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STRUCTURE-ACTIVITY RELATIONSHIP OF PHENYLETHANOID GLYCOSIDES ON THE INHIBITION OF AMYLOID β AGGREGATION

Eri Kidachi,[a] Manami Kurisu,[a] Yusaku Miyamae,[b] Mizuho Hanaki,[c] Kazuma Murakami,[c] Kazuhiro Irie,[c] and Hideyuki Shigemori[d,*]

[a]Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan; [b]Graduate School of Biostudies, Kyoto University, Kitashirakawa Oiwake-cho, Sakyo-ku, Kyoto 606-8502, Japan; [c]Graduate School of Agriculture, Kyoto University, Kitashirakawa Oiwake-cho, Sakyo-ku, Kyoto 606-8502, Japan; [d]Faculty of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan; E-mail: shigemori.hideyuk.fn@u.tsukuba.ac.jp

Abstract – The structure-activity relationship of the inhibitory activity of Aβ aggregation and antioxidant activity for phenylethanoid glycosides, acteoside (**1**), oraposide (**2**), and their derivatives which isolated from *Orobanche minor* was investigated. Acteoside (**1**) and oraposide (**2**) containing two catechol moieties exhibit strong activities for inhibition of Aβ aggregation and antioxidant. These results suggested that existence of the catechol moieties of the phenylethanoid glycosides were important roles for these activities.

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia. The pathological hallmarks of AD is extracellular deposits of aggregated amyloid β protein (Aβ) and intracellular neurofibrillary tangles and loss of neurons.[1] The amyloid cascade hypothesis proposes that excessive accumulation of 40- or 42-mer Aβ is the key step in AD.[2] Aβ is produced from the amyloid precursor protein (APP) by sequential proteolytic cleavage of APP first by β-secretase and then by γ-secretase.[3,4] Aβ42 plays a more important role in the pathogenesis of AD than Aβ40 because of its stronger aggregative ability and neurotoxicity.[1] So, inhibition of Aβ42 aggregation is an attractive therapeutic strategy for AD.

Our research groups have already reported that polyphenolic compounds such as caffeoylquinic acids and acteoside (**1**) exhibited Aβ inhibitory effects on Aβ aggregation, suggesting that the catechol moiety is

important role for the activity.[5-8] In addition, the decrease of reactive oxygen species (ROS) is involved in one of the neuroprotective mechanism of action of Aβ has been reported.[9] Therefore, in this study, we investigated SAR of inhibitory activity of Aβ aggregation and antioxidant activity by acteoside (**1**) and its derivatives.

RESULTS AND DISCUSSION

Inhibitory effect of compounds 1-10 on Aβ42 aggregation

Thioflavin-T (Th-T) fluorescence assay was performed to evaluate the inhibitory effects on Aβ aggregation of acteoside (**1**) and its derivatives (Figure 1). The inhibitory effects on Aβ aggregation by acteoside (**1**) and its derivatives are shown in Table 1. The IC_{50} values were calculated from the inhibitory rate (%) of each compound toward Aβ42 aggregation after 48 h by using the Th-T assay. Acteoside (**1**, IC_{50} 11.3 μM) and oraposide (**2**, IC_{50} 8.2 μM) indicated inhibitory effects on the aggregation of Aβ42, while acteoside-tetramethylether (Me-acteoside) (**7**, IC_{50} >100 μM), oraposide-tetramethylether (Me-oraposide) (**8**, IC_{50} >100 μM), and cistanoside D (**5**, IC_{50} >100 μM) did not show inhibitory activity toward Aβ aggregation, and caffeic acid (**9**, IC_{50} 93.8 μM) and hydroxytyrosol (**10**, IC_{50} 92.0 μM) showed very weak inhibitory activities. In addition, 3'''-*O*-methylcrenatoside (**6**, IC_{50} 28.0 μM) which one place of phenolic hydroxyl group was methylated showed moderate activity.

