

Detection of Dietary Chalcone and Flavonoid Metabolites in Mouse Using UPLC–MS/MS and Their Modulatory Effects on Amyloid β Aggregation

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Abstract

Amyloid β -protein ($A\beta_{42}$) aggregates have been demonstrated to induce cognitive decline and neurodegeneration in Alzheimer's disease (AD). Thus, functional food ingredients that inhibit $A\beta_{42}$ aggregation are valuable for AD prevention. Although several food ingredients have been studied for their antiaggregation activity, information on their bioavailability in the brain, incorporated forms, and relevance to AD etiology is limited. Here, we first detected the sulfate- and glucuronic acid-conjugated forms of green perilla-derived chalcone (**1**) and taxifolin (**2**), which inhibit $A\beta_{42}$ aggregation, in the brain, small intestine, and plasma of mice (**1** and **2** were administered orally) using ultraperformance liquid chromatography–mass spectrometry (UPLC–MS/MS). We observed that the conjugated metabolites (**4**: sulfate and **5**: glucuronide) of **1** prevented the fibrillization and oligomerization of $A\beta_{42}$. These findings imply that the conjugated metabolites of **1** can prove beneficial for AD treatment.

Keywords: aggregation, Alzheimer disease, amyloid β -protein, chalcone, flavonoid, glucuronidation, metabolite, oligomer, sulfation, UPLC–MS/MS.

1. INTRODUCTION

The pathogenesis of Alzheimer's disease (AD)¹ is related to the accumulation of amyloid β -protein (A β), which is produced from the A β precursor protein.^{2,3} The 40-mer- and 42-mer-A β (A β 40 and A β 42, respectively) proteins form metastable oligomers during fibrilization, which exhibit neurotoxicity and lead to the progressive and gradual decline of memory function.⁴⁻⁷ Despite its lower content, A β 42 exhibits a stronger ability to aggregate and exhibit neurotoxicity than A β 40.⁸ The mechanism of amyloid propagation is generally explained by a uniform stacking model, e.g., a nucleation-dependent polymerization model⁹ or a template-dependent dock-lock model.¹⁰ The former features a nucleation phase with a two-phase process, whereas the latter features an elongation phase with a plateau (saturation) phase. Small molecules that decrease the nucleation velocity and/or amounts of elongated aggregates may prove valuable for the development of drugs (lead compounds) for AD treatment, and their healthy intake as dietary ingredients would be ideal.

Numerous studies have been conducted on modulators with anti-A β aggregation properties. Our group has specifically investigated the antiaggregation properties of various natural products, including functional food ingredients and herbal medicine, and have identified three structural requirements for achieving inhibitory activity against A β 42 aggregation.¹¹ These requirements include the following: (i) Possessing an α,β -unsaturated carbonyl structure (e.g., catechol, 2-pyrone, and 4-pyrone) capable of forming a Michael adduct¹²⁻¹⁴ or a Schiff base¹⁵ with the side chains of Lys16 and 28. (ii) Being a planar compound formed from conjugated systems, achieved through the presence of an α,β -unsaturated carbonyl group and/or polycyclic aromatic compounds. This planar structure allows for interactions with the aromatic rings of Phe19 and 20 involved in the intermolecular β -sheet region.^{13,16} (iii) Containing carboxylic acid derivatives, such as triterpenoids or anthraquinoids,¹⁷ which can generate a salt bridge with the side chains of Lys16 and 28. This knowledge may help in conveniently assessing if a dietary ingredient in food exhibits antiaggregation activity.

Dietary polyphenols have gained increased attention because of their benefits against various diseases as well as the contribution of their metabolites in intestinal absorption and phase II metabolism.¹⁸ For example, resveratrol 3-*O*- β -D-glucuronide and resveratrol 4'-*O*- β -D-glucuronide have been demonstrated to more extensively inhibit the growth of human adenocarcinoma (Caco-2) than the aglycone forms.¹⁹ Resveratrol 3-sulfate, resveratrol 3-*O*- β -D-glucuronide, and resveratrol 4'-*O*- β -D-glucuronide have been detected in the colorectum of cancer patients taking resveratrol.²⁰ Some of these metabolites also exhibit antiobesity activities.²¹ These findings indicate that the

bioavailabilities of resveratrol and its metabolites depend on their distribution mechanism to tissues and the type of metabolites contributing to the activity. These findings highlight the importance of understanding the pharmacokinetics of dietary ingredients in the context of drug development.

