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Note

Structure–Activity Relationship for (+)-Taxifolin Isolated from Silymarin as an Inhibitor of Amyloid β Aggregation

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Silymarin, the seed extract of *Silybium marianum*, has preventive effects against Alzheimer's disease-like pathogenesis *in vivo*. We isolated (+)-taxifolin (4) from silymarin as an inhibitor of aggregation of the 42-residue amyloid β-protein. Structure-activity relationship studies revealed the 3′,4′-dihydroxyl groups to be critical to the anti-aggregative ability, whereas the 7-hydroxyl group and the stereochemistry at positions 2 and 3 were not important.

Key words: Alzheimer's disease; amyloid β; aggregation; (+)-taxifolin; silymarin

Alzheimer's disease (AD) is characterized by amyloid fibril in senile plaques which mainly consist of 40- and 42-residue amyloid β-proteins (Aβ40 and Aβ42).[1,2] Aβ42 has been considered a principal cause of AD-like pathogenesis because of its strong aggregative ability and neurotoxicity.[3] It is widely accepted that a soluble oligomeric assembly of Aβ42 induces neuronal death and cognitive dysfunction.[4,5]

Such polyphenols as curcumin,[6,7] resveratrol,[8] and (−)-epigallocatechin-3-gallate (EGCG)[9] have been reported to show preventive effects on the aggregation and neurotoxicity of Aβ42. Some of these compounds are in clinical or preclinical trials.[10] Since polyphenols can be found in daily foods or supplements,[11] they are promising as preventive medicines or therapeutic agents for AD.

Silymarin, a seed extract of *Silybum marianum* containing flavonolignane diastereomers,[12] has long been used as an anti-hepatotoxic medicine without notable adverse effects,[13] and in particular, is efficacious against the damage induced by alcohol and disturbances in the function of the gastrointestinal tract.[14] Our group has recently reported that silymarin reduced such AD-like pathologies as senile plaques, neuroinflammation, behavioral dysfunction, and Aβ oligomer formation in a well-established AD mouse model (J20 line).[15] We report in this paper the structure–activity relationship for (+)-taxifolin (**4**) isolated as one of the active components of silymarin against Aβ42 aggregation; this is defined as the change of the Aβ42 monomer into amyloid fibril by way of an oligomer and protofibril.

Silymarin (lot no. 228–216; LKT Laboratories, St. Paul, MN, USA) was fractionated by column chromatography, eluting with 5% MeOH/$CHCl_3$ on Wakogel C-200 (Wako, Osaka, Japan), to give two major fractions containing flavonoids. The first fraction was chromatographed by high-performance liquid chromatography (HPLC) in a YMC-Pack ODS-A column (20 mm i.d. × 150 mm; YMC, Kyoto, Japan), using 50% MeOH/H_2O, to yield silibinin A (**1**, 16 mg from 240 mg of silymarin, 6.7%)[16] and silibinin B (**2**, 25 mg from 240 mg of silymarin, 10%),[16] and using 40% MeOH/H_2O to yield silydianin (**3**, 31 mg from 370 mg of silymarin, 8.4%.[17] The second fraction was separated in a YMC-Pack ODS-AL column (20 mm i.d. × 150 mm; YMC) using 40% MeOH/H_2O to give (+)-taxifolin (**4**, 6.7 mg from 310 mg of silymarin, 2.2%),[18] isosilychristin (**5**, 3.9 mg from 310 mg of silymarin, 1.3%),[19] and silychristin (**6**, 26 mg from 310 mg of silymarin, 8.4%)[20] (Fig. 1A). The structures of these compounds were confirmed by ^{1}H-NMR (AVANCE III 500, ref. tetramethylsilane, Bruker, Germany),[16–20] EI-MS (JMS-600H, 70 eV, 300 μA, JEOL, Tokyo, Japan), and specific optical rotation (P-2200, Jasco, Tokyo, Japan).

