HETEROCYCLES, Vol. 104, No. 11, 2022, pp. 2025 - 2036. © 2022 The Japan Institute of Heterocyclic Chemistry
Received, 2nd July, 2022, Accepted, 9th September, 2022, Published online, 21st September, 2022
DOI: 10.3987/COM-22-14711

INHIBITION OF AMYLOID-β AGGREGATION BY *p*-TERPHENYLS FROM THE MUSHROOM *POLYOZELLUS MULTIPLEX* AND THEIR NEUROPROTECTIVE EFFECTS

Shoko Nakabayashi,[a] Ayaka Ishikura,[a] Koji Fujihara,[a] Shuntaro Hirabayashi,[a] Shin Koike,[b] Hiroaki Sasaki,[a] Yuki Ogasawara,[b] Kiyotaka Koyama,[a] and Kaoru Kinoshita[a]*

[a]Department of Pharmacognosy and Phytochemistry, Meiji Pharmaceutical University, Noshio 2-522-1, Kiyose-shi, Tokyo 204-8588, Japan. [b]Department of Analytical Biochemistry, Meiji Pharmaceutical University, Noshio 2-522-1, Kiyose-shi, Tokyo 204-8588, Japan. E-mail: kaoru@my-pharm.ac.jp

Abstract – The main pathogenesis of Alzheimer's disease (AD) is related to the accumulation of amyloid-β (Aβ) peptides in the brain that leads to neuronal cell death. In this study, we identified compounds in a methanol extract of the fruiting body of *Polyozellus multiplex* that inhibited Aβ aggregation and are neuroprotective. Seven compounds against $Aβ_{40}$ aggregation were obtained through bioactivity-guided fractionation of the extract, including polyozellin (**1**), kynapcin-12 (**2**), NSC617425 (**3**), cycloleucomelone (**4**), Bl-V (**5**), succinic acid (**6**), and protocatechuic acid (**7**). Compounds **1**–**5** inhibited $Aβ_{40}$ aggregation in a dose-dependent manner. Moreover, compounds **2**–**5** protected SH-SY5Y cells from Aβ toxicity. Therefore, these compounds are potential agents in AD treatment.

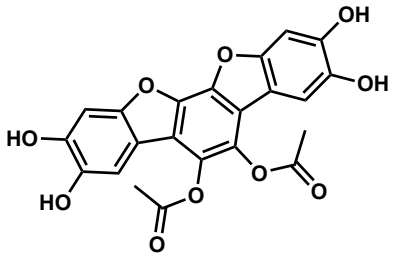
Dementia is a neurodegenerative disease caused by acquired organic brain damage. The disease is characterized by a persistent loss of cognitive function that interferes with daily and social life. Alzheimer's disease (AD) is the most frequent cause of dementia[1]; its pathological features include neuronal loss (mainly in the cerebral cortex), neurofibrillary tangles, and the occurrence of senile plaques in the brain parenchyma. Senile plaques are composed of amyloid-β (Aβ) peptides.[2] Normally, Aβ peptides are degraded and/or eliminated. However, excessive accumulation of Aβ forms senile plaques, eventually leading to AD, which is known as the amyloid cascade hypothesis.[3] Aβ is a partial fragment of amyloid precursor protein (APP), which is produced by the cleavage of β-secretase 1 (BACE1) and γ-secretase and is subsequently secreted into the extracellular milieu.[4-6] Aβ undergoes degradation by various extracellular pathways. Therefore,

regulation of BACE1 and γ-secretase activity and activation of the Aβ degradation pathway may be an important strategy for the treatment of AD.[7] In 2021, the US Food & Drug Administration announced the approval of a new drug, aducanumab (brand name Aduhelm™), to treat AD.[8] Aducanumab, an antibody that reduces Aβ deposition,[9,10] has proven to be clinically useful for the amyloid cascade hypothesis. However, further research is necessary on Aβ inhibitors, as well as BACE1 inhibitors, which have not been clinically used to date. In our laboratory, we screened 110 extracts of various mushrooms for their inhibitory effects on Aβ aggregation. We have already reported on meroterpenoids isolated from the fruiting bodies of *Boletinus asiaticus*[11] and *Albatrellus yasudae*[12,13] that inhibit Aβ aggregation and/or BACE1. In addition to these mushrooms, a methanol extract from the fruiting body of *Polyozellus multiplex* showed inhibitory activity against Aβ aggregation. The edible black mushroom *P. multiplex* belongs to the Thelephoraceae family; it is often harvested in Japan, Korea, and North America. *p*-Terphenyl compounds isolated from *P. multiplex* have been reported to have anti-inflammatory,[14] anti-tumorigenic,[15] and anti-prolylendopeptidase[16-19] effects. The four *p*-terphenyls identified from the *P. multiplex* extracts, polyozellin, polyozellic acid, thelephoric acid, and kynapcin-12, have been reported to have BACE1 inhibitory activity and neuroprotective effects [20] and are expected to have anti-AD activity. However, no reports evaluating the Aβ aggregation inhibitory activity of *P. multiplex* are known. Recently, $A\beta_{42}$ aggregation inhibitory activity was reported for *p*-terphenyl compounds isolated from *Boletopsis leucomelas* of the same family as *P. multiplex*.[21] Based on this, it would be meaningful to evaluate the Aβ aggregation inhibitory activity of *p*-terphenyl compounds isolated from *P. multiplex* to explore their potential anti-AD activity. Here, seven compounds were isolated from this extract through bioactivity-guided separation and evaluated for their $A\beta_{40}$ aggregation inhibitory activity, BACE1 inhibitory activity, and neuroprotective effects. Furthermore, their structure-activity relationships (SARs) were examined.