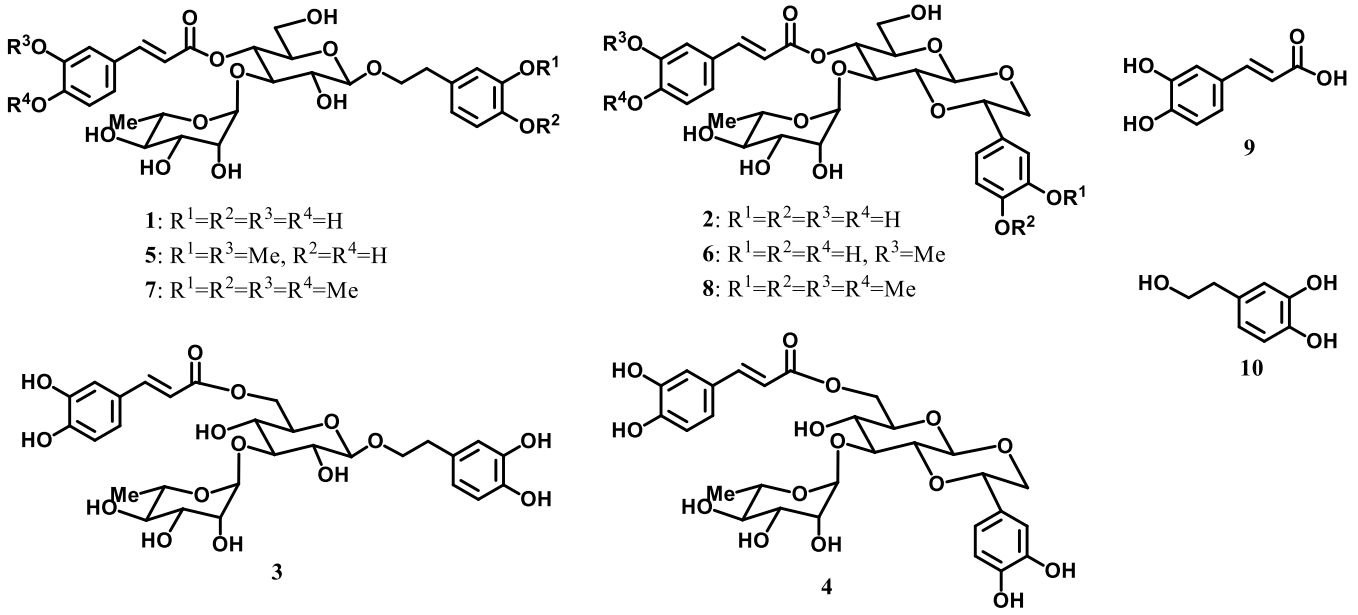

Figure 1. Structures of test compounds **1**-**10**

Table 1. IC_{50} values of **1**-**10** for Aβ aggregation inhibitory activity and DPPH radical scavenging activity

	IC_{50} (μM)	
Compounds	Aβ aggregation	DPPH
acteoside (**1**)	11.3	15.2
oraposide (**2**)	8.2	24.5
isoacteoside (**3**)	33.5	20.0
isocrenatoside (**4**)	27.4	29.0
cistanoside D (**5**)	>100	>100
3'''-*O*-methylcrenatoside (**6**)	28.0	54.2
Me-acteoside (**7**)	>100	>100
Me-oraposide (**8**)	>100	>100
caffeic acid (**9**)	93.8	38.7
hydroxytyrosol (**10**)	92.0	44.6
3,4-di-*O*-caffeoylquinic acid[a]	30.2	-
epigallocatechin gallate[b]	-	13.5

[a,b] positive controls for Aβ aggregation and DPPH assays, respectively

Inhibitory effect of acteoside (1), oraposide (2) and their derivatives 7 and 8 on Aβ42 aggregation with TCEP

