

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 (A β 40 and A β 42).^{1,2} A β 42 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 A β 42 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 A β 42. 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 *Silybium 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 A β 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 A β 42 aggregation; this is defined as the change of the A β 42 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/CHCl₃ 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. \times 150 mm; YMC, Kyoto, Japan), using 50% MeOH/H₂O, 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/H₂O 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. \times 150 mm; YMC) using 40% MeOH/H₂O 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 ¹H-NMR (AVANCE III 500, ref. tetramethylsilane, Bruker, Germany),^{16–20} EI-MS (JMS-600H, 70 eV, 300 μ A, JEOL, Tokyo, Japan), and specific optical rotation (P-2200, Jasco, Tokyo, Japan).

The effects of these flavonoids on A β 42 aggregation were examined by using thioflavin-T (Th-T), a reagent that fluoresces when bound to A β 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 A β 42 aggregation, meaning the potent inhibition of A β 42 aggregation by **4**. The analysis of TEM showed that the fibril formation of A β 42 was inhibited by **4**; shorter or slighter fibrils (Fig. 2B). (+)-Taxifolin (**4**) also disaggregated the preformed fibrils of A β 42 (Fig. 1C). The inhibitory effect of **4** on A β 42 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: A β , amyloid β ; AD, Alzheimer’s disease; HPLC, high-performance liquid chromatography; Th-T, thioflavin-T; TEM, transmission electron microscopy

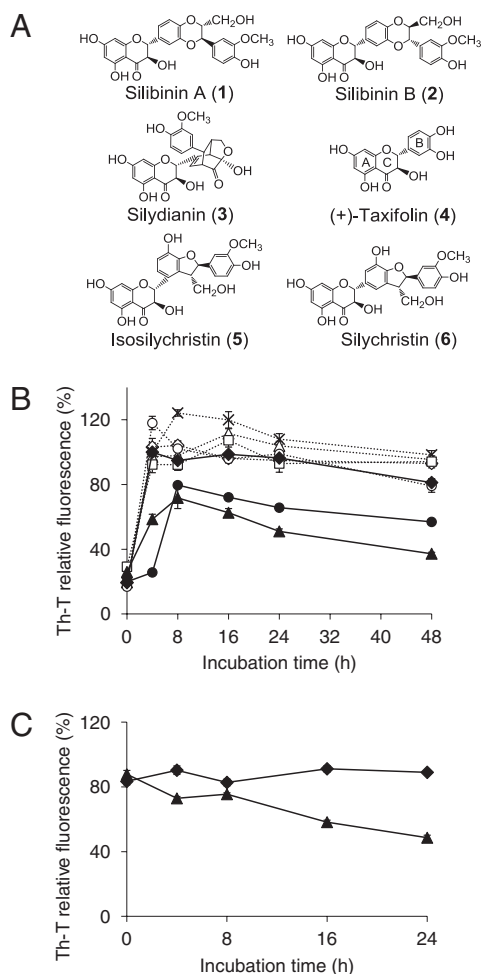


Fig. 1. Identification of (+)-Taxifolin (**4**) from Silymarin as One of the Active Components against A β 42 Aggregation.

A, Structure of the flavonoids isolated from silymarin. EI-MS and optical rotation data are as follows: 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 A β 42 aggregation was estimated by the Th-T method. A β 42 (25 μ M) was incubated with or without each flavonoid (50 μ M) 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 μ M 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. ◆ A β 42 without flavonoids; ● A β 42 with silymarin; ◊ A β 42 with **1**; △ A β 42 with **2**; □ A β 42 with **3**; ▲ A β 42 with **4**; ○ A β 42 with **5**; × A β 42 with **6**. Data are presented as the mean \pm SEM (n = 8). C, The disaggregation of A β 42 fibrils by (+)-taxifolin (**4**) was estimated by the Th-T method. A β 42 (25 μ M) was incubated at 37 °C in PBS (pH 7.4) for 48 h for preparing the A β 42 fibrils, to which were then added **4** (50 μ M) before incubating at 37 °C for 24 h. ◆ A β 42 without flavonoid; ▲ A β 42 with **4**. Data are presented as the mean \pm 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. \times 150 mm; YMC) with a linear gradient of 50–100% CH₃CN/H₂O 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 ¹H-NMR¹⁸) 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 A β 42 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 A β 42 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 A β 42 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 A β 42 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 ¹H-NMR chemical shift (11.7 ppm in (CD₃)₂CO). The practical implication of this result is that the hydroxyl group at position 5 did not contribute to the inhibition of A β 42 aggregation by **4**. Although methylated **4** at position 3 was not also obtained (Fig. 2), the report²³) that luteolin without a hydroxyl group at position 3 inhibited A β 42 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 A β 42 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 CH₂Cl₂ 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

