

## INHIBITION OF AMYLOID- $\beta$ AGGREGATION BY *p*-TERPHENYLS FROM THE MUSHROOM *POLYOZELLUS MULTIPLEX* AND THEIR NEUROPROTECTIVE EFFECTS

Shoko Nakabayashi,<sup>a</sup> Ayaka Ishikura,<sup>a</sup> Koji Fujihara,<sup>a</sup> Shuntaro Hirabayashi,<sup>a</sup> Shin Koike,<sup>b</sup> Hiroaki Sasaki,<sup>a</sup> Yuki Ogasawara,<sup>b</sup> Kiyotaka Koyama,<sup>a</sup> and Kaoru Kinoshita<sup>a\*</sup>

<sup>a</sup>Department of Pharmacognosy and Phytochemistry, Meiji Pharmaceutical University, Noshio 2-522-1, Kiyose-shi, Tokyo 204-8588, Japan. <sup>b</sup>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- $\beta$  (A $\beta$ ) 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 A $\beta$  aggregation and are neuroprotective. Seven compounds against A $\beta$ <sub>40</sub> aggregation were obtained through bioactivity-guided fractionation of the extract, including polyozellin (1), kynapcin-12 (2), NSC617425 (3), cycloleucomelone (4), BI-V (5), succinic acid (6), and protocatechuic acid (7). Compounds 1–5 inhibited A $\beta$ <sub>40</sub> aggregation in a dose-dependent manner. Moreover, compounds 2–5 protected SH-SY5Y cells from A $\beta$  toxicity. Therefore, these compounds are potential agents in AD treatment.

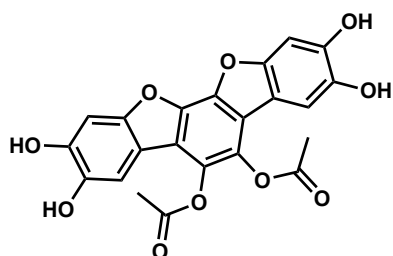
Dementia is a neurodegenerative disease caused by acquired organic brain damage. The disease is characterized by a persistent loss of cognitive function that interferes with daily and social life. Alzheimer’s disease (AD) is the most frequent cause of dementia<sup>1</sup>; its pathological features include 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- $\beta$  (A $\beta$ ) peptides.<sup>2</sup> Normally, A $\beta$  peptides are degraded and/or eliminated. However, excessive accumulation of A $\beta$  forms senile plaques, eventually leading to AD, which is known as the amyloid cascade hypothesis.<sup>3</sup> A $\beta$  is a partial fragment of amyloid precursor protein (APP), which is produced by the cleavage of  $\beta$ -secretase 1 (BACE1) and  $\gamma$ -secretase and is subsequently secreted into the extracellular milieu.<sup>4-6</sup> A $\beta$  undergoes degradation by various extracellular pathways. Therefore,

regulation of BACE1 and  $\gamma$ -secretase activity and activation of the A $\beta$  degradation pathway may be an important strategy for the treatment of AD.<sup>7</sup> In 2021, the US Food & Drug Administration announced the approval of a new drug, aducanumab (brand name Aduhelm™), to treat AD.<sup>8</sup> Aducanumab, an antibody that reduces A $\beta$  deposition,<sup>9,10</sup> has proven to be clinically useful for the amyloid cascade hypothesis. However, further research is necessary on A $\beta$  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 A $\beta$  aggregation. We have already reported on meroterpenoids isolated from the fruiting bodies of *Boletinus asiaticus*<sup>11</sup> and *Albatrellus yasudae*<sup>12,13</sup> that inhibit A $\beta$  aggregation and/or BACE1. In addition to these mushrooms, a methanol extract from the fruiting body of *Polyozellus multiplex* showed inhibitory activity against A $\beta$  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,<sup>14</sup> anti-tumorigenic,<sup>15</sup> and anti-prolylendopeptidase<sup>16-19</sup> 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<sup>20</sup> and are expected to have anti-AD activity. However, no reports evaluating the A $\beta$  aggregation inhibitory activity of *P. multiplex* are known. Recently, A $\beta$ <sub>42</sub> aggregation inhibitory activity was reported for *p*-terphenyl compounds isolated from *Boletopsis leucomelas* of the same family as *P. multiplex*.<sup>21</sup> Based on this, it would be meaningful to evaluate the A $\beta$  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 $\beta$ <sub>40</sub> aggregation inhibitory activity, BACE1 inhibitory activity, and neuroprotective effects. Furthermore, their structure-activity relationships (SARs) were examined.

