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INHIBITORY ACTIVITY OF HISPIDIN DERIVATIVES ISOLATED FROM *INONOTUS OBLIQUUS* ON AMYLOID β AGGREGATION

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Abstract – Alzheimer's disease (AD), the most common neurodegenerative disease, is characterized by the aggregation of the 42-mer amyloid β-protein (Aβ42). The inhibition of Aβ aggregation is one of the therapeutic strategies for AD. We recently reported that caffeoylquinic acids and phenylethanoid glycosides possessing two or more catechol moieties strongly inhibit the aggregation of Aβ. We report on the inhibitory effect of hispidin derivatives, which were isolated from *Inonotus obliquus*, on the aggregation of Aβ42. Hispidin derivatives exhibited anti-aggregation activity in a dose-dependent manner. The structure–activity relation of hispidin derivatives validated that the presence of a catechol moiety is essential for the inhibitory activity.

INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative disease affecting a large number of elderly people. Currently, there is no fundamental cure for AD. Recently, the amyloid hypothesis was proposed as a pathogenic mechanism for AD progression.[1] It is characterized by the self-assembly of 40- or 42-mer amyloid β-proteins (Aβ40 or Aβ42) into senile plaques. The Aβ peptides are produced from the amyloid β precursor protein (APP) by the β- and γ-secretases.[2,3] Aβ40 and Aβ42 aggregate as oligomers and fibrils, respectively, through the formation of β-sheet structures and are deposited in the cerebral cortex. They cause neuronal cell death and atrophy of the hippocampus, finally leading to AD.

Aβ42 has a greater propensity to oligomerize and higher neurotoxicity than Aβ40, and therefore, Aβ42 plays a more important role in the onset of AD.[1,4] Consequently, the inhibition of Aβ aggregation by small molecules from natural products contributes to the prevention and treatment of AD.

In our previous research, we revealed that bioactive polyphenols containing catechol moieties, such as caffeoylquinic acids (CQAs) and phenylethanoid glycosides, inhibit the aggregation of Aβ42 and suggested that catechol moieties are essential for the inhibition of aggregation.[5,6]

Inonotus obliquus, a medicinal mushroom found in cold regions like Siberia, is used as a traditional medicine for cancer with minimal side effects.[7] Several bioactive compounds have been isolated from this mushroom.[8] However, the effect of *I. obliquus* on AD is not well understood. Hispidin derivatives, which are the related compounds of hispidin (**5**), are one class of bioactive compounds isolated from *I. obliquus* and are known to possess strong antioxidant activity.[9] As these compounds contain catechol moieties, they can potentially show inhibitory activity on Aβ aggregation.

In this research, we isolated several hispidin derivatives from *I. obliquus* and investigated their anti-aggregation activity on Aβ.

RESULTS AND DISCUSSION

Isolation of hispidin derivatives from *I. obliquus*

I. obliquus (dry weight: 200 g) was extracted with MeOH, and the MeOH extract (5.0 g) was partitioned between EtOAc and H_2O. The EtOAc-soluble material (2.6 g) was partitioned between hexane and 90% MeOH. The 90% MeOH-soluble material (1.6 g) was separated *via* gel filtration and reverse-phase HPLC to yield phelligridin D (**1**; 0.9 mg), phelligridin E (**2**; 0.1 mg), phelligridin G (**3**; 2.6 mg), and phellibaumin A (**4**; 0.3 mg). The structures of compounds **1**–**4** were confirmed by NMR spectral analyses.[10-12]