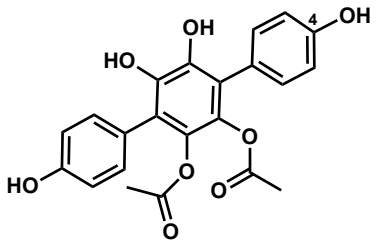
Extraction, isolation, structural determination, and inhibitory activity of Aβ aggregation

The methanol extract from the fruiting body of *P. multiplex* showed potent inhibitory activity against $A\beta_{40}$ aggregation (an $A\beta_{40}$-aggregation rate of 25.1%) at 10 μg/mL. Bioactivity-guided separation was performed using silica gel column chromatography (Si. C. C.) to yield seven fractions, A–G, and an insoluble fraction, H. The seven fractions A–G and the insoluble fraction H were evaluated for their inhibitory activity against $A\beta_{40}$ aggregation at 10 μg/mL (A: 3.18 g, 49.0%; B: 5.80 g, 78.3%; C: 1.52 g, 59.3%; D: 0.31 g, 34.5%; E: 4.50 g, 30.3%; F: 33.50 g, 22.5%; G: 1.56 g, 14.7%; H: 23.91 g, 14.5%). Because of its relatively high $A\beta_{40}$-aggregation inhibitory activity and large quantity, fraction F (22.5%) was further separated. The structures were elucidated as seven compounds **1**–**7** using mass spectrometry (MS) and comprehensive nuclear magnetic resonance (NMR) spectra, and were identified as polyozellin

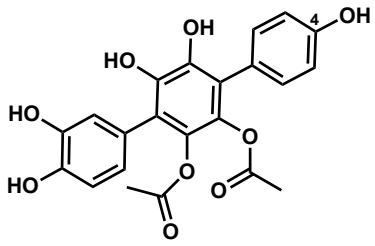
(**1**),[22,23] kynapcin-12 (**2**),[19,24] NSC617425 (**3**),[25] cycloleucomelone (**4**),[26] Bl-V (**5**),[27] succinic acid (**6**),[28] and protocatechuic acid (**7**)[28] (Figures, 1, S2, S3). This is the first report on the isolation of compounds **4**–**7** from *P. multiplex*. Compounds **1**–**5** were classified as *p*-terphenyl compounds.



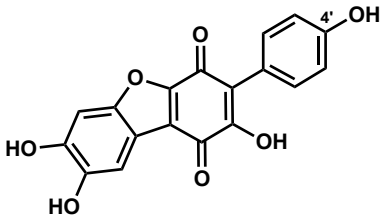
polyozellin (**1**)



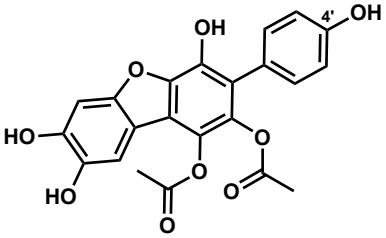
kynapcin-12 (**2**)



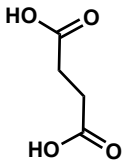
NSC617425 (**3**)



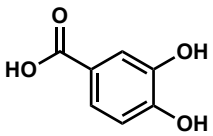
cycloleucomelone (**4**)



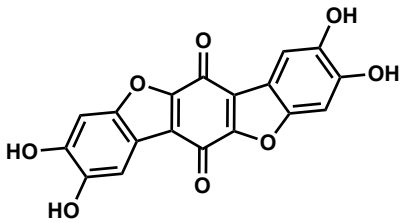
Bl-V (**5**)



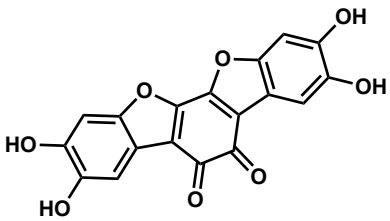
succinic acid (**6**)



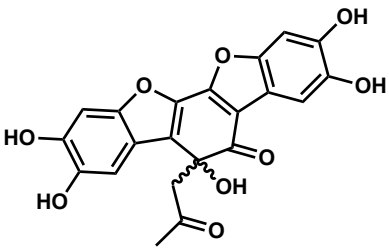
protocatechuic acid (**7**)