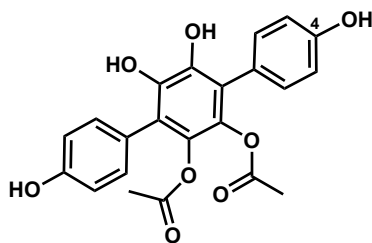
### **Extraction, isolation, structural determination, and inhibitory activity of A $\beta$ aggregation**

The methanol extract from the fruiting body of *P. multiplex* showed potent inhibitory activity against A $\beta$ <sub>40</sub> aggregation (an A $\beta$ <sub>40</sub>-aggregation rate of 25.1%) at 10  $\mu$ g/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 $\beta$ <sub>40</sub> aggregation at 10  $\mu$ g/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 $\beta$ <sub>40</sub>-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

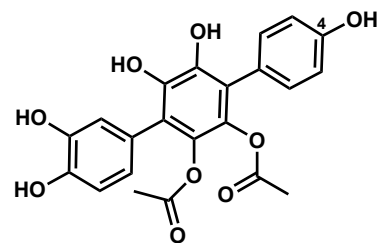
(1),<sup>22,23</sup> kynapcin-12 (2),<sup>19,24</sup> NSC617425 (3),<sup>25</sup> cycloleucomelone (4),<sup>26</sup> BI-V (5),<sup>27</sup> succinic acid (6),<sup>28</sup> and protocatechuic acid (7)<sup>28</sup> (Figure, 1, S2, S3). This is the first report on the isolation of compounds 4–7 from *P.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



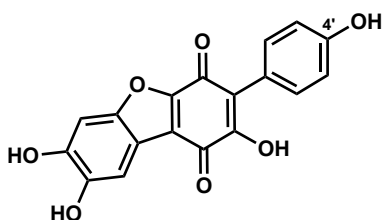
polyozellin (1)



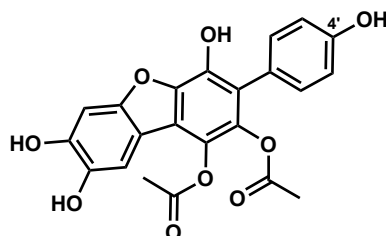
kynapcin-12 (2)



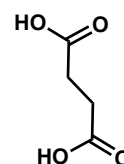
NSC617425 (3)



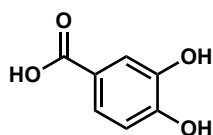
cycloleucomelone (4)



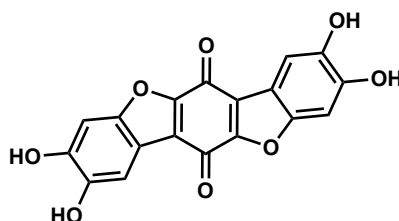
BI-V (5)



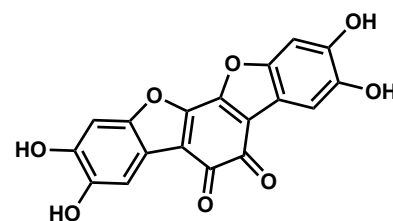
succinic acid (6)



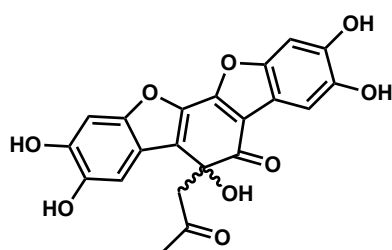
protocatechuic acid (7)



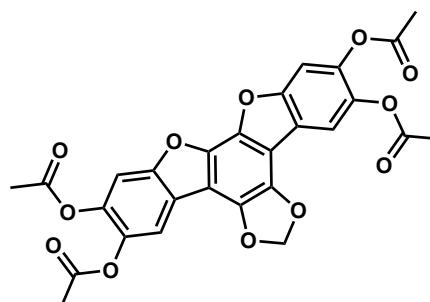
thelephoric acid (8)



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 BI-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<sub>50</sub>) values of compounds **1–7** were 0.615, 6.76, 6.45, 2.17, 3.61, >100, and >100  $\mu$ M, respectively (Table 1, S5). Compounds **1–5** inhibited A $\beta$ <sub>40</sub> aggregation in a dose-dependent manner. The results of compound **5** supported the report of Kodori et al.<sup>21</sup>