Inhibitory effect of compounds 1–9 on Aβ aggregation

Thioflavin-T (Th-T) fluorescence assays were performed to evaluate the inhibitory effects of phelligridin D (**1**) and related compounds (Figure 1) on Aβ aggregation. The inhibitory activity of compounds **1**–**9** is shown in Figure 2 and Table 1. The IC_{50} value was calculated from the rate of inhibition (%) of each compound on Aβ aggregation after 24 h. Phelligridin D (**1**; IC_{50} 7.3 μM), phelligridin E (**2**; IC_{50} 11.5 μM), phelligridin G (**3**; IC_{50} 1.8 μM), and phellibaumin A (**4**; IC_{50} 2.2 μM) inhibited the aggregation of Aβ. The activities of hispidin (**5**; IC_{50} 14.2 μM), 3,4-dihydroxybenzylideneacetone (**6**; 3,4-DBA; IC_{50} 18.1 μM), and caffeic acid (**7**; IC_{50} 25.3 μM) containing one catechol moiety were weaker than compounds **1**–**4** containing two or three catechol moieties. However, 3,4-di-*O*-methylcaffeic acid (**8**; IC_{50} > 100 μM)

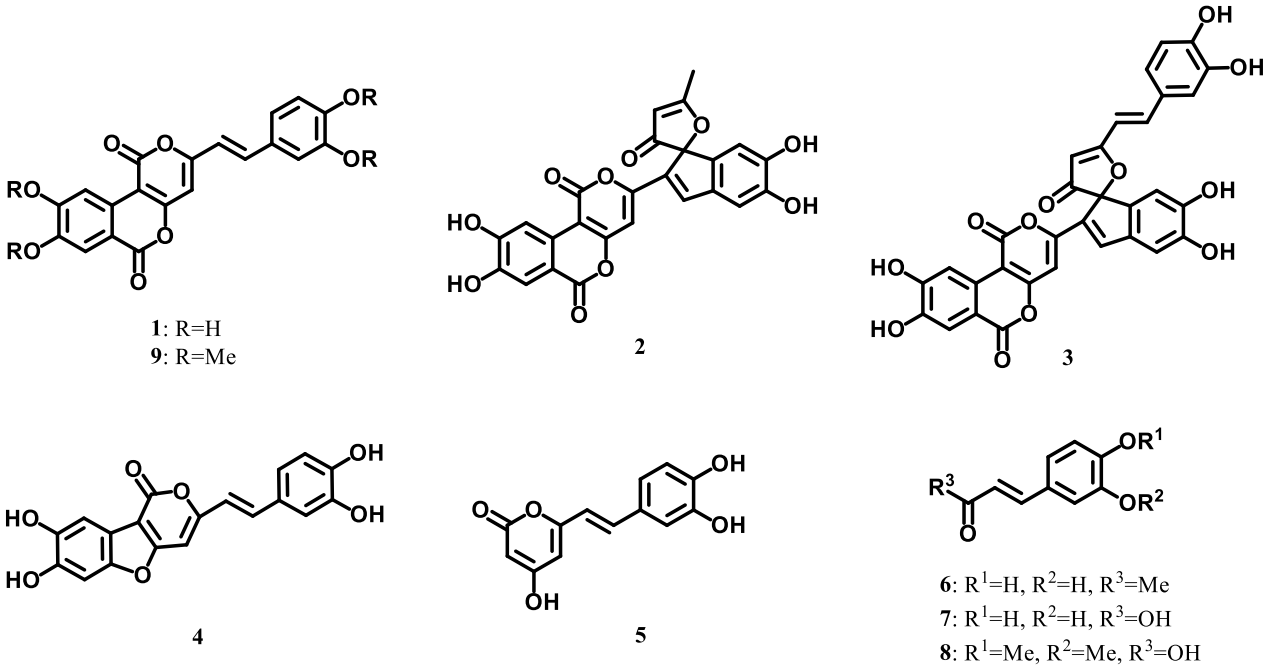

Figure 1. Structures of test compounds **1–9**

and Me-phelligridin D (**9**; IC_{50} > 100 μM), whose phenolic hydroxyl groups are methylated, did not show an inhibitory effect on the aggregation of Aβ.

