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INHIBITION OF AMYLOID β AGGREGATION BY *p*-TERPHENYL DERIVATIVES ISOLATED FROM *BOLETOPSIS LEUCOMELAS*

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Abstract – The number of patients with Alzheimer's disease (AD) is increasing rapidly. Amyloid polypeptides are thought to be involved in the pathogenesis of the disease. Amyloid-β (Aβ), which aggregates in the brain, is cytotoxic and is considered the cause of AD. Therefore, inhibiting the aggregation of amyloid polypeptides and degrading existing amyloid aggregates is a promising approach for the treatment and prevention of the disease. In the present study, we report the inhibition of Aβ42 aggregation by *p*-terphenyl derivatives **1**–**4** isolated from *Boletopsis leucomelas* and the hydrolysis derivative **5** and acetyl derivative **6** of compound **3**. *p*-Terphenyl derivatives exhibited aggregation-inhibiting activity in a dose-dependent manner. All of the *p*-terphenyl derivatives used in this study demonstrated activity, suggesting that daily consumption of foods containing *p*-terphenyl derivatives may be effective for the prevention and treatment of AD.

INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative disease, and it affects many elderly people. Currently, there is no cure for AD. Recently, the amyloid hypothesis has been proposed as a possible mechanism for the pathogenesis of AD.[1] The amyloid hypothesis refers to the self-assembly of 40- or 42-mer amyloid-β proteins (Aβ40 or Aβ42) to form senile plaques. Aβ peptides are produced from amyloid precursor protein (APP) by β- and γ-secretases.[2,3] Aβ40 and Aβ42 form oligomers and fibrils, respectively,

and are deposited in the cerebral cortex in a β-sheet structure, causing neuronal cell death and hippocampal atrophy, eventually leading to AD.

It is likely that Aβ42 plays a more important role in the pathogenesis of AD because it has a stronger tendency to oligomerize and is more neurotoxic than Aβ40.[1,4] Therefore, the inhibition of Aβ aggregation by small molecules derived from natural products may contribute to the prevention and treatment of AD. In addition, catechol is likely to be involved in the inhibition of Aβ aggregation, and a recent structure-activity relationship study using three tyrosol ligands demonstrated that catechol has aggregation-inhibiting activity, which is a result of hydrogen bonding between the hydroxy group of catechol and Glu22. The hydrogen bond may stabilize the Aβ-ligand interaction.[5]

We have previously reported that caffeoylquinic acids, phenylethanoid glycosides, and hispidin derivatives inhibit Aβ42 aggregation.[6-9] Recently, we demonstrated that kukoamines A and B, schizotenuin A, lycopic acid, rosmarinic acid, clovamide, and A-type procyanidins exhibit Aβ aggregation-inhibiting activity.[10-15] All of these compounds contain catechol moieties, suggesting that catechol-type polyphenols may inhibit the aggregation of amyloid proteins.

Boletopsis leucomelas is an edible mushroom that forms black nodules. Several bioactive compounds, including polyphenols such as thelephoric acid, have been isolated from this mushroom[16] and *p*-terphenyl derivatives such as thelephoric acid have been reported to inhibit the enzyme β-secretase (BACE1), which cleaves the Aβ precursor protein (APP).[17] However, to the best of our knowledge, there are no reports on the inhibition of Aβ42 aggregation by *p*-terphenyl derivatives. *p*-Terphenyl derivatives isolated from *B. leucomelas* contain a catechol moiety, which may have an inhibitory effect on Aβ aggregation.

In the present study, we isolated and derivatized several *p*-terphenyl derivatives from *B. leucomelas* and investigated their inhibitory activity against Aβ aggregation.