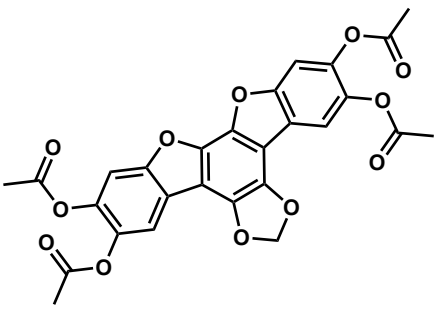
thelephoric acid (**8**)



polyozellic acid (**9**)



acetone adduct from polyozellic acid (**10**)



11,12-(methylenedioxy)polyozellic acid acetate (**11**)

Figure 1. Structures of compounds **1**–**11**

Based on thin layer chromatography (TLC), fraction D (34.5%) and E (30.3%) contained polyozellin (**1**), kynapcin-12 (**2**), and Bl-V (**5**). Fraction G (14.7%) showed a similar pattern with fraction F and contained more polyozellin (**1**). The insoluble fraction H (14.5%) contained mainly polyozellin (**1**) (Figure S15). The half-maximal inhibitory concentration (IC_{50}) values of compounds **1**–**7** were 0.615, 6.76, 6.45, 2.17, 3.61, >100, and >100 μM, respectively (Tables 1, S5). Compounds **1**–**5** inhibited $A\beta_{40}$ aggregation in a dose-dependent manner. The results of compound **5** supported the report of Kodori et al.[21]

Table 1. Aβ aggregation and BACE1 inhibitory activities of compounds **1**–**11**

	Aβ aggregation IC_{50} (μM, n=3)	BACE1 IC_{50} (μM, n=3)
1	0.615	4.78[d]
2	6.76	15.79[d]
3	6.45	>100
4	2.17	66.62
5	3.61	>100
6	>100	>100
7	>100	>100
8	12.62	3.50[d]
9	1.29	4.78[d]
10	1.96	34.49
11	21.61	>100
myricetin[a]	9.9[c]	2.8[e]
inhibitor IV[b]	>100[c]	0.015[c]

[a]Myricetin was used as a positive control for the inhibitory activity of Aβ aggregation.

[b]Inhibitor IV was used as a positive control for BACE1 inhibitory activity.

[c,d,e]Data were referred to reference,[13,20,29] respectively.

Structure-activity relationship (SAR)

To determine the SAR of inhibitory activity on $A\beta_{40}$ aggregation, *p*-terphenyl compounds **8**–**11**, thelephoric acid (**8**), polyozellic acid (**9**), an acetone adduct from polyozellic acid (**10**), and 11,12-(methylenedioxy)polyozellic acid acetate (**11**), from our previous work were used (Figure 1).[15] The IC_{50} value of compounds **8**–**11** were 12.62, 1.29, 1.96, and 21.61 μM, respectively (Tables 1, S5). Compounds **8**–**10** exhibited inhibitory activity against $A\beta_{40}$ aggregation in a dose-dependent manner. Based on these

assay results, there are some consistent characteristics of compounds with inhibitory activity on $A\beta_{40}$ aggregation. The first is the presence of catechol moieties. Compounds **1**–**5** and **8**–**10** contain one or two catechol moieties and tend to be relatively more active than compound **11**, which has acetylated catechol moieties. Sato et al.[30] reported a site-specific inhibitory mechanism against $A\beta_{42}$ aggregation through a catechol-type flavonoid, (+)-taxifolin, which targets Lys16 and Lys28 after autoxidation. Furthermore, catechol-bearing compounds have been reported to have antioxidative and anti-inflammatory properties.[31,32] However, compound **7**, which contained a catechol moiety but no *p*-terphenyl structure, was active. This suggests that the *p*-terphenyl structure is important for $A\beta_{40}$ aggregation inhibition. The second characteristic is the presence of furan rings. Compounds **1**, **4**, **5**, and **8**–**11** contain one or two furan rings. Compounds **1**, **9**, and **10**, with two furan rings in the same direction, tended to be more active than compound **8**, with two furan rings in the opposite direction. Compound **11** was less active than compounds **1**, **9**, and **10**, although two furan rings in the same direction are present. The presence of two acetyl groups in the central ring (compounds **1**–**3** and **5**) does not appear to be important for $A\beta_{40}$ aggregation inhibitory activity compared to the other compounds that showed activity.