From the above results, the importance of the catechol moiety is suggested in the active expression for Aβ aggregation inhibition. So, we performed Th-T assay in the presence of reducing agent, tris(2-carboxyethyl)phosphine (TCEP), to examine whether or not there is change in activity by controlling *o*-benzoquinone structure formation. As a result, under the presence of TCEP, the fluorescence intensities of acteoside (**1**) and oraposide (**2**) were increased, so that Aβ aggregation inhibitory activities were decreased (Figures 2A and 2B). On the other hand, there is no significant difference the fluorescence intensities of Me-acteoside (**7**) and Me-oraposide (**8**), in the presence of TCEP (Figures 2C and 2D). These results imply that the *o*-benzoquinone formation plays an important role for the inhibition of Aβ aggregation.

DPPH radical scavenging activity of compounds 1-10

DPPH radical scavenging activity assay was performed to investigate the antioxidant activity of compounds **1**-**10** (Table 1). Acteoside (**1**, IC_{50} 15.2 μM), oraposide (**2**, IC_{50} 24.5 μM), isoacteoside (**3**, IC_{50} 20.0 μM), and isocrenatoside (**4**, IC_{50} 29.0 μM), which have two catechol moieties, showed strong activity. On the other hand, Me-acteoside (**7**, IC_{50} >100 μM), Me-oraposide (**8**, IC_{50} >100 μM), and cistanoside D (**5**, IC_{50} >100 μM) did not show antioxidant activity. 3'''-*O*-Methylcrenatoside (**6**, IC_{50} 54.2 μM), caffeic acid (**9**, IC_{50} 38.7 μM), and hydroxytyrosol (**10**, IC_{50} 44.6 μM) showed moderate activities. These results imply that the catechol moiety plays an important role for the antioxidant activity.