The effects of these flavonoids on Aβ42 aggregation were examined by using thioflavin-T (Th-T), a reagent that fluoresces when bound to Aβ aggregates, and transmission electron microscopy (TEM), as previously described.[21,22] As shown in Fig. 1B, only (+)-taxifolin (**4**) among the isolated flavonoids strongly reduced the Th-T relative fluorescence induced by Aβ42 aggregation, meaning the potent inhibition of Aβ42 aggregation by **4**. The analysis of TEM showed that the fibril formation of Aβ42 was inhibited by **4**; shorter or slighter fibrils (Fig. 2B). (+)-Taxifolin (**4**) also disaggregated the preformed fibrils of Aβ42 (Fig. 1C). The inhibitory effect of **4** on Aβ42 aggregation was almost equal to that of silymarin (Fig. 1B). A quantification analysis by HPLC revealed that silymarin used in this work

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Abbreviations: Aβ, amyloid β; AD, Alzheimer's disease; HPLC, high-performance liquid chromatography; Th-T, thioflavin-T; TEM, transmission electron microscopy

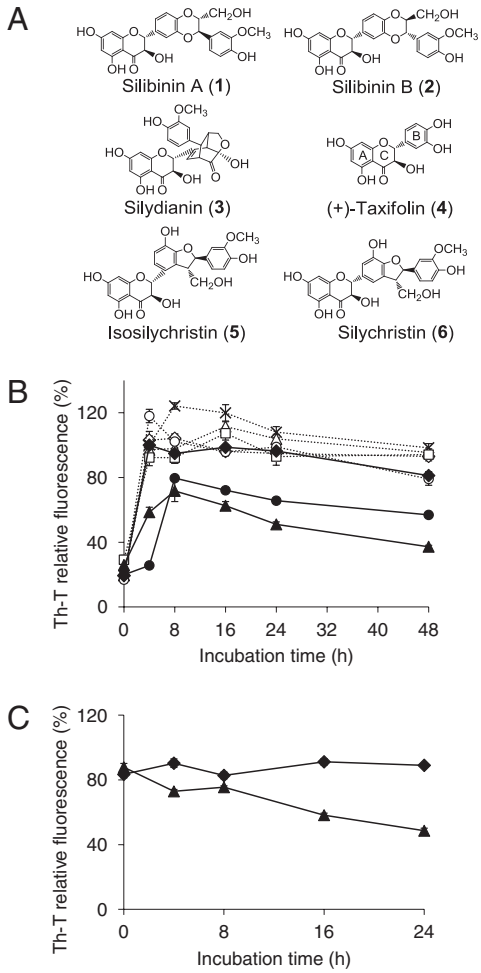

Fig. 1. Identification of (+)-Taxifolin (**4**) from Silymarin as One of the Active Components against Aβ42 Aggregation.