We investigated the effects of compounds **1**–**9** on Aβ42 fibrillogenesis using transmission electron microscopy (TEM). Typical fibril formation was observed in Aβ alone, while the formation of fibril was significantly inhibited after 24 h on treatment with phelligridin G (**3**). Compounds **1**, **2**, **4**, and **5** also inhibited the fibrillogenesis of Aβ42, while compounds **6** and **7** less inhibited the fibril formation of Aβ42 and compounds **8** and **9** did not show the inhibitory activity (Figure 3).

We investigated the potency of hispidin derivatives toward the inhibition of Aβ aggregation and the structure–activity relation of compounds **1**–**9** using a Th-T assay (Figure 2 and Table 1). Moreover, the modulation of the inhibitory effect of compounds **1**–**9** on the fibril formation of Aβ42 was observed using TEM (Figure 3).

The results of the Th-T assay and TEM indicate that 3,4-di-*O*-methylcaffeic acid (**8**) and Me-phelligridin D (**9**) showed the lowest activity among compounds **1**–**9**, suggesting that phenolic hydroxyl groups play a significant role in the inhibition of Aβ aggregation. Furthermore, hispidin (**5**), 3,4-dihydroxybenzylideneacetone (**6**), and caffeic acid (**7**) exhibited lower anti-aggregation activity than phelligridins D (**1**), E (**2**), and G (**3**) and phellibaumin A (**4**), indicating that compounds with multiple catechol moieties exhibit stronger activity than those containing a single catechol moiety. This finding supports our previous reports on the inhibitory effects of CQAs and phenylethanoid glycosides.[5,6,13] In a