RESULTS AND DISCUSSION

Isolation of *p*-terphenyl derivatives from *B. leucomelas*

B. leucomelas (58.6 g, wet weight) was extracted in MeOH, and the MeOH extract (5.0 g) was partitioned with EtOAc and H_2O. The EtOAc-soluble material (1.1 g) was separated by silica gel chromatography to yield compounds **1** (161.2 mg; Bl-III), **2** (19.2 mg; sarcodan), **3** (297.7 mg; Bl-IV), and **4** (12.4 mg; Bl-V). Compound **3** was hydrolyzed with NaOH aq. in MeOH to afford **5**, and was separately acetylated with acetic anhydride in pyridine to afford compound **6**. The structures of compounds **1**–**6** (Figure 1) were confirmed by NMR spectral data.[16,18-20]

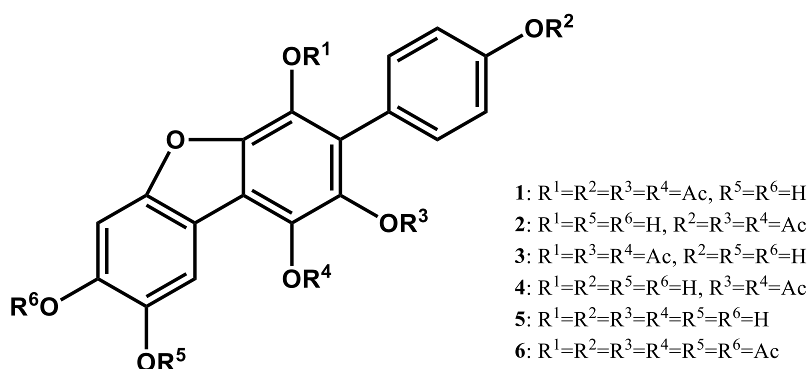

Figure 1. Structures of compounds **1–6**

Inhibitory effect of compounds 1–6 on Aβ42 aggregation

A thioflavin-T (Th-T) fluorescence assay was performed to evaluate the inhibitory effect of compounds **1**–**6** on Aβ42 aggregation (Figure 2). The IC_{50} values were calculated from the percentage inhibition (%) of each compound on Aβ42 aggregation after 24 h; **1**: 1.2 μM, **2**: 1.3 μM, **3**: 0.9 μM, **4**: 0.5 μM, **5**: 1.6 μM, and **6**: 7.2 μM. Compounds **1**–**5**, which have one or two catechol moieties, and compound **6**, which does not have a catechol moiety, all demonstrated the same level of activity. The IC_{50} value of clovamide, which has already been reported to have aggregation inhibitory activity in our previous result,[13] is 5.7 μM, and the compound of this study has the same or higher activity.

Transmission electron microscopy (TEM) was used to examine the effect of compounds **1**–**6** on the fibril formation of Aβ42. Typical fibril formation was observed in Aβ42 alone. However, treatment with compounds **1**–**6** significantly inhibited fibril formation after 24 h (Figure 3).

The Th-T assay was used to investigate the efficacy of the *p*-terphenyl derivatives and the structure-activity relationships of **1**–**6** that lead to the inhibition of Aβ42 aggregation (Figure 2). Furthermore, TEM was used to observe the modulation of the inhibitory effect of **1**–**6** on Aβ42 fibril formation (Figure 3). The Th-T assay and TEM results demonstrate that compounds **1**–**5** exhibit comparable activity. Several studies have demonstrated that the catechol moiety is important for Aβ42 aggregation inhibitory activity. The catechol moiety readily autoxidizes to form *o*-benzoquinone, which can covalently bind to nucleophilic amino acid residues in amyloid proteins (Michael addition and Schiff base formation) and destabilize the β-sheet structure.[21-23] As compounds **1**–**5** contain catechol moieties, this mechanism can be used to explain their inhibitory effect on Aβ42 aggregation.