Inhibitory activity against BACE1

In this study, compounds **3**–**7**, **10**, and **11** were evaluated for their inhibitory activities against BACE1. The IC_{50} values of compounds **3**–**7**, **10**, and **11** were >100, 66.62, >100, >100, >100, 34.49, and >100 μM, respectively (Tables 1, S6). Compounds **4** and **10** showed weak BACE1 inhibitory activities in a dose-dependent manner. The BACE1 inhibitory activity of compounds **1**, **2**, **8**, and **9** have reported by Chon et al.[20]

Protective effect of compounds on $A\beta_{42}$ toxicity in SH-SY5Y cells

Since compounds **1**–**5** inhibited $A\beta_{40}$ aggregation, their neuroprotective effects against $A\beta_{42}$ toxicity were also evaluated using human neuroblastoma SH-SY5Y cells. Compounds **6** and **7**, which did not inhibit $A\beta_{40}$ aggregation, were evaluated as well. To avoid measuring a reduction in $A\beta_{42}$ production due to the potential toxicity of these compounds, the effect of these compounds on SH-SY5Y cells was measured before the $A\beta_{42}$ measurement and evaluated using doses below those observed to have no adverse effects for each compound (Figure S16). The results showed that SH-SY5Y cell viability after treatment with 10 μM $A\beta_{42}$ was reduced to 31.7% of the control group, whereas the cell viabilities with co-incubation of compounds **1**–**7** and 10 μM $A\beta_{42}$ were 29.0 ± 10.6% at 3.1 μM, 63.3 ± 2.60% at 25.0 μM, 74.7 ± 1.5% at 13.0 μM, 85.8 ± 6.8% at 25.0 μM, 51.6 ± 3.7% at 13.0 μM, 40.7 ± 2.6% at 13.0 μM, and 37.7 ± 7.9% at 13.0 μM, respectively (Figure 2). Cell viability was improved with the addition of compounds **2**–**5**, and furthermore,

compounds **2**–**4** were dose-dependent. Compounds **2**–**5** also have catechol moieties, which might be important for neuroprotection as well as Aβ$_{40}$ aggregation inhibitory activity. Although compound **1** has a catechol moiety, it showed no neuroprotective effects against Aβ$_{42}$ toxicity. This is because the cytotoxicity of compound **1** was strong, so it is possible that there was no concentration that had activity but no toxicity.

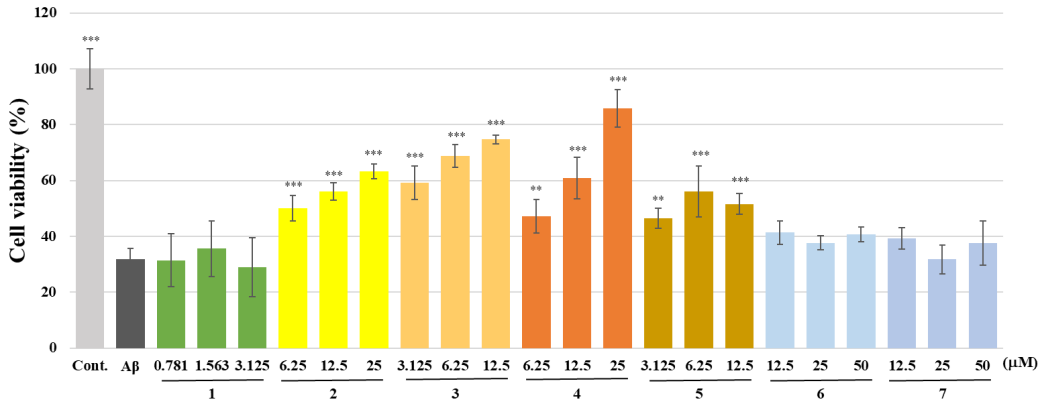


Figure 2. Protective effects of seven compounds on SH-SY5Y cells against Aβ toxicity Cont. indicates the control group (cells in the absence of Aβ and compounds **1**–**7**). Aβ indicates the group treated with 10 μM Aβ but no compound. **1**–**7** indicate the groups treated with each compound (**1**–**7**) and 10 μM Aβ. Statistical significance was analyzed using one-way ANOVA followed by Dunnett's test. Values are expressed as mean ± SD, n=3, $^{**}p < 0.01$, $^{***}p < 0.001$ as compared with Aβ.

Based on SAR, the presence of the *p*-terphenyl structure and the C4 or C4′-linked hydroxyl group tended to enhance the protective effect against Aβ$_{42}$ toxicity in SH-SY5Y cells, since no neuroprotective effect was observed with compounds **1**, **6**, and **7**. Hanaki et al. reported on the inhibition of Aβ$_{42}$ aggregation by flavonoids with non-catechol structures, and kaempferol with a C4′-linked hydroxy group may prevent the elongation phase in the aggregation stage of Aβ$_{42}$.[33] We isolated five candidate compounds, **1**–**5**, for the treatment of AD from the methanol extract of the fruiting body of *P. multiplex* and these compounds exhibited inhibitory activities against Aβ$_{40}$ aggregation. Compounds **2**–**5** protected against Aβ$_{42}$ toxicity in SH-SY5Y cells. These results suggest that compounds **2**–**5** might be developed into therapeutic agents that can prevent AD symptoms by protecting neurons after the deposition of Aβ$_{42}$ in the brain. Furthermore, because compounds **2**–**5** also inhibited Aβ$_{40}$ aggregation, they might be expected to be prophylactic agents against AD and mild cognitive impairment. Compound **1** exhibited inhibitory activity against both BACE1[20] and Aβ$_{40}$ aggregation. According to previous studies based on the amyloid cascade hypothesis, most compounds reported to have the potential for AD therapy have been limited to either inhibitors of Aβ aggregation or BACE1. In general, no relationship has been found between the inhibition of Aβ aggregation and BACE1. However, there has been a finding similar to the present study for *A. yasudae*.[13] Therefore,