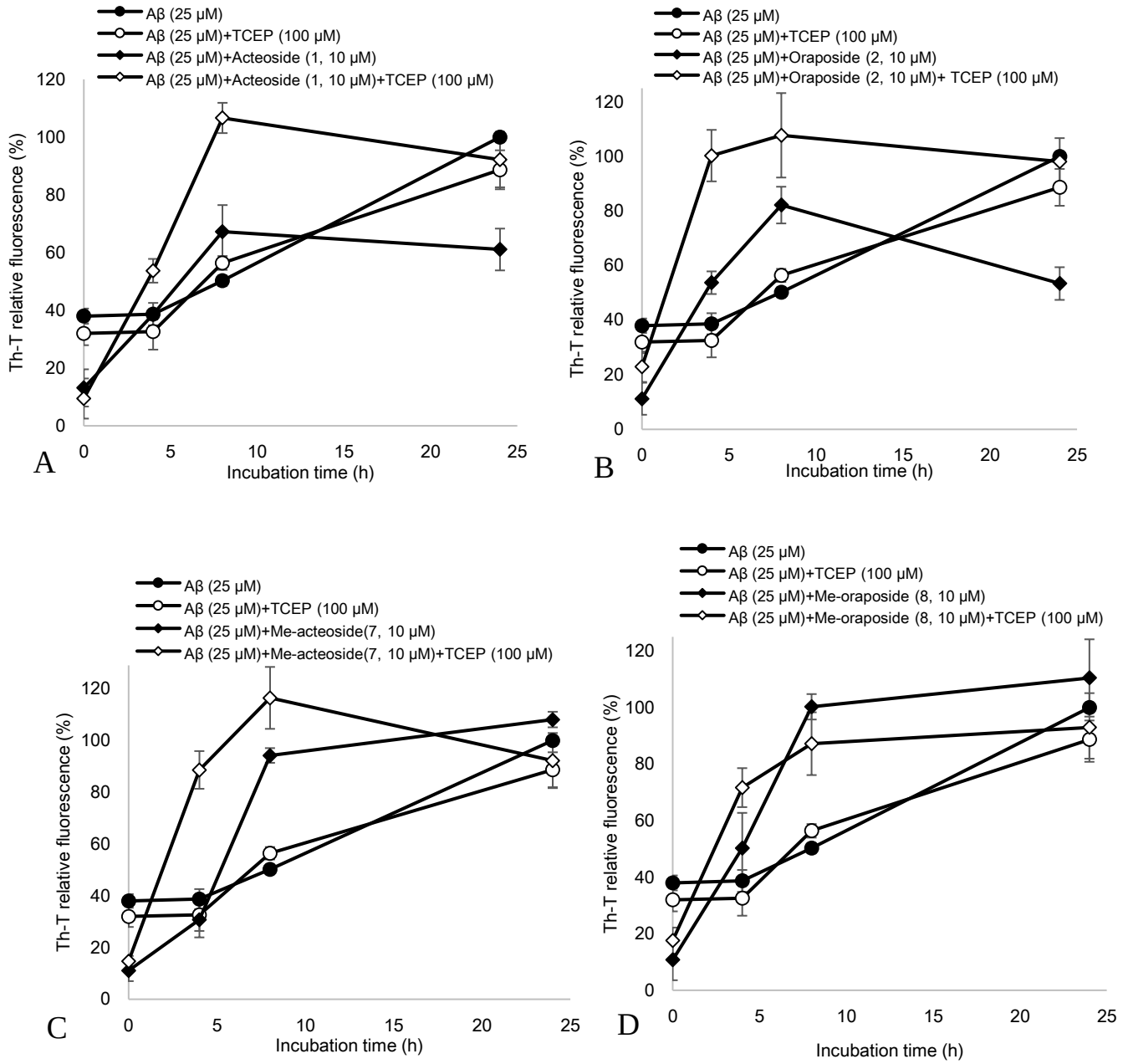

Figure 2. Results of Th-T assay of **1**, **2**, **7**, and **8** in the presence of TCEP

Aβ42 (25 μM) was observed by Th-T fluorescence in the presence of 10 μM (A) acteoside (**1**), (B) oraposide (**2**), (C) Me-acteoside (**7**) and (D) Me-oraposide (**8**) in the presence of 100 μM TCEP. The fluorescence intensity was measured at 420 nm excitation and 485 nm emission. Values represent the mean ± SD (n = 6).

Based on the structure-activity relationship of compounds **1**-**10**, acteoside (**1**) and oraposide (**2**) with two catechol moieties showed Aβ aggregation inhibitory activity. These results do not contradict with our previous reports.[5-8] Our research groups presumed that auto-oxidation of catechol structure would be connected with the hypothesis for Aβ aggregation inhibitory activity. Th-T assay results using the

reducing agent such as TCEP supported this hypothesis. Interaction between catechol structure and Aβ may have destabilized the β-sheet structure in amyloidogenic polypeptides. Since acteoside (**1**) and oraposide (**2**) with two catechol moieties exhibit strong activities for inhibition of Aβ aggregation and antioxidant, this hypothesis may also be applied to these compounds for Aβ aggregation inhibitory agents. In the previous research it has suggested that Aβ-induced oxidative stress played an important role in the pathogenesis or progression of AD.[10] Aβ induces oxidative stress[11] and oxidative stress promoted the production of Aβ.[12] According to the structure-activity relationship for antioxidant activity of the isolated phenylethanoid glycosides, acteoside (**1**), oraposide (**2**), isoacteoside (**3**), and isocrenatoside (**4**), with two catechol moieties, showed strong activity (Table 1). The antioxidative ability of polyphenols such as caffeoylquinic acids and phenylethanoids might be effective to inhibit the formation of Aβ fibril by suppressing the radical-mediated aggregation of Aβ.[7,13] These results suggest that there is a close relationship between Aβ aggregation inhibitory activity and antioxidant activity. Further analysis will be required to elucidate the inhibitory mechanism of acteoside (**1**) and its derivatives against Aβ aggregation.