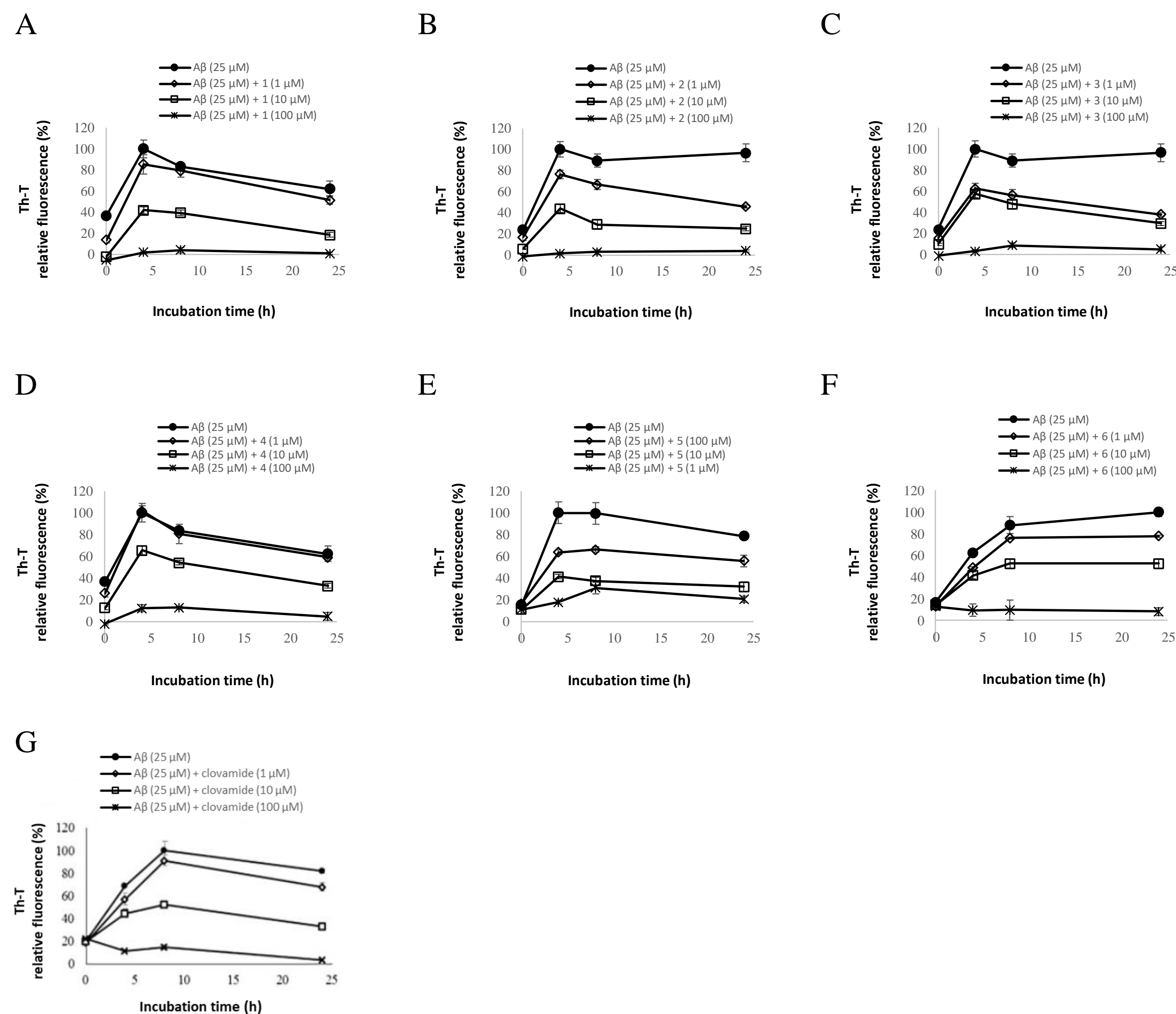

Figure 2. Effect of compounds **1**–**6** on aggregation of Aβ42. Fibril formation of 25 μM of Aβ42 was monitored by Th-T fluorescence and presence of 1, 10, and 100 μM of **A**: **1**, **B**: **2**, **C**: **3**, **D**: **4**, **E**: **5**, **F**: **6** and G: clovamide. Fluorescence intensity was measured at an excitation wavelength 420 nm and emission wavelength of 485 nm, n=7.