A, Structure of the flavonoids isolated from silymarin. EI-MS and optical rotation data are as follow: silibinin A (**1**), m/z 482 $[M]^+$, $[\alpha]_D$ +26.0 (c 0.25, MeOH, 26 °C);[19] silibinin B (**2**), m/z 482 $[M]^+$, $[\alpha]_D$ +12.0 (c 0.19, MeOH, 26 °C);[19] silydianin (**3**), m/z 482 $[M]^+$, $[\alpha]_D$ +231 (c 0.0050, MeOH, 27 °C);[19] (+)-taxifolin (**4**), m/z 304 $[M]^+$, $[\alpha]_D$ +22.2 (c 0.12, MeOH, 29 °C);[19] isosilychristin (**5**), m/z 482 $[M]^+$, $[\alpha]_D$ +207 (c 0.0050, MeOH, 26 °C);[19] silychristin (**6**), m/z 482 $[M]^+$, $[\alpha]_D$ +112 (c 0.30, MeOH, 26 °C).[28] B, The effect of each flavonoid on Aβ42 aggregation was estimated by the Th-T method. Aβ42 (25 μM) was incubated with or without each flavonoid (50 μM) in phosphate-buffered saline (PBS, 50 mM sodium phosphate, 100 mM NaCl, pH 7.4) at 37 °C for 48 h. Each flavonoid was dissolved in ethanol at 5.0 mM before use, and diluted with PBS (50 μM final concentration). The molecular weight of silymarin was defined as 482, which was that of the main components (silybinin A and B, silydianin, isosilychristin, and silychristin) in silymarin. ◆ Aβ42 without flavonoids; ● Aβ42 with silymarin; ◇ Aβ42 with **1**; △ Aβ42 with **2**; □ Aβ42 with **3**; ▲ Aβ42 with **4**; ○ Aβ42 with **5**; × Aβ42 with **6**. Data are presented as the mean ± SEM ($n = 8$). C, The disaggregation of Aβ42 fibrils by (+)-taxifolin (**4**) was estimated by the Th-T method. Aβ42 (25 μM) was incubated at 37 °C in PBS (pH 7.4) for 48 h for preparing the Aβ42 fibrils, to which were then added **4** (50 μM) before incubating at 37 °C for 24 h. ◆ Aβ42 without flavonoid; ▲ Aβ42 with **4**. Data are presented as the mean ± SEM ($n = 8$).

contained 1.2% (+)-taxifolin (**4**) which was slightly less than the isolated yield (2.2%, purity 99.6%). The low content rate of **4** does not exclude the presence of other active components in silymarin.

We identified the hydroxyl groups of (+)-taxifolin (**4**) involved in the inhibitory effect by preparing four *O*-methyl derivatives of **4** by a diazomethane treatment (Fig. 2A). In brief, a 0.20 mM ether/EtOH solution (35 mL/15 mL) of *N*-methyl–*N*-nitroso-*p*-toluenesulfonamide was heated at 70 °C. To the solution was added a potassium hydroxide solution (one gram of potassium hydroxide in 15 mL of water) to yield diazomethane which was condensed in a cold tube as a yellow ether solution. (+)-Taxifolin (**4**, 65 mg, 0.21 mmol) was dispersed in benzene (1.0 mL) and diethylether (3.0 mL), to which an aliquot of a diazomethane solution (12 mL) was added at 0 °C, and the mixture was stood at the same temperature for 1.5 h. The solution was evaporated *in vacuo*, and part of the residue was separated by preparative thin-layer chromatography and followed by HPLC in a YMC-Pack ODS-A column (20 mm i.d. × 150 mm; YMC) with a linear gradient of 50–100% CH_3CN/H_2O for 30 min to yield (+)-7-*O*-methyl-(**7**, 18 mg, 28% yield), (+)-7,3′-di-*O*-methyl-(**8**, 6.3 mg, 9.7% yield), (+)-7,4′-di-*O*-methyl-(**9**, 7.3 mg, 11% yield), and (+)-7,3′,4′-tri-*O*-methyltaxifolin (**10**, 2.1 mg, 3.2% yield, Fig. 2A). Their structures were confirmed by ^{1}H-NMR[18] and EI-MS to be identical to those reported previously. The Th-T assay showed that (+)-7-*O*-methyl-taxifolin (**7**) prevented the aggregation of Aβ42 in a manner similar to **4**, whereas (+)-7,3′-di-*O*-methyl-(**8**), (+)-7,4′-di-*O*-methyl-(**9**), and (+)-7,3′,4′-tri-*O*-methyltaxifolin (**10**) did not (Fig. 2A). The TEM images of Aβ42 fibrils treated with **7**, but not with **8**, were similar to those treated with **4** (Fig. 2B). These results indicate the 3′,4′-dihydroxyl groups on the B-ring of **4** to be important to prevent Aβ42 aggregation, while the 7-hydroxyl group was not critical. This is consistent with the findings that only **4** had a catechol moiety among the flavonoids isolated from silymarin in this study. These findings do not contradict the report by Akaishi *et al.* that the 3′,4′-dihydroxyl group, and not the 7-hydroxyl group, was essential to the inhibitory effect of fisetin (a quercetin analog without the 5-hydroxyl group) on Aβ42 fibril formation.[23]