compounds that inhibit both activities, such as compound **1**, may be useful as new prophylactic agents for the treatment of AD. Aβ peptides aggregate and form oligomers, protofibrils, and mature fibrils with various structures. It has been reported that some toxic aggregates are non-toxic conformations to neurons.[34-37] It is necessary for prophylactic and therapeutic drugs to suppress the aggregation of neurotoxic Aβ. One limitation of this study is that the thioflavin-T (Th-T) assay cannot distinguish the difference between toxic and non-toxic conformations due to the characteristics of the assay. In addition, compound **1** might need the addition of modifying groups to reduce its cytotoxicity.

CONCLUSION

In conclusion, in this study, we demonstrated that *p*-terphenyl compounds from a methanol extract of the fruiting body of *P. multiplex*, particularly compounds **1**–**5**, have potential as prophylactic and therapeutic agents for the treatment of AD. In addition to the *p*-terphenyl compounds mentioned above, meroterpenoids isolated from the fruiting bodies of *B. asiaticus* and *A. yasudae* inhibit Aβ aggregation and/or BACE1. Therefore, mushrooms may be valuable natural resources for the treatment of AD.

EXPERIMENTAL

General experimental procedures

Column chromatography was performed using silica gel 60N (63–210 μm; Kanto Chemical, Tokyo, Japan), ODS silica gel YMC-GEL ODS-A (75 μm; YMC Co., Ltd., Kyoto, Japan), Chromatorex COOH MB100-75/200 (100 μm; Fuji Silysia Chemical Ltd., Aichi, Japan), and Sephadex LH-20 (GE Healthcare, Uppsala, Sweden). Preparative high-performance liquid chromatography (HPLC) was performed on a Jasco PU-2080 Plus equipped with a Jasco UV-2075 Plus UV detector at 254 nm (Jasco Co., Tokyo, Japan). The HPLC columns were Inertsustain C_{18} column (10ϕ × 250 mm; GL Sciences Inc., Tokyo, Japan), Senshu Pak PEGASIL Silica SP100 (10ϕ × 250 mm; Senshu Scientific Co., Ltd.), and Inertsustain phenyl 5 μm column (10ϕ × 250 mm; GL Sciences Inc., Tokyo, Japan).

Fungal material

P. multiplex was collected on Mt. Mizugaki in Yamanashi Prefecture, Japan. A voucher specimen (PM-2018) was deposited in Sep. 2018. Species identification was confirmed by one of the authors (K. Koyama).

Extraction and isolation

Dried and fractured fruiting body of *P. multiplex* (734.0 g) was extracted three times each with $CHCl_3$ and MeOH at room temperature. The MeOH extracts were evaluated for their inhibitory activity against Aβ aggregation with an Aβ aggregation rate of 25.1% at 10 μg/mL. The MeOH extract (75.0 g, 25.1%) was

further fractionated by Si. C. C. using a stepwise gradient ($CHCl_3$-MeOH; 100:1, 75:1, 50:1, 20:1, 10:1) to yield seven fractions (A–G) and an insoluble fraction (H). Fractions A–H were evaluated for their inhibitory activity against Aβ aggregation at 10 μg/mL (A: 3.18 g, 49.0%; B: 5.80 g, 78.3%; C: 1.52 g, 59.3%; D: 0.31 g, 34.5%; E: 4.50 g, 30.3%; F: 33.50 g, 22.5%; G: 1.56 g, 14.7%; H: 23.91 g, 14.5%).

Fraction F (33.50 g, 22.5%) was further fractionated by Si. C. C. using a stepwise gradient ($CHCl_3$-MeOH; 50:1, 20:1, 10:1, 5:1, 2:1) to yield five fractions (F1–F5). Fractions F1–F5 were evaluated for their inhibitory activity against Aβ aggregation at 10 μg/mL (F1: 0.12 g, 25.3%; F2: 0.17 g, 32.5%; F3: 2.20 g, 14.2%; F4: 3.07 g, 9.8%; F5: 18.70 g, 13.9%).

Fraction F3 (2.20 g, 14.2%) was further fractionated by a Sephadex LH-20 with $CHCl_3$-MeOH-acetone (1:1:1) to yield six fractions (F3a–F3f) and an insoluble fraction (fraction F3g). Fractions F3a–F3g were evaluated for their inhibitory activity against Aβ aggregation at 10 μg/mL (F3a: 72.2 mg, 28.5%; F3b: 324.2 mg, 25.9%; F3c: 1.42 g, 5.1%; F3d: 262.7 mg, 18.9%; F3e: 18.4 mg, 23.2%; F3f: 15.8 mg, 5.6%; F3g: 35.8 mg, 5.6%). Fraction F3f was obtained as polyozellin (**1**, 15.8 mg).