**Table 1.** A $\beta$  aggregation and BACE1 inhibitory activities of compounds **1–11**

	A $\beta$ aggregation IC <sub>50</sub> ( $\mu$ M, n=3)	BACE1 IC <sub>50</sub> ( $\mu$ M, n=3)
<b>1</b>	0.615	4.78 <sup>d</sup>
<b>2</b>	6.76	15.79 <sup>d</sup>
<b>3</b>	6.45	>100
<b>4</b>	2.17	66.62
<b>5</b>	3.61	>100
<b>6</b>	>100	>100
<b>7</b>	>100	>100
<b>8</b>	12.62	3.50 <sup>d</sup>
<b>9</b>	1.29	4.78 <sup>d</sup>
<b>10</b>	1.96	34.49
<b>11</b>	21.61	>100
myricetin <sup>a</sup>	9.9 <sup>c</sup>	2.8 <sup>e</sup>
inhibitor IV <sup>b</sup>	>100 <sup>c</sup>	0.015 <sup>c</sup>

<sup>a</sup>Myricetin was used as a positive control for the inhibitory activity of A $\beta$  aggregation.

<sup>b</sup>Inhibitor IV was used as a positive control for BACE1 inhibitory activity.

<sup>c,d,e</sup>Data were referred to reference,<sup>13,20,29</sup> respectively.

### Structure-activity relationship (SAR)

To determine the SAR of inhibitory activity on A $\beta$ <sub>40</sub> 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).<sup>15</sup> The IC<sub>50</sub> value of compounds **8–11** were 12.62, 1.29, 1.96, and 21.61  $\mu$ M, respectively (Table 1, S5). Compounds **8–10** exhibited inhibitory activity against A $\beta$ <sub>40</sub> aggregation in a dose-dependent manner. Based on these

assay results, there are some consistent characteristics of compounds with inhibitory activity on A $\beta$ <sub>40</sub> 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.<sup>30</sup> reported a site-specific inhibitory mechanism against A $\beta$ <sub>42</sub> 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.<sup>31,32</sup> 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 $\beta$ <sub>40</sub> 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 $\beta$ <sub>40</sub> aggregation inhibitory activity compared to the other compounds that showed activity.

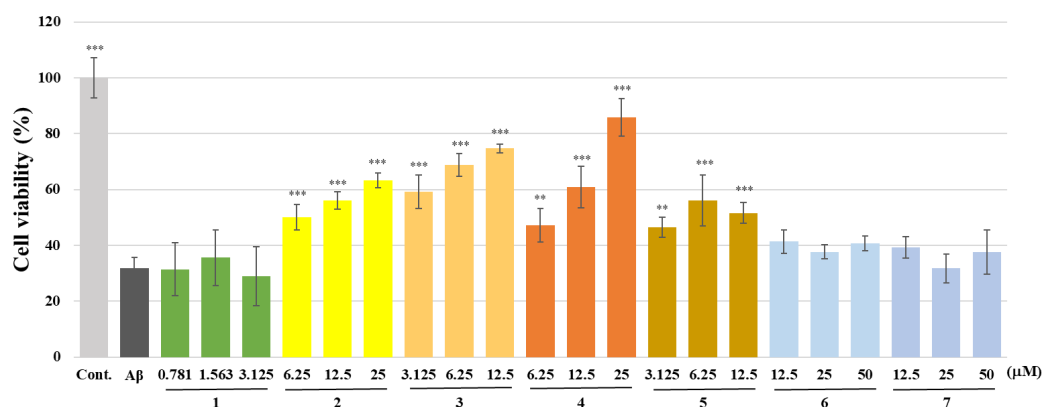
### **Inhibitory activity against BACE1**

In this study, compounds **3–7**, **10**, and **11** were evaluated for their inhibitory activities against BACE1. The IC<sub>50</sub> values of compounds **3–7**, **10**, and **11** were >100, 66.62, >100, >100, >100, 34.49, and >100  $\mu$ M, respectively (Table 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.<sup>20</sup>