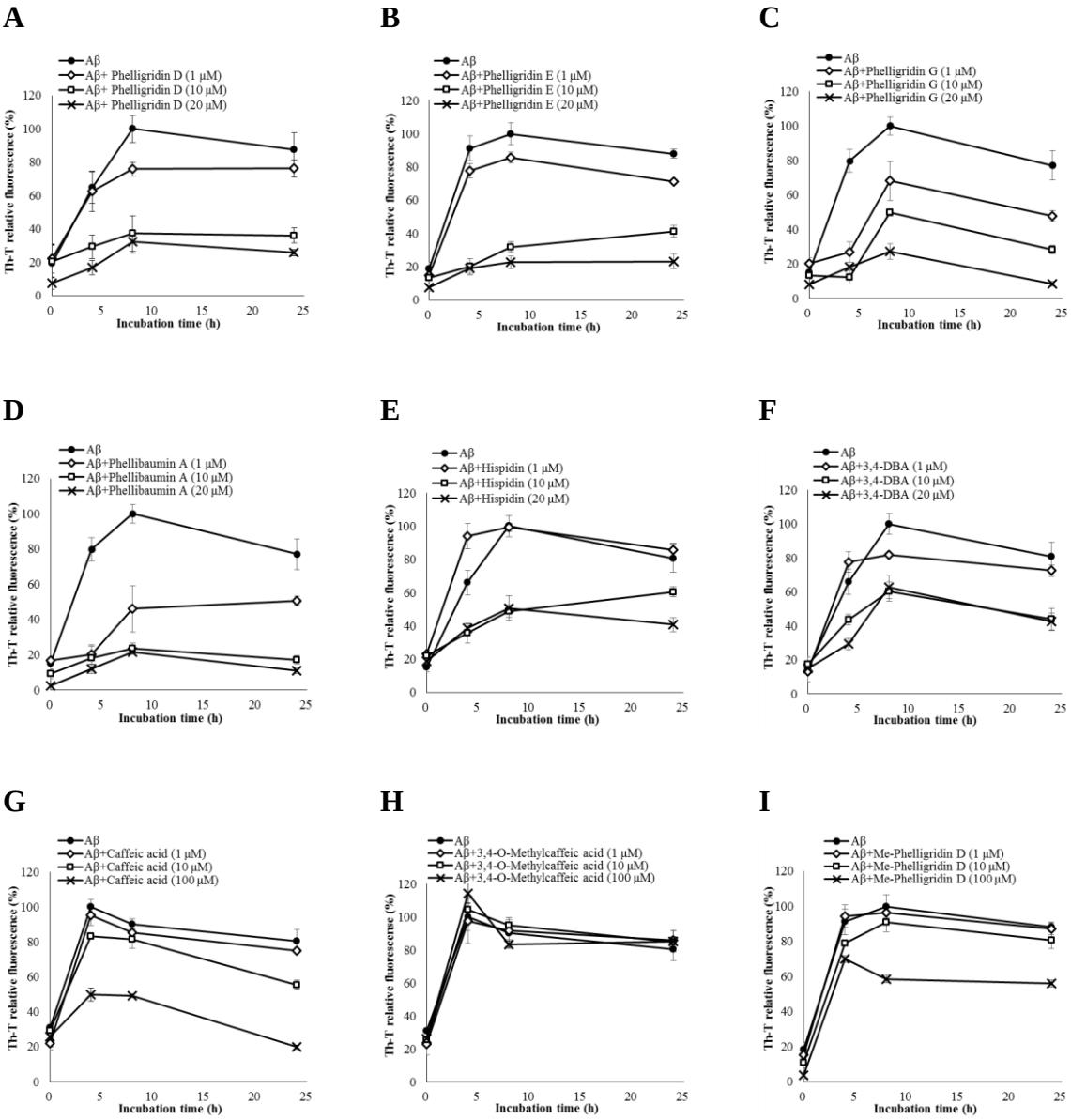

Figure 2. Effect of compounds **1**–**9** on aggregation of Aβ. Fibril formation of 25 μM of Aβ was monitored by Th-T fluorescence and presence of 1, 10, and 20 μM of (**A**) phelligridin D (**1**), (**B**) phelligridin E (**2**), (**C**) phelligridin G (**3**), (**D**) phellibaumin A (**4**), (**E**) hispidin (**5**), (**F**) 3,4-dihydroxybenzylideneacetone (**6**), (**G**) caffeic acid (**7**), (**H**) 3,4-di-*O*-methylcaffeic acid (**8**), and (**I**) Me-phelligridin D (**9**). Fluorescence intensity was measured at an excitation wavelength 420 nm and emission wavelength of 485 nm, $n = 6$.

previous structure–activity relation study, we suggested that the caffeoyl group in CQAs and phenylethanoid glycosides is essential for the inhibitory activity. We inferred that the auto-oxidization of the catechol moiety in the caffeoyl group is implicated in the mechanism of the inhibition of Aβ aggregation. The catechol moiety is possibly autoxidized to form *o*-benzoquinone, followed by the adduct formation of *o*-benzoquinone with some Aβ42 residues. Such covalent modification may destabilize the

