

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