Fraction F3c (1.42 g, 5.1%) was subjected to four chromatography steps: (1) ODS C. C. with MeOH-H_2O (50:0, 100:0), (2) Si-COOH. C. C. with $CHCl_3$-MeOH (50:1, 20:1, 10:1, 5:1, 2:1, 0:100), (3) preparative HPLC by ODS C. C. eluted with MeOH-H_2O (45:55) to obtain NSC617425 (**3**) (5.6 mg, t_R 8.0 min) and kynapcin-12 (**2**) (33.0 mg, t_R 11.2 min), (4) preparative HPLC by ODS C. C. eluted with MeOH-H_2O (5:5) to obtain Bl-V (**5**) (9.1 mg, t_R 11.2 min), and (5) preparative HPLC by ODS C. C. eluted with MeOH-H_2O (2:8) to obtain succinic acid (**6**) (13.4 mg, t_R 8.0 min).

Fraction F3d (262.7 mg, 18.9%) was subjected to three chromatography steps: (1) Sephadex LH-20 with $CHCl_3$-MeOH-acetone (1:1:1), (2) Si-COOH. C. C. with $CHCl_3$-MeOH (50:1, 0:100), and (3) ODS C. C. with MeOH-H_2O (80:20, 100:0) to obtain protocatechuic acid (**7**, 3.5 mg).

Fraction F4 (3.07 g, 9.8%) was fractionated by Sephadex LH-20 with $CHCl_3$-MeOH-acetone (1:1:1) to yield eleven fractions (F4a–F4k) and an insoluble fraction (F4l). Fractions F4a–F4l were evaluated for their inhibitory activity against Aβ aggregation at 10 μg/mL (F4a: 499.3 mg, 52.2%; F4b: 779.6 mg, 43.0%; F4c: 305.0 mg, 43.2%; F4d: 120.2 mg, 39.1%; F4e: 590.1 mg, 35.3%; F4f: 158.9 mg, 25.7%; F4g: 227.6 mg, 11.7%; F4h: 73.3 mg, 9.1%; F4i: 36.2 mg, 5.4%; F4j: 19.3 mg, 13.7%; F4k: 18.1 mg, 6.1%; F4l: 546.8 mg, 12.8%). Fractions F4g and F4h (300.9 mg) were fractionated by Sephadex LH-20 with $CHCl_3$-MeOH-acetone (1:1:1) to obtain cycloleucomelone (**4**, 12 mg).

Thioflavin-T (Th-T assay)

Similar data were previously obtained for $Aβ_{40}$ and $Aβ_{42}$ using both test conditions, and thus $Aβ_{40}$ was used in this study.[38] Th-T assays of extracts, fractions, and isolated compounds were performed using thioflavine-T (FUJIFILM Wako Pure Chemical Co., Ltd., Japan) and $Aβ_{40}$ (Peptide Institute, Japan). The

aggregation of Aβ was evaluated using a slight modification of the Th-T method developed by Naiki and co-workers.[35] In brief, 80 μL of 50 mM sodium phosphate buffer containing 100 mM NaCl at pH 7.4 was aliquoted into a 0.5 mL tube, followed by the addition of 10 μL of each test sample that was dissolved with DMSO. Then, 10 μL of $A\beta_{40}$ (250 μM in 0.02% NH_4OH) was added to the tube so that the final concentration was $A\beta_{40}$ 25 μM, respectively. All procedures were performed on ice. The resultant solution was incubated at 37 °C for 24 h, and then mixed with 300 μL of 5.0 μM Th-T in 5.0 mM Gly-NaOH buffer at pH 8.5. The mixture was transferred to black 96-well plates at 100 μL per well and then gently vortexed for 30 min. Fluorescence intensity was measured at 440 nm excitation and 485 nm emission using a Synergy HTX Multi-Mode Reader (BioTek, USA). The Aβ aggregation rate was calculated by the following equation: Aβ aggregation rate (%) = (S – B)/(C – B) × 100, where C is the fluorescence intensity of a DMSO control (assay buffer, 25 μM $A\beta_{40}$, and DMSO containing no test sample) after 24 h of incubation, B is the fluorescence intensity of a blank (assay buffer, 25 μM $A\beta_{40}$, and DMSO containing no test sample) without incubation, S is the fluorescence intensity of the tested samples (assay buffer, 25 μM $A\beta_{40}$, and DMSO containing test sample) after 24 h of incubation. Myricetin (Tokyo Chemical Industry Co., Ltd., Japan) was used as a positive control. The IC_{50} values were calculated by GraphPad Prism version 5.02 (GraphPad Software, USA).