Compound **6**, which contains no catechol moieties, demonstrated weaker activity than that of compounds **1**–**5**. There have been a large number of reports on the anti-aggregation ability and mechanism of action of catechol-type flavonoids such as (+)-taxifolin, myricetin, quercetin, (+)-catechin, epigallocatechin gallate, and rosmarinic acid on amyloid-forming proteins. In addition, several non-catechol-type flavonoids and curcuminoids have also demonstrated potent inhibitory effects on Aβ42 aggregation.

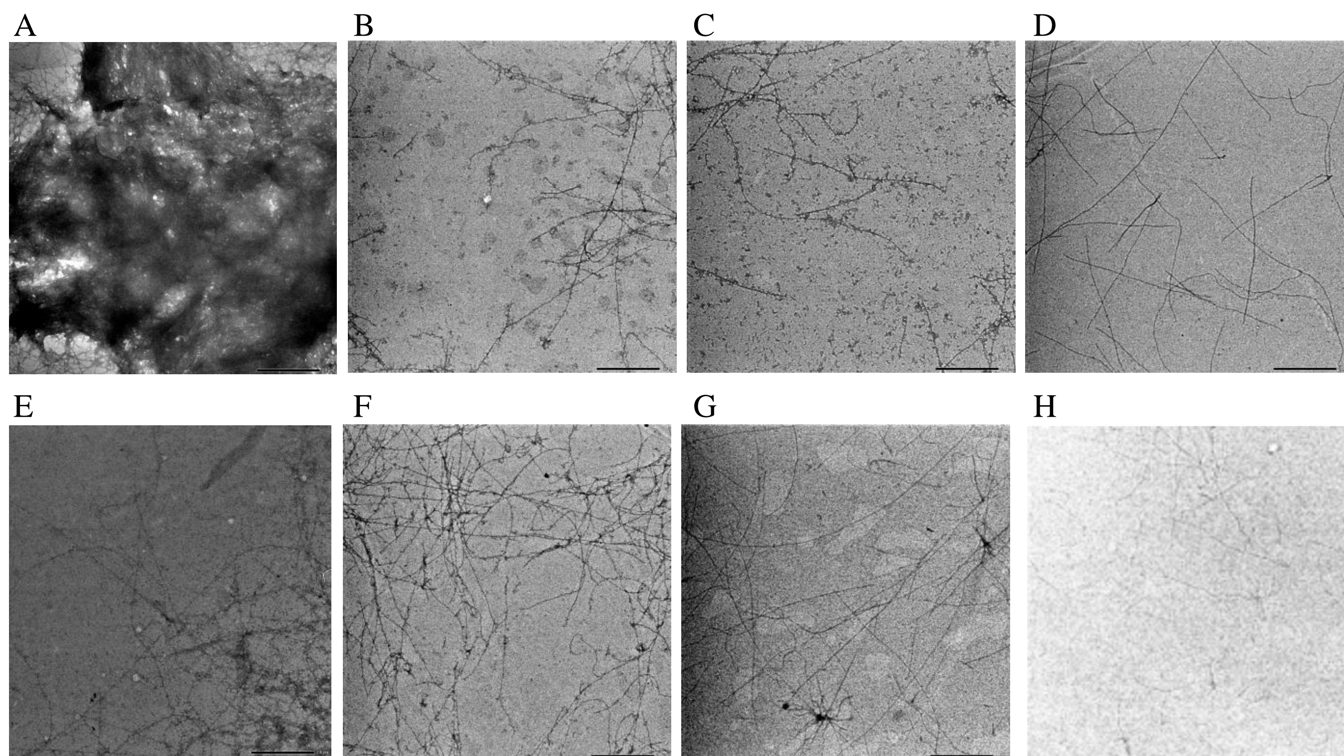

Figure 3. Effects of compounds **1**–**6** on Aβ42 fibrillogenesis by transmission electron micrographs. Fibril formation was observed after 24 h incubation in 50 μM PBS buffer. Scale bars: 1 μm. A: Aβ42 (25 μM), **B**: Aβ42 (25 μM) + **1** (100 μM), **C**: Aβ42 (25 μM) + **2** (100 μM), **D**: Aβ42 (25 μM) + **3** (100 μM), **E**: Aβ42 (25 μM) + **4** (100 μM), **F:** Aβ42 (25 μM) + **5** (100 μM), **G:** Aβ42 (25 μM) + **6** (100 μM) and **H:** Aβ42 (25 μM) + clovamide (100 μM).