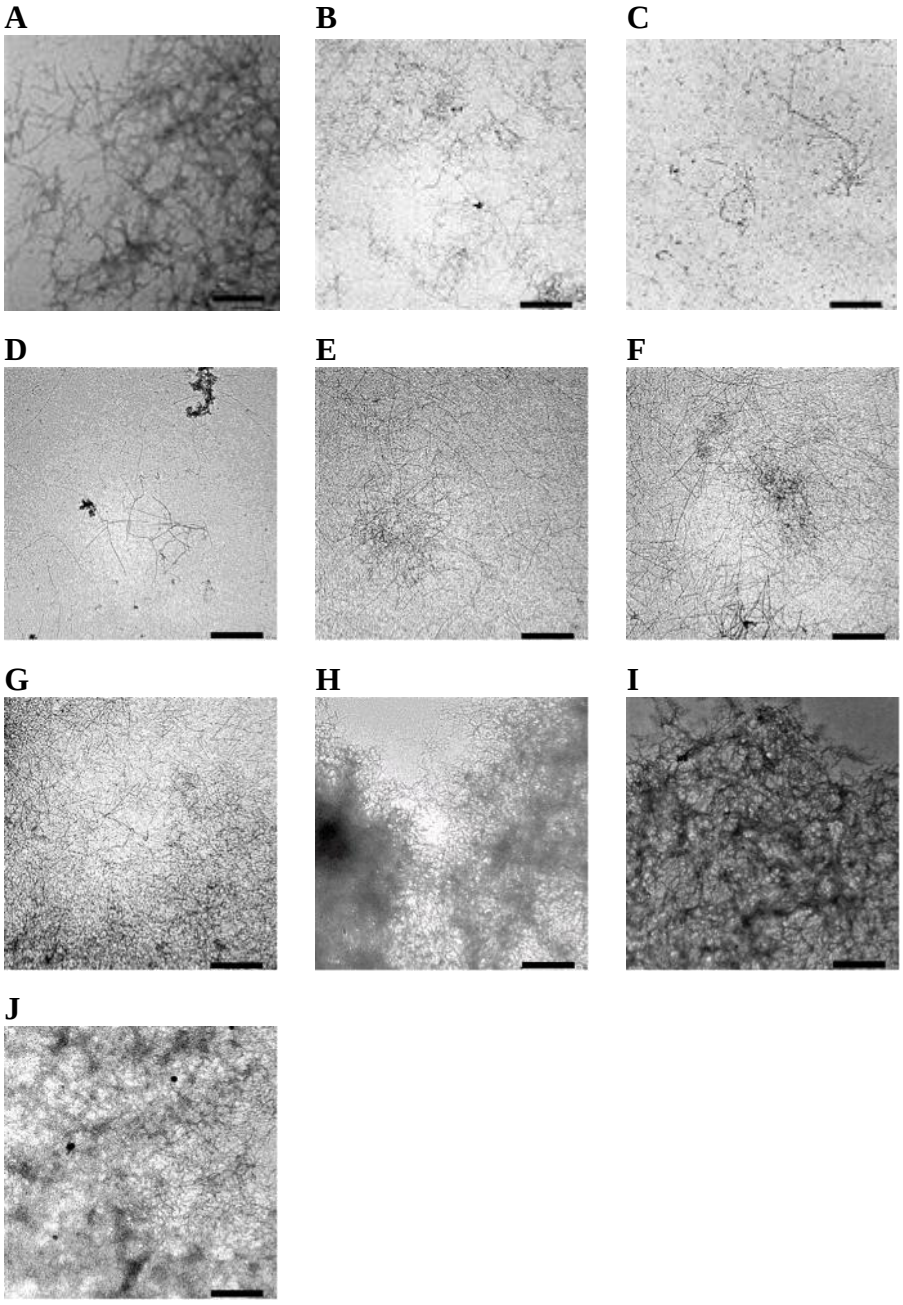

Figure 3. Effects of compounds **1**-**9** on Aβ fibrillogenesis by transmission electron micrographs (TEM). The fibril formation was observed after 24 h incubation in 50 mM PBS buffer. Scale bars: 1 μm (**A**) 25 μM Aβ42, (**B**) 25 μM Aβ42 + 100 μM phelligridin D (**1**), (**C**) 25 μM Aβ42 + 100 μM phelligridin E (**2**), (**D**) 25 μM Aβ42 + 100 μM phelligridin G (**3**), (**E**) 25 μM Aβ42 + 100 μM phellibaumin A (**4**), (**F**) 25 μM Aβ42 + 100 μM hispidin (**5**), (**G**) 25 μM Aβ42 + 100 μM 3,4-DBA (**6**), (**H**) 25 μM Aβ42 + 100 μM caffeic acid (**7**), (**I**) 25 μM Aβ42 + 100 μM 3,4-di-*O*-methylcaffeic acid (**8**), and (**J**) 25 μM Aβ42 + 100 μM Me-phelligridin D (**9**)

β-sheet structure in amyloidgenic polypeptides.[14] As the hispidin derivatives contain catechol moieties, this mechanism may also be used for analyzing the inhibitory effects of hispidin derivatives on Aβ42 aggregation.