BACE1 FRET assay

BACE1 assays were performed using the BACE1 FRET assay kit, Red (Thermo Fisher Scientific, USA).[39] 9 μL of test sample, 9 μL of 722 nM BACE1 substrate (Rh-EVNNLDAEFK-Quencher in 50 mM ammonium bicarbonate), and 9 μL of BACE1 enzyme (1 U/mL) were mixed on black 384-well plates and incubated for 3 h at room temperature. Fluorescence intensity was measured at 540 nm excitation and 590 nm emission using a Synergy HTX Multi-Mode Reader (BioTek, USA). The BACE1 inhibition rate was calculated by the following equation: BACE1 inhibition rate (%) = [1 – {(S – S_0) – (B – B_0)/(C – C_0) – (B – B_0)}] × 100, where C is the fluorescence of a DMSO control (enzyme, substrate, and assay buffer with DMSO) after 3 h of incubation, C_0 is the fluorescence of the DMSO control at 1 h after incubation, B is the fluorescence of a no-enzyme control (substrate and assay buffer with DMSO) after 3 h of incubation, B_0 is the fluorescence of the non-enzyme control at 1 h after incubation, S is the fluorescence of the tested samples (enzyme, sample solution, and substrate) after 3 h of incubation, and S_0 is the fluorescence of the tested samples at 1 h after incubation. β-secretase inhibitor IV (Merck, Germany) was used as a positive control. The IC_{50} values were calculated by GraphPad Prism version 5.02 (GraphPad Software, USA).

Cell culture

Human neuroblastoma SH-SY5Y cells were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium/Ham's F-12 medium (DMEM/F12) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin and 15 mM HEPES. The cells were cultured and maintained in T75 flasks at 37 °C in 5% CO_2.

Protective effect on SH-SY5Y against Aβ toxicity assay

Each compound was dissolved in DMSO. The final DMSO concentration in the culture medium was 0.1%. The assay was conducted in Type I collagen-coated 96-well cell culture plates (Nippi Inc., Tokyo, Japan), and 9.6×10^3 cells/well were seeded. After incubation for 72 h, the medium was removed. Each test sample and 250 μM $A\beta_{42}$ in 0.02% NH_4OH (final concentration of $A\beta_{42}$: 10 μM) was added in DMEM/F12 medium containing 2% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. After incubation for 24 h at 37 °C in 5% CO_2, cell viability was determined using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). The plate was incubated for 3 h at 37 °C in 5% CO_2, then the absorbance at 450 nm was measured using the Synergy HTX Multi-Mode Reader while blank wells containing no-cells, no-$A\beta_{42}$ peptides, and test samples were also prepared and measured at 450 nm in the same manner. The relative cell viability was calculated by the following equation: relative cell viability (%) = $(S–B_S)/(C–B_C) \times 100$, where C is the absorbance at 450 nm of DMSO control (9.6×10^3 cells/well, 10 μM $A\beta_{42}$, and DMSO containing no test samples), S is the absorbance at 450 nm of DMSO control (9.6×10^3 cells/well, 10 μM $A\beta_{42}$, and DMSO containing test sample), B_C is the absorbance at 450 nm of DMSO control (no cells, no $A\beta_{42}$ peptide, and DMSO containing no test samples), B_S is the absorbance at 450 nm of DMSO control (no cells, no $A\beta_{42}$ peptide, and DMSO containing test samples), each absorbance was measured in the same procedure.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Dementia Drug Resource Development Center (DRC) Project, the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan (S1511016) and JSPS KAKENHI Grant Number JP19K07143.

REFERENCES AND NOTES

1. S. Gauthier, P. Rosa-Neto, J. A. Morais, and C. Webster, 'World Alztheimer Report 2021', *Alzheimer's Disease Int.*, 2021, pp. 1-314, London, UK.
2. J. Cummings, G. Lee, A. Ritter, and K. Zhong, *Alzheimers Dement.*, 2018, **4**, 195.
3. J. A. Hardy and G. A. Higgins, *Science*, 1992, **256**, 184.
4. S. Sinha and I. Lieberburg, *Proc. Natl. Acad. Sci., USA*, 1999, **96**, 11049.