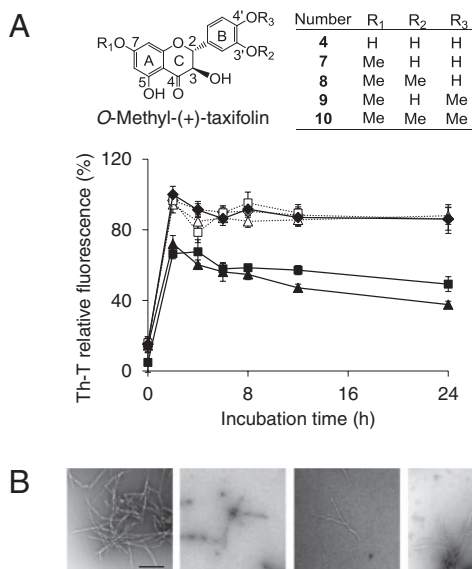


Fig. 2. Structure–Activity Relationships of (+)-Taxifolin (4).

A, Methylated (+)-taxifolins and their inhibitory effects on A β 42 aggregation determined by the Th-T assay. A β 42 (25 μ M) was incubated with or without each methylated (+)-taxifolin (50 μ M) in PBS (pH 7.4) at 37 °C for 48 h. \blacklozenge A β 42 without flavonoid; \blacktriangle A β 42 with 4; \blacksquare A β 42 with 7; \blacklozenge A β 42 with 8; \blacktriangle A β 42 with 9; and \square A β 42 with 10. EI-MS data for the methylated (+)-taxifolins are as follows: 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 \pm SEM ($n = 8$). B, The TEM analysis of A β 42 fibrils treated with the methylated (+)-taxifolins was performed under an H-7650 electron microscope (Hitachi, Ibaraki, Japan). Scale bar, 200 nm. Left, A β 42 without a flavonoid; left middle, A β 42 with 4; right middle, A β 42 with 7; right, A β 42 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 H₂O₂ under alkaline conditions quantitatively yielded the epoxide which was cyclized and deprotected under HCl/MeOH to give (\pm)-taxifolin (59% yield). The enantiomers were separated by HPLC in a CHERALCEL OJ-RH column (10 mm i.d. \times 150 mm; Daicel Corporation, Osaka, Japan) by using 15% CH₃CN/H₂O 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), $[\alpha]_D^{25} +17.3$ (c 0.10, MeOH, 16 °C, lit.¹⁹) $[\alpha]_D^{25} +19.0$, c 0.1, MeOH); (–)-taxifolin, $[\alpha]_D^{25} -16.2$ (c 0.10, MeOH, 16 °C). As shown in Fig. 3, the inhibitory ability against A β 42 aggregation was almost the same between these two enantiomers. In addition, no difference in the inhibitory activity against A β 40 or A β 42 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 A β 42 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 A β 42 aggregation. Although the inhibition of A β 42 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

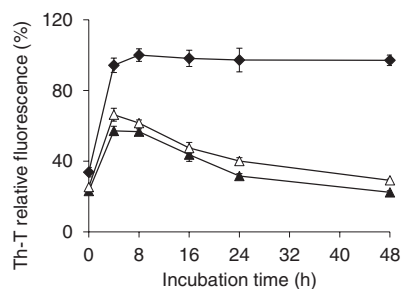


Fig. 3. Effects of the Stereochemistry at Position 3 of (+)-Taxifolin (4) on A β 42 Aggregation.

The effects of (–)-taxifolin on A β 42 aggregation were evaluated by the Th-T test. A β 42 (25 μ M) was incubated with or without each enantiomer (50 μ M) in PBS (pH 7.4) at 37 °C for 48 h. \blacklozenge A β 42 without flavonoid; \blacktriangle A β 42 with (+)-taxifolin (4); and \triangle A β 42 with (–)-taxifolin. Data are presented as the mean \pm SEM ($n = 8$).

sometimes influenced by several factors; for example, the outside temperature and batch of A β 42. 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-A β 42 aggregation. To the best of our knowledge, this is the first report that 4, belonging to flavanols containing a single bond between C2 and C3 on the C-ring, had a preventive effect on A β 42 aggregation. On the other hand, flavonoids (myricetin, quercetin, *etc.*), most of which have previously been reported to inhibit A β 42 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 A β 42 aggregation. A further study to clarify its inhibitory mechanism is in progress in our laboratory.

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