Previous studies have suggested that π-π stacking interactions between amino acid residues of Aβ42 and the aromatic rings of inhibitors and hydrogen bonding are the factors controlling the inhibition of Aβ42 β-sheet formation.[24] Irie *et al.* reported that the mechanism of inhibition of Aβ aggregation by the non-catechol structural compounds morin and datiscetin involves an important interaction between the aromatic rings Phe19 and Phe20 in Aβ42 aggregates due to their inherent planarity.[25] Morin and datiscetin are planar compounds similar to curcumin, as they contain an aromatic B ring in the C ring and an α,β-unsaturated ketone. In this study, compounds **1**–**6** also have an furan moiety with a fixed planar structure, which may intercalate with Phe19 and Phe20 in Aβ42 aggregates, leading to the inhibition of Aβ42 aggregation.

Docking simulations using benzoquinone, which is structurally similar to compounds **1**-**6**, also report π-π stacking interactions between Leu34 and Met35.[26] It can be inferred that compound **6** uses this mechanism to inhibit Aβ42 aggregation.

Thus, compounds **1**–**5** prevent Aβ42 aggregation by covalent binding of Aβ42 to Lys16 and Lys28 as well as non-covalent interactions with His13, His14, Phe19, and Phe20 residues, while compound **6** prevented aggregation only by non-covalent interactions with His13, His14, Phe19, and Phe20 residues. The formation of covalent bonds with the Lys residues of Aβ42 is expected to inhibit aggregation more

effectively than milder interactions. This is consistent with the report that the formation of covalent bonds with the Lys residues of Aβ42 would be more effective for inhibiting aggregation than the moderate interaction with His13, His14, Phe19, and Phe20 residues through π-π stacking.[25]

To the best of our knowledge, this is the first report demonstrating the inhibitory effect of *p*-terphenyl derivatives on Aβ42 aggregation. Therefore, the use of *p*-terphenyl derivatives may be a promising therapeutic approach to inhibit Aβ-mediated pathology in AD progression. In addition, the high content (*ca.* 1–2%) of *p*-terphenyl derivatives in *B. leucomelas* may add value to *B. leucomelas* as an edible mushroom.

EXPERIMENTAL

General Procedures

^{1}H and ^{13}C NMR spectra were obtained with a Bruker Avance 500 spectrometer in CD_3OD. The resonances at δ_H 3.35 and δ_C 49.8 were used as internal references for the ^{1}H and ^{13}C NMR spectra. ESI-MS and HRESI-MS spectra were recorded with a Waters Synapt G2 mass spectrometer.

Mushroom Material

Boletopsis leucomelas was collected from Kamiina-gun, Nagano, Japan and was identified by co-author Dr. K. Hosaka.

Isolation of the *p*-terphenyl derivatives from *B. leucomelas*

The fruiting body (58.6 g, wet weight) of *B. leucomelas* was extracted in MeOH (200 mL×3), and the MeOH extract (BL, 5.0 g) was partitioned using EtOAc (250 mL×1, 200 mL×2) and H_2O (250 mL). The EtOAc soluble material (BL-EA, 1.1 g) was transferred to a silica gel column (Wakogel C-300, 2.2×37 cm, $CHCl_3$/MeOH, 100:0→98:2→95:5→9:1→8:2→0:100) and partitioned into a further 15 fractions (BL-EA-1–15). Compound **1** (161.2 mg) was obtained from BL-EA-8 and compound **3** (297.7 mg) from BL-EA-11. BL-EA-10 was purified using a silica gel column (Wakogel C-300, 1.0×12 cm, $CHCl_3$/MeOH, 9:1→0:10) to yield compound **2** (19.2 mg). BL-EA-13 was purified using a silica gel column (Cosmosil 75C 18-OPN, 1.0×13 cm, H_2O/MeOH, 1:1→4:6→0:10) to yield compound **4** (12.4 mg). The structures of compounds **1**–**4** were confirmed by NMR spectral analysis.[17,19-21]

