Silymarin, the seed extract of *Silybum 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).2,1) 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/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. × 150 mm; YMC, Kyoto, Japan), using 50% MeOH/H₂O, to yield silibinin A (1, 16 mg from 240 mg of silymarin, 6.7%)10) and silibinin B (2, 25 mg from 240 mg of silymarin, 10%),10) and using 40% MeOH/H₂O to yield silydianin (3, 31 mg from 370 mg of silymarin, 8.4%).11) The second fraction was separated in a YMC-Pack ODS-AL column (20 mm i.d. × 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) EIMS (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 fibrils, 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
The disaggregation of Aβ fibrils by (+)-taxifolin (4) was estimated by the Th-T method. Aβ fibrils were then added 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 mM 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. Aβ fibrils 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 Aβ 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 Aβ 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).

(2A). In brief, a 0.20 mM ether/EtOH solution (35 mL/15 mL) of N-methyl-N-nitroso-p-toluene sulfonamide 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 diethyl ether (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% 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-NMR 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′-dihydroxy 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′-dihydroxy group, and not the 7-hydroxyl group, was essential to the inhibitory effect of fisetin (a quercetin analog without the 5-hydroxyl group) on Aβ 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 CD3CO). 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 report23 that lutecin 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 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. 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: silybinin A (1), m/z 482 [M]+, [α]220 +260 (c 0.23, MeOH, 25 °C);20 silybinin B (2), m/z 482 [M]+, [α]220 +120 (c 0.19, MeOH, 26 °C);20 silydianin (3), m/z 482 [M]+, [α]220 +231 (c 0.005, MeOH, 23 °C);20 (+)-taxifolin (4), m/z 304 [M]+, [α]220 +112 (c 0.30, MeOH, 26 °C).20 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 mM 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.

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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 Aβ 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 Aβ 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).
phenolic hydroxyl groups were protected with methoxymethyl groups (73% yield). A cross-aldol reaction of these two enantiomers was performed under HCl/MeOH to give (−)-taxifolin (50% yield). The enantiomers were separated by HPLC (24% yield). A further study to clarify its inhibitory mechanism is in progress in our laboratory.

Acknowledgments

This study was partly supported by Grants in Aid for Scientific Research (A) (Grant no. 21248015 to K. I.), and (C) (no. 22603006 to K. M.), and by a Fund for the Promotion of Science for Young Scientists (Grant no. 224068 to M. S.) from The Ministry of Education, Culture, Sports, Science and Technology of Japan, and by funds from the Asahi Group Foundation (to K. I.) and from the Kato Memorial Bioscience Foundation (to K. I.) and by a Fund for the Promotion of Science for Young Scientists (Grant no. 21248015 to K. I.). We thank Prof. Nobutaka Fujii and Dr. Shinya Oishi from the Graduate School of Pharmaceutical Sciences at Kyoto University for use of the MALDI-TOF-MS. M. S. is a Research Fellow of the Japan Society for the Promotion of Science.

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