5. K. Ono and M. Yamada, *J. Neurochem*., 2011, **117**, 19.
6. K. L. Viola and W. L. Klein, *Acta Neuropathol.*, 2015, **129**, 183.
7. D. J. Selkoe and J. Hardy, *EMBO Mol. Med*., 2016, **8**, 595.
8. U. S. Food and Drug Administration(2021, June 7). FDA Grants Accelerated Approval for Alzheimer's Drug [Press release].
9. J. Sevigny, P. Chiao, T. Bussière, P. H. Weinreb, L. Williams, M. Maier, R. Dunstan, S. Salloway, T. Chen, Y. Ling, J. O'Gorman, F. Qian, M. Arastu, M. Li, S. Chollate, M. S. Brennan, O. Quintero-Monzon, R. H. Scannevin, H. M. Arnold, T. Engber, K. Rhodes, J. Ferrero, Y. Hang, A. Mikulskis, J. Grimm, C. Hock, R. M. Nitsch, and A. Sandrock, *Nature*, 2016, **537**, 50.
10. FDA. Prescribing information of Aduhelm. Retrieved from https://www.accessdata.fda.gov/drugsatfda_docs/label/2021/761178s000lbl.pdf.
11. G. Yatsu, Y. Kino, H. Sasaki, J. I. Satoh, K. Kinoshita, and K. Koyama, *J. Nat. Prod*., 2019, **82**, 1797.
12. M. Akiba, K. Kinoshita, Y. Kino, J. I. Sato, and K. Koyama, *Bioorg. Med. Chem. Lett*., 2020, **30**, 126808.
13. Y. Masuda, K. Fujihara, S. Hayashi, H. Sasaki, Y. Kino, H. Kamauchi, M. Noji, J. I. Satoh, T. Takanami, K. Kinoshita, and K. Koyama, *J. Nat. Prod*., 2021, **84**, 1748.
14. S. H. Lee, K. S. Song, D. H. Sohn, and G. S. Seo, *Arch. Pharm. Res.*, 2011, **34**, 91.
15. I. Nagasawa, A. Kaneko, T. Suzuki, K. Nishio, K. Kinoshita, M. Shiro, and K. Koyama, *J. Nat. Prod*., 2014, **77**, 963.
16. J. Y. Kwak, I. K. Rhee, K. B. Lee, J. S. Hwang, I. D. Yoo, and K. S. Song, *J. Microbiol. Biotechnol*., 1999, **9**, 798.
17. S. I. Kim, I. H. Park, and K. S. Song, *J. Antibiot.*, 2002, **55**, 623.
18. K. S. Song and I. Raskin, *J. Nat. Prod*., 2002, **65**, 76.
19. H. J. Lee, I. K. Rhee, K. B. Lee, I. D. Yoo, and K. S. Song, *J. Antibiot.*, 2000, **53**, 714.
20. S. H. Chon, E. J. Yang, T. Lee, and K. S. Song, *Food Funct*., 2016, **7**, 3834.
21. A. Kobori, K. Hosaka, and H. Shigemori, *Heterocycles*, 2022, **104**, 925.
22. J. S. Hwang, K. S. Song, W. G. Kim, T. H. Lee, H. Koshino, and I. D. Yoo, *J. Antibiot.*, 1997, **50**, 773.
23. S. Takahashi, T. Kawano, N. Nakajima, Y. Suda, N. Usukhbayar, K. I. Kimura, and H. Koshino, *Bioorg. Med. Chem. Lett.*, 2018, **28**, 9303.
24. S. Takahashi, A. Yoshida, S. Uesugi, Y. Hongo, K. Kimura, K. Matsuoka, and H. Koshino, *Bioorg. Med. Chem. Lett.*, 2014, **24**, 3373.
25. C. Tringali, M. Piattelli, C. Geraci, G. Nicolosi, and C. Rocco, *Can. J. Chem*., 1987, **65**, 2369.
26. E. Jägers, E. Hillen-Maske, and W. Steglich, *Z. Naturforsch*. *B*, 1987, **42**, 1349.
27. A. Takahashi, R. Kudo, G. Kusano, and S. Nozoe, *Chem. Pharm. Bull.*, 1992, **40**, 3194.

28. Spectral Database for Organic Compounds (SDBS), http://riodb01.ibase.aist.go.jp/sdbs/ (accessed 2022).
29. Y. Shimmyo, T. Kihara, A. Akaike, T. Niidome, and H. Sugimoto, *Biochim. Biophys. Acta*, 2008, **1780**, 819.
30. 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.
31. T. Satoh, K. Kosaka, K. Itoh, A. Kobayashi, M. Yamamoto, Y. Shimojo, C. Kitajima, J. Cui, J. Kamins, S. Okamoto, M. Izumi, T. Shirasawa, and S. A. Lipton, *J. Neurochem*., 2008, **104**, 1116.
32. P. S. Son, S. A. Park, H. K. Na, D. M. Jue, S. Kim, and Y. J. Surh, *Carcinogenesis*, 2010, **31**, 1442.
33. M. Hanaki, K. Murakami, K. Akagi, and K. Irie, *Bioorg. Med. Chem.*, 2016, **24**, 304.
34. K. J. Barnham, C. L. Masters, and A. I. Bush, *Nat. Rev. Drug Discov*., 2004, **3**, 205.
35. H. Naiki and F. Gejyo, *Methods Enzymol.*, 1999, **309**, 305.
36. K. Ono, *Neurochem. Int.*, 2018, **119**, 57.
37. M. Necula, R. Kayed, S. Milton, and C. G. Glabe, *J. Biol. Chem*., 2007, **282**, 10311.
38. M. Hirohata, K. Ono, H. Naiki, and M. Yamada, *Neuropharmacology*, 2005, **49**, 1088.
39. H. Sasaki, K. Miki, K. Kinoshita, K. Koyama, L. D. Juliawaty, S. A. Achmad, E. H. Hakim, M. Kaneda, and K. Takahashi, *Bioorg. Med. Chem. Lett*., 2010, **20**, 4558.