Table 1. Effects of compounds **1**–**9** on aggregation of Aβ

Compounds	IC_{50} value (μM)
phelligridin D (**1**)	7.3
phelligridin E (**2**)	11.5
phelligridin G (**3**)	1.8
phellibaumin A (**4**)	2.2
hispidin (**5**)	14.2
3,4-dihydroxybenzylideneacetone (**6**, 3,4-DBA)	18.1
caffeic acid (**7**)	25.3
3,4-di-*O*-methylcaffeic acid (**8**)	> 100
Me-phelligridin D (**9**)	> 100

Gazit proposed that the inhibition of the self-assembly of amyloid structures by polyphenols could originate from the π–π stacking interaction between Aβ42 and polyphenols.[15] The π-orbital of the aromatic ring of catechol moiety in the hispidin derivatives could contribute to π–π stacking formation, leading to the inhibition of Aβ42 aggregation. To elucidate the inhibitory mechanism of hispidin derivatives against Aβ aggregation, further analysis is required.

To the best of our knowledge, this is the first report demonstrating the inhibitory effects of hispidin derivatives on Aβ42 aggregation. Therefore, the hispidin derivatives are a promising therapeutic option for inhibiting Aβ-mediated pathology in the progression of AD.

EXPERIMENTAL

General Procedures

NMR and ESI-MS were measured on a Bruker Avance 500 spectrometer and Waters Synapt G2 mass spectrometer, respectively. Hispidin (**5**) and 3,4-dihydroxybenzylideneacetone (**6**) were purchased from Wako Pure Chemical Industries, Ltd. (Japan), while caffeic acid (**7**) and 3,4-di-*O*-methylcaffeic acid (**8**) were purchased from Nacalai Tesque, Inc. (Japan) and Tokyo Chemical Industry Co., Ltd. (Japan), respectively. Me-phelligridin D (**9**) was synthesized by Prof. Kigoshi's group (University of Tsukuba).

Isolation of the hispidin derivetives from *I. obliquus*

I. obliquus (dry weight: 200 g) was extracted with MeOH (900 mL × 3), and the MeOH extract (IO, 5.0 g) was partitioned with EtOAc (500 mL × 3) and H_2O (500 mL). The EtOAc soluble material (IOEA, 2.6 g) was partitioned with hexane (500 mL × 3) and 90% MeOH (500 mL). Furthermore, 90% MeOH soluble material (IOEA90M, 1.6 g) was divided into 15 fractions (IOEA90M-1~15) by gel filtration

column (Sephadex LH-20, 4.0 × 100 cm, MeOH). IOEA90M-9 (23.0 mg) was purified by HPLC column (TSKgel ODS-80Ts; 7.8 × 300 mm, MeOH/H_2O/CF_3CO_2H (TFA), 5:95:0.1→50:50:0.1→90:10:0.1→100:0:0.1) to yield phelligridin D (**1**, 0.9 mg, t_R 41.0 min) and phelligridin E (**2**, 0.1 mg, t_R 36.7 min).[13] And then IOEA90M-10 (23.9 mg) was purified by HPLC column (TSKgel ODS-80Ts; 7.8 × 300 mm, MeOH/H_2O/TFA, 5:95:0.1→65:35:0.1→90:10:0.1→100:0:0.1) to yield phelligridin D (**1**, 3.9 mg, t_R 28.6 min) and phellibaumin A (**4**, 0.3 mg, t_R 26.8 min).[14] IOEA90M-13 and 14 was combined (13.4 mg) and purified by HPLC column (TSKgel ODS-80Ts; 7.8 × 300 mm, MeOH/H_2O, 5:95→60:40→90:10→100:0) to yield phelligridin G (**3**, 2.6 mg, t_R 28.3 min).[15]