(+)-Taxifolin (**4**) was not methylated at position 5 by diazomethane, implying that the hydroxyl group at position 5 could not be involved in the intermolecular interaction. Indeed, the hydroxyl group at position 5 of **4** could have participated in the intramolecular hydrogen bond with the carbonyl oxygen on the C-ring, this being deduced from the ^{1}H-NMR chemical shift (11.7 ppm in $(CD_3)_2CO$). The practical implication of this result is that the hydroxyl group at position 5 did not contribute to the inhibition of Aβ42 aggregation by **4**. Although methylated **4** at position 3 was not also obtained (Fig. 2), the report[23] that luteolin without a hydroxyl group at position 3 inhibited Aβ42 aggregation suggests that the hydroxyl group at position 3 of **4** would not participate in the inhibitory activity.

Furthermore, to examine the effect of the stereochemistry of the hydroxyl group at position 3 on the C-ring of (+)-taxifolin (**4**), the 2,3-(*R,R*)-*trans* form, on the inhibition of Aβ42 aggregation, the (−)-taxifolin, 2,3-(*S,S*)-*trans* form was synthesized basically according to the method of Roschek *et al.*,[24] except for using 3,4-trihydroxybenzaldehyde as a substrate. Briefly, vanillin (0.10 g, 0.63 mmol) dissolved in CH_2Cl_2 was demethylated by being treated with 1 M boron tribromide in dichloromethane (2.6 mL, 2.6 mmol) at 4 °C for 1 h to quantitatively give 3,4-dihydroxybenzaldehyde. The

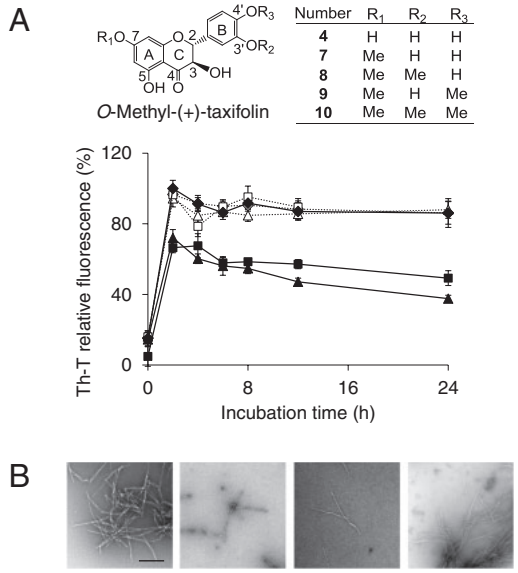

Fig. 2. Structure–Activity Relationships of (+)-Taxifolin (**4**).

A, Methylated (+)-taxifolins and their inhibitory effects on Aβ42 aggregation determined by the Th-T assay. Aβ42 (25 μM) was incubated with or without each methylated (+)-taxifolin (50 μM) in PBS (pH 7.4) at 37 °C for 48 h. ◆ Aβ42 without flavonoid; ▲ Aβ42 with **4**; ■ Aβ42 with **7**; ◇ Aβ42 with **8**; △ Aβ42 with **9**; and □ Aβ42 with **10**. EI-MS data for the methylated (+)-taxifolins are as follow: **7**, m/z 318 $[M]^+$; **8**, m/z 332 $[M]^+$; **9**, m/z 332 $[M]^+$; and **10**, m/z 346 $[M]^+$. Data are presented as the mean ± SEM ($n = 8$). B, The TEM analysis of Aβ42 fibrils treated with the methylated (+)-taxifolins was performed under an H-7650 electron microscope (Hitachi, Ibaraki, Japan). Scale bar, 200 nm. Left, Aβ42 without a flavonoid; left middle, Aβ42 with **4**; right middle, Aβ42 with **7**; right, Aβ42 with **8**.