EXPERIMENTAL

Chemicals

We isolated various kinds of phenolic compounds (Figure 1). By the first extraction, we got acteoside (**1**), oraposide (**2**), isoacteoside (**3**), and isocrenatoside (**4**) from the whole plant of *O. minor* (1.0 kg fresh weight). The plant was extracted with MeOH (1 L×3), and the MeOH extract (51.2 g) was partitioned between EtOAc (1 L×3) and H_2O (1 L). The EtOAc soluble portion (9.0 g from 9.7 g) was divided into 14 fractions using silica gel column chromatography (4.4×30 cm, $CHCl_3$/MeOH, 95:5 to 0:100). A fraction (1.0 g from 6.9 g) was divided into 9 fractions using ODS column chromatography (2.2×35 cm, MeOH/H_2O, 30:70 to 100:0). A fraction was separated by HPLC on TSK-gel ODS-120A (7.8×300 mm, TOSOH; MeOH/H_2O, 35:65 to 100:0) to give acteoside (**1**, 23.8 mg), oraposide (**2**, 21.8 mg), isoacteoside (**3**, 2.4 mg), and isocrenatoside (**4**, 3.0 mg). By the second extraction, cistanoside D (**5**) and 3'''-*O*-methylcrenatoside (**6**) from the whole plant of *O. minor* (1.0 kg fresh weight). The plant extracted with MeOH (1 L×3), and the MeOH extract (63.5 g) was partitioned between EtOAc (1 L×3) and H_2O (1 L). The EtOAc soluble portion (7.0 g from 10.5 g) was divided into 12 fractions using silica gel column chromatography (4.4×30 cm, Hexane/EtOAc, 9:1 to 1:1 and $CHCl_3$/MeOH, 95:5 to 0:100). A fraction (70 mg from 87 mg) was separated by reversed-phase HPLC on TSK-gel ODS-120A (7.8×300 mm, TOSOH; MeOH/H_2O, 40:60 to 90:10) to give cistanoside D (**5**, 3.1 mg) and 3'''-*O*-methylcrenatoside (**6**, 6.5 mg). Acteoside-tetramethylether (**7**) and oraposide-tetramethylether (**8**) were synthesized as previously described.[8] Caffeic acid (**9**) and hydroxytyrosol (**10**) were purchased from Sigma (St. Louis, MO, USA).

Th-T assay

Thioflavin-T (Th-T) fluorescence assay was performed to evaluate the inhibitory effects on Aβ aggregation of acteoside (**1**) and its derivatives. In brief, Aβ42 was dissolved in 0.1% NH_4OH at 250 μM. The Aβ42 solution was diluted 10-fold with sodium phosphate-buffered saline (PBS: 50 mM sodium phosphate and 100 mM NaCl, pH 7.4), and the solution incubated at 37 °C with or without acteoside (**1**) and its derivatives. At each time, 2.5 μL volume of peptide solution was added to 250 μL of 1 mM Th-T in 50 mM Gly-NaOH (pH 8.5). The fluorescence intensity was measured at 420 nm excitation and 485 nm emission using Multilabel Counter (Wallac 1420 ARVO MX, Perkin Elmer, Inc. Waltham, MA, USA). Tris(2-carboxyethyl)phosphine (TCEP) was dissolved in PBS at 100 mM, then diluted 10-fold with PBS before use. Th-T relative fluorescence was calculated as percentage of Aβ42 alone whose maximum value was taken as 100%.

DPPH radical scavenging activity assay

DPPH radical scavenging activity assay was performed according to the previous studies.[14] In brief, 10 μL of each test compound solution was added to 190 μL of reaction mixture [0.2 mM DPPH radical solution, 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 6.1]. The solution was mixed and incubated for 10 min at room temperature, and the absorbance was measured at 520 nm on Ultramark Microplate Reader (Bio-Rad Laboratories, Inc. Hercules, CA, USA). DPPH radical scavenging activity was calculated as percentage decrease in the absorbance of control.

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