Th-T assay

The aggregative ability of Aβ42 was evaluated by the Th-T method developed by Niki *et al*.[16] The procedure was described elsewhere.[17] Th-T fluorescence assays were performed to evaluate the inhibitory effects of compounds **1**–**9** on Aβ aggregation. In brief, Aβ42 was dissolved in 0.1% NH_4OH at a concentration of 250 μM. The Aβ42 solution was diluted tenfold with 50-mM sodium phosphate (pH = 7.4), and the solution was incubated at 37 °C with or without samples. The peptide solution (2.5 μL) was added to 250 μL of 5-μM Th-T in 5-mM Gly–NaOH (pH = 8.5). The fluorescence intensity was measured at an excitation wavelength of 420 nm and an emission wavelength of 485 nm using a Wallac 1420 ARVO MX multidetection microplate reader (PerkinElmer). Th-T relative fluorescence was calculated as percentage of Aβ42 alone whose maximum value was taken as 100%. And IC_{50} value was calculated from the inhibitory rate (%) of each compound on Aβ42 aggregation after 24 h incubation.

TEM

The fibrillogenesis of Aβ42 was examined using TEM. Four μL of each sample after Th-T assay were spotted on a glow-discharged Formvar-carbon-coated grid, incubated for 2 min, and washed twice with 4 μL of pure water. Finally, the grid was negatively stained twice for 1 min each with 4 μL of 0.4% silicotungstic acid and the solution was removed. Following air drying for 5 min, each sample was examined using a JEM-1400 electron microscope (JEOL).

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REFERENCES

1. J. Hardly and D. J. Selkoe, *Science*, 2002, **297**, 353.
2. G. G. Glenner and C. W. Wong, *Biochem. Biophys. Res. Commun*., 1984, **120**, 885.
3. C. L. Masters, G. Simms, N. A. Weinman, G. Multhaup, B. L. McDonald, and K. Beyreuther, *Proc. Natl. Acad. Sci. USA*, 1985, **82**, 4245.
4. C. Haass and D. J. Selkoe, *Nat. Rev. Mol. Cell Biol.*, 2007, **8**, 101.
5. Y. Miyamae, M. Kurisu, K. Murakami, J. Han, H. Isoda, K. Irie, and H. Shigemori, *Bioorg. Med. Chem.*, 2012, **20**, 5844.
6. M. Kurisu, Y. Miyamae, K. Murakami, J. Han, H. Isoda, K. Irie, and H. Shigemori, *Biosci. Biotechnol. Biochem.*, 2013, **77**, 1329.
7. M. Saar, *J. Ethnopharmacol.*, 1991, **31**, 175.
8. W. Zheng, K. Miao, Y. Liu, Y. Zhao, M. Zhang, S. Pan, and Y. Dai, *Microbiol. Biotechnol.*, 2010, **87**, 1237.
9. Y. Cui, D. S. Kim, and K. C. Park, *J. Ethnopharmacol.*, 2005, **96**, 79.
10. S. Mo, S. Wang, G. Zhou, Y. Yang, Y. Li, X. Chen, and J. Shi, *J. Nat. Prod*., 2004, **67**, 823.
11. C. Wu, Z. Lin, L. Wang, D. Guo, S. Wang, Y. Liu, H. Yuan, and H. Lou, *Bioorg. Med. Chem. Lett*., 2011, **21**, 3261.
12. Y. Wang, S. Mo, S. Wang, S. Li, Y. Yang, and J. Shi, *Org. Lett*., 2005, **7**, 1675.
13. E. Kidachi, M. Kurisu, Y. Miyamae, M. Hanaki, K. Murakami, K. Irie, and H. Shigemori, *Heterocycles*, 2016, **92**, 1976.
14. M. Sato, K. Murakami, M. Uno, Y. Nakagawa, S. Katayama, K. Akagi, Y. Masuda, K. Takegoshi, and K. Irie, *J. Biol. Chem.*, 2013, **288**, 23212.
15. E. Gazit, *FASEB J*., 2002, **16**, 77.
16. H. Naiki and F. Gejyo, *Methods Enzymol*., 1999, **309**, 305.
17. K. Murakami, K. Irie, A. Morimoto, H. Ohigashi, M. Shindo, M. Nagao, T. Shimizu, and T. Shirasawa, *J. Biol. Chem.*, 2003, **278**, 46179.