phenolic hydroxyl groups were protected with methoxymethyl groups (73% yield). On the other hand, the phenolic hydroxyl groups of 2,4,6-trihydroxyacetophenone were also protected with methoxymethyl groups (24% yield). A cross-aldol reaction of these two products in KOH/MeOH (83% yield) and subsequent treatment with H_2O_2 under alkaline conditions quantitatively yielded the epoxide which was cyclized and deprotected under HCl/MeOH to give (±)-taxifolin (59% yield). The enantiomers were separated by HPLC in a CHERALCEL OJ-RH column (10 mm i.d. × 150 mm; Daicel Corporation, Osaka, Japan) by using 15% CH_3CN/H_2O containing 0.10% acetic acid.[25] The ratio of the enantiomers was almost 1:1, based on the isolated yield of each enantiomer: (+)-taxifolin (**4**), $[\alpha]_D$ +17.3 (*c* 0.10, MeOH, 16 °C, lit.[19] $[\alpha]_D$ +19.0, *c* 0.1, MeOH); (−)-taxifolin, $[\alpha]_D$ −16.2 (*c* 0.10, MeOH, 16 °C). As shown in Fig. 3, the inhibitory ability against Aβ42 aggregation was almost the same between these two enantiomers. In addition, no difference in the inhibitory activity against Aβ40 or Aβ42 aggregation between (+)-catechin (2,3-*trans* form) and (−)-epicatechin (2,3-*cis* form) has been reported.[26] Furthermore, quercetin with a C2–C3 double bond on the C-ring has been reported to inhibit Aβ42 aggregation.[27] These findings suggest the stereochemistry at positions 2 and 3 not to play an important role in the inhibitory effects of **4** against Aβ42 aggregation. Although the inhibition of Aβ42 aggregation by **4** in Fig. 3 seemed slightly stronger than that in Figs. 1 and 2, the fluorescence intensity in the Th-T assay between different figures is

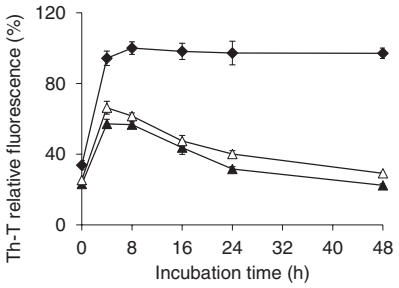

Fig. 3. Effects of the Stereochemistry at Position 3 of (+)-Taxifolin (**4**) on Aβ42 Aggregation.

The effects of (−)-taxifolin on Aβ42 aggregation were evaluated by the Th-T test. Aβ42 (25 μM) was incubated with or without each enantiomer (50 μM) in PBS (pH 7.4) at 37 °C for 48 h. ◆ Aβ42 without flavonoid; ▲ Aβ42 with (+)-taxifolin (**4**); and △ Aβ42 with (−)-taxifolin. Data are presented as the mean ± SEM ($n = 8$).

sometimes influenced by several factors; for example, the outside temperature and batch of Aβ42. However, similar results were obtained by another independent experiment.

In conclusion, we found (+)-taxifolin (**4**) with a catechol moiety on the B-ring from silymarin (a mixture of flavonoid-related compounds) to be one of the active components for anti-Aβ42 aggregation. To the best of our knowledge, this is the first report that **4**, belonging to flavanonols containing a single bond between C2 and C3 on the C-ring, had a preventive effect on Aβ42 aggregation. On the other hand, flavonoids (myricetin, quercetin, *etc*.), most of which have previously been reported to inhibit Aβ42 aggregation,[27] belong to flavonols containing a double bond between C2 and C3 on the C-ring. The structure-activity relationship of **4** clarified the requisite moiety (a catechol structure on the B-ring) for inhibitory activity against Aβ42 aggregation. A further study to clarify its inhibitory mechanism is in progress in our laboratory.

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