### **Protective effect of compounds on A $\beta$ <sub>42</sub> toxicity in SH-SY5Y cells**

Since compounds **1–5** inhibited A $\beta$ <sub>40</sub> aggregation, their neuroprotective effects against A $\beta$ <sub>42</sub> toxicity were also evaluated using human neuroblastoma SH-SY5Y cells. Compounds **6** and **7**, which did not inhibit A $\beta$ <sub>40</sub> aggregation, were evaluated as well. To avoid measuring a reduction in A $\beta$ <sub>42</sub> production due to the potential toxicity of these compounds, the effect of these compounds on SH-SY5Y cells was measured before the A $\beta$ <sub>42</sub> 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  $\mu$ M A $\beta$ <sub>42</sub> was reduced to 31.7% of the control group, whereas the cell viabilities with co-incubation of compounds **1–7** and 10  $\mu$ M A $\beta$ <sub>42</sub> were 29.0  $\pm$  10.6% at 3.1  $\mu$ M, 63.3  $\pm$  2.60% at 25.0  $\mu$ M, 74.7  $\pm$  1.5% at 13.0  $\mu$ M, 85.8  $\pm$  6.8% at 25.0  $\mu$ M, 51.6  $\pm$  3.7% at 13.0  $\mu$ M, 40.7  $\pm$  2.6% at 13.0  $\mu$ M, and 37.7  $\pm$  7.9% at 13.0  $\mu$ M, 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 A $\beta$ <sub>40</sub> aggregation inhibitory activity. Although compound **1** has a catechol moiety, it showed no neuroprotective effects against A $\beta$ <sub>42</sub> 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 A $\beta$  toxicity. Cont. indicates the control group (cells in the absence of A $\beta$  and compounds **1–7**). A $\beta$  indicates the group treated with 10  $\mu$ M A $\beta$  but no compound. **1–7** indicate the groups treated with each compound (**1–7**) and 10  $\mu$ M A $\beta$ . Statistical significance was analyzed using one-way ANOVA followed by Dunnett’s test. Values are expressed as mean  $\pm$  SD, n=3, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001 as compared with A $\beta$ .

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 A $\beta$ <sub>42</sub> 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 A $\beta$ <sub>42</sub> 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 A $\beta$ <sub>42</sub>.<sup>33</sup> 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 A $\beta$ <sub>40</sub> aggregation. Compounds **2–5** protected against A $\beta$ <sub>42</sub> 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 A $\beta$ <sub>42</sub> in the brain. Furthermore, because compounds **2–5** also inhibited A $\beta$ <sub>40</sub> aggregation, they might be expected to be prophylactic agents against AD and mild cognitive impairment. Compound **1** exhibited inhibitory activity against both BACE1<sup>20</sup> and A $\beta$ <sub>40</sub> 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 A $\beta$  aggregation or BACE1. In general, no relationship has been found between the inhibition of A $\beta$  aggregation and BACE1. However, there has been a finding similar to the present study for *A. yasudae*.<sup>13</sup> Therefore,

compounds that inhibit both activities, such as compound **1**, may be useful as new prophylactic agents for the treatment of AD. A $\beta$  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.<sup>34-37</sup> It is necessary for prophylactic and therapeutic drugs to suppress the aggregation of neurotoxic A $\beta$ . 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 A $\beta$  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  $\mu\text{m}$ ; Kanto Chemical, Tokyo, Japan), ODS silica gel YMC-GEL ODS-A (75  $\mu\text{m}$ ; YMC Co., Ltd., Kyoto, Japan), Chromatorex COOH MB100-75/200 (100  $\mu\text{m}$ ; 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<sub>18</sub> column (10 $\phi$   $\times$  250 mm; GL Sciences Inc., Tokyo, Japan), Senshu Pak PEGASIL Silica SP100 (10 $\phi$   $\times$  250 mm; Senshu Scientific Co., Ltd.), and Inertsustain phenyl 5  $\mu\text{m}$  column (10 $\phi$   $\times$  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<sub>3</sub> and MeOH at room temperature. The MeOH extracts were evaluated for their inhibitory activity against A $\beta$  aggregation with an A $\beta$  aggregation rate of 25.1% at 10  $\mu\text{g}/\text{mL}$ . The MeOH extract (75.0 g, 25.1%) was

further fractionated by Si. C. C. using a stepwise gradient (CHCl<sub>3</sub>-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 A $\beta$  aggregation at 10  $\mu$ g/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<sub>3</sub>-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 A $\beta$  aggregation at 10  $\mu$ g/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<sub>3</sub>-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 A $\beta$  aggregation at 10  $\mu$ g/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<sub>2</sub>O (50:0, 100:0), (2) Si-COOH. C. C. with CHCl<sub>3</sub>-MeOH (50:1, 20:1, 10:1, 5:1, 2:1, 0:100), (3) preparative HPLC by ODS C. C. eluted with MeOH-H<sub>2</sub>O (45:55) to obtain NSC617425 (**3**) (5.6 mg, *t<sub>R</sub>* 8.0 min) and kynapcin-12 (**2**) (33.0 mg, *t<sub>R</sub>* 11.2 min), (4) preparative HPLC by ODS C. C. eluted with MeOH-H<sub>2</sub>O (5:5) to obtain Bl-V (**5**) (9.1 mg, *t<sub>R</sub>* 11.2 min), and (5) preparative HPLC by ODS C. C. eluted with MeOH-H<sub>2</sub>O (2:8) to obtain succinic acid (**6**) (13.4 mg, *t<sub>R</sub>* 8.0 min).