Hydrolysis of compound 3

Compound **3** (30 mg) was reacted with NaOH aq (2 mL) in MeOH (2 mL). The mixture was reacted at room temperature for 30 min and then EtOAc (6 mL) and H_2O (2 mL) were added. The EtOAc layer was concentrated and dried to afford compound **5** (19.9 mg, 97% yield).[16] ^{1}H NMR (CD_3OD) δ_H: 6.81 (2H,

d, J = 6.6 Hz), 7.06 (1H, s), 7.26 (2H, d, J = 6.6 Hz), and 7.36 (1H, s); ^{13}C NMR (CD_3OD) δ_C: 99.0, 108.8, 115.6, 116.0 (2C), 117.0, 118.5, 126.5, 132.4, 133.4 (2C), 133.8, 138.5, 133.5, 142.7, 146.5, 152.0, and 157.6; ESI-MS (positive ion) *m/z* 341 $[M+H]^+$, HR-ESI-MS (positive ion) *m/z* 341.0661 $[M+H]^+$ (calcd for $C_{18}H_{13}O_7$, 341.0659).

Acetylation of compound 3

Compound **3** (53 mg) was reacted with acetic anhydride (0.6 mL) in pyridine (0.5 mL) at room temperature for 24 h. The reaction solution was concentrated *in vacuo* to afford **6** (47 mg, 86% yield).[20] ^{1}H NMR ($CDCl_3$) δ_H: 7.16 (2H, d, J = 8.6 Hz), 7.33 (2H, d, J = 8.6 Hz), 7.49 (1H, s), and 7.58 (1H, s); ^{13}C NMR ($CDCl_3$) δ_C: 19.7, 19.9, 20.2, 20.3 (2C), 20.8, 107.8, 116.3, 117.8, 118.8, 121.9 (2C), 128.1 (2C), 130.3, 130.4 (2C), 134.4, 136.1, 139.2, 142.6, 146.1, 150.5, 152.9, 167.8, 168.0 (3C), 168.3, and 168.9; ESI-MS (positive ion) *m/z* 615 $[M+Na]^+$, HR-ESI-MS (positive ion) *m/z* 615.1115 $[M+Na]^+$ (calcd for $C_{30}H_{24}O_{13}Na$, 615.1131).

Th-T assay

The aggregation ability of Aβ42 was assessed by the Th-T method developed by Niki *et al.*[27] The procedure is described elsewhere.[28] To assess the inhibitory effect of compounds **1**–**6** on Aβ aggregation, a Th-T fluorescence assay was performed. Briefly, 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 incubated at 37 °C with and without the sample. The peptide solution (2.5 μL) was added to 250 μL of 5 μM Th-T in 5 μM Gly-NaOH (pH=8.5). The fluorescence intensity was measured using a Wallac 1420 ARVO MX multi-detection microplate reader (PerkinElmer) at an excitation wavelength of 420 nm and emission wavelength of 485 nm. The relative fluorescence of Th-T was calculated as a percentage of Aβ42 alone, with a maximum value of 100%. The IC_{50} value was calculated from the inhibition rate (%) of each compound on Aβ42 aggregation after 24 h of incubation.

TEM

TEM was used to examine the fibrillation of Aβ42. Aβ42 (25 μM) was treated with compounds **1**–**6** (100 μM each), deposited on carbon-coated Formvar grids, incubated at room temperature for 2 min, washed twice with H_2O, and air-dried for 5 min. After 24 h of incubation, the samples were observed using a JEOL JEM-1400 electron microscope.

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