Fraction F3d (262.7 mg, 18.9%) was subjected to three chromatography steps: (1) Sephadex LH-20 with CHCl<sub>3</sub>-MeOH-acetone (1:1:1), (2) Si-COOH. C. C. with CHCl<sub>3</sub>-MeOH (50:1, 0:100), and (3) ODS C. C. with MeOH-H<sub>2</sub>O (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<sub>3</sub>-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 A $\beta$  aggregation at 10  $\mu$ g/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<sub>3</sub>-MeOH-acetone (1:1:1) to obtain cycloleucomelone (**4**, 12 mg).

### **Thioflavin-T (Th-T assay)**

Similar data were previously obtained for A $\beta$ <sub>40</sub> and A $\beta$ <sub>42</sub> using both test conditions, and thus A $\beta$ <sub>40</sub> was used in this study.<sup>38</sup> Th-T assays of extracts, fractions, and isolated compounds were performed using thioflavine-T (FUJIFILM Wako Pure Chemical Co., Ltd., Japan) and A $\beta$ <sub>40</sub> (Peptide Institute, Japan). The



aggregation of A $\beta$  was evaluated using a slight modification of the Th-T method developed by Naiki and co-workers.<sup>35</sup> In brief, 80  $\mu$ L 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  $\mu$ L of each test sample that was dissolved with DMSO. Then, 10  $\mu$ L of A $\beta$ <sub>40</sub> (250  $\mu$ M in 0.02% NH<sub>4</sub>OH) was added to the tube so that the final concentration was A $\beta$ <sub>40</sub> 25  $\mu$ M, respectively. All procedures were performed on ice. The resultant solution was incubated at 37 °C for 24 h, and then mixed with 300  $\mu$ L of 5.0  $\mu$ M Th-T in 5.0 mM Gly-NaOH buffer at pH 8.5. The mixture was transferred to black 96-well plates at 100  $\mu$ L 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 A $\beta$  aggregation rate was calculated by the following equation: A $\beta$  aggregation rate (%) = (S – B)/(C – B)  $\times$  100, where C is the fluorescence intensity of a DMSO control (assay buffer, 25  $\mu$ M A $\beta$ <sub>40</sub>, and DMSO containing no test sample) after 24 h of incubation, B is the fluorescence intensity of a blank (assay buffer, 25  $\mu$ M A $\beta$ <sub>40</sub>, and DMSO containing no test sample) without incubation, S is the fluorescence intensity of the tested samples (assay buffer, 25  $\mu$ M A $\beta$ <sub>40</sub>, 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<sub>50</sub> 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).<sup>39</sup> 9  $\mu$ L of test sample, 9  $\mu$ L of 722 nM BACE1 substrate (Rh-EVNNLDAEFK-Quencher in 50 mM ammonium bicarbonate), and 9  $\mu$ L 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<sub>0</sub>) – (B – B<sub>0</sub>)/(C – C<sub>0</sub>) – (B – B<sub>0</sub>)}]  $\times$  100, where C is the fluorescence of a DMSO control (enzyme, substrate, and assay buffer with DMSO) after 3 h of incubation, C<sub>0</sub> 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<sub>0</sub> 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<sub>0</sub> is the fluorescence of the tested samples at 1 h after incubation.  $\beta$ -secretase inhibitor IV (Merck, Germany) was used as a positive control. The IC<sub>50</sub> 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<sub>2</sub>.

## 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  $9.6 \times 10^3$  cells/well were seeded. After incubation for 72 h, the medium was removed. Each test sample and 250 µM Aβ<sub>42</sub> in 0.02% NH<sub>4</sub>OH (final concentration of Aβ<sub>42</sub>: 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<sub>2</sub>, 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<sub>2</sub>, then the absorbance at 450 nm was measured using the Synergy HTX Multi-Mode Reader while blank wells containing no-cells, no-Aβ<sub>42</sub> 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 ( $9.6 \times 10^3$  cells/well, 10 µM Aβ<sub>42</sub>, and DMSO containing no test samples), S is the absorbance at 450 nm of DMSO control ( $9.6 \times 10^3$  cells/well, 10 µM Aβ<sub>42</sub>, and DMSO containing test sample), B<sub>C</sub> is the absorbance at 450 nm of DMSO control (no cells, no Aβ<sub>42</sub> peptide, and DMSO containing no test samples), B<sub>S</sub> is the absorbance at 450 nm of DMSO control (no cells, no Aβ<sub>42</sub> peptide, and DMSO containing test samples), each absorbance was measured in the same procedure.

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