Despite the recognized benefits of flavonoids in the brain,²² only a few studies have investigated the involvement of metabolites of flavonoids and chalcones, which are secondary metabolites of flavonoids (quercetin-3-*O*- β -D-glucuronide²³ and 3-*O*-methyl-epicatechin-5-*O*- β -D-glucuronide,²⁴ both of which have been identified in the brain of Sprague–Dawley rats), in AD intervention. Furthermore, the available information on the bioavailability of flavonoids in the brain of mice is limited. In this study, we analyzed the dietary chalcone and flavonoid metabolites. First, we focused on the bioavailability of 2',3'-dihydroxy-4',6'-dimethoxychalcone (DDC, **1**: Figure 1), a component of green perilla (*Perilla frutescens* var. *crispa* f. *viridis*).^{15,25} DDC has been shown to inhibit A β 42 oligomerization through a lysine-targeting mechanism both *in vitro* and *in vivo*. Second, taxifolin (**2**: Figure 1), a mixture of flavonoids isolated from silymarin in the blessed milk thistle (*Silybum marianum*)²⁶ or found in small amounts in red onions,²⁷ was selected because it demonstrated pleiotropic neuroprotective effects against cerebral amyloid angiopathy (CAA) by restoring impaired cerebral blood flow²⁸ and suppressing triggering receptors expressed on myeloid cells 2-associated inflammation.²⁹ These findings highlight the need for future clinical trials to evaluate the efficacy and safety of taxifolin in patients with CAA.³⁰ Third, apomorphine (**3**: Figure 1), a dopamine agonist that is already being used for therapeutic purposes and is known to inhibit A β 42 aggregation,¹³ was used because its bioavailability in humans has been well studied.^{31,32}

Ultraperformance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) was used to detect the sulfate-, glucuronic acid-, and/or methyl-conjugated forms of the compounds administered to C57BL/6 mice via oral (**1** and **2**) and subcutaneous (**3**) routes. Notably, this study focuses on the preparation and characterization of specific metabolites of **1**, namely the sulfate and glucuronide forms (**4** and **5**, respectively; Figure 1), following phase II metabolism. This study aimed to investigate the function of known metabolites (sulfate and glucuronide), which were indeed detected, rather than the functions of all detected compounds. Moreover, the inhibitory effects of sulfate and glucuronide of **1** on A β 42 aggregation were evaluated using a combination of thioflavin-T (Th-T) fluorescence assay and transmission electron microscopy (TEM) to assess fibrilization. Dot blotting was also performed to evaluate oligomerization.

2. MATERIALS AND METHODS

2.1. Animal Experiments

All experimental procedures were performed in accordance with specified guidelines for the care and use of laboratory animals and were approved by the Animal Care and Use Committee of Chiba University (No. 30-239). We employed 3–4-week-old C57/BL6 male mice (Japan SLC, Shizuoka, Japan). The animals were housed in a $24.0^{\circ}\text{C} \pm 2^{\circ}\text{C}$ room with $55\% \pm 5\%$ relative humidity under a 12-h light/dark cycle, with *ad libitum* access to food and drinking water. Compounds **1** (3 mg), as synthesized previously,¹⁵ and **2** (3 mg), as synthesized previously,¹² suspended in 0.3 mL of polypropylene glycol were orally administered to mice starved overnight (100 mg/kg). Compound **2** was employed as a racemic mixture. Compound **3** (3 mg; Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) suspended in 0.3 mL of 0.9% NaCl was subcutaneously administered to mice starved overnight (100 mg/kg). The control mice were administered either polypropylene glycol or 0.9% NaCl. The blood, brain, and small intestine samples of the mice were collected under anesthesia 15 min (**1** and **2**) or 30 min (**3**) after the administration. Most of the intact forms of **1** and **2** were metabolized or degraded in the case of collection 3 hr after administration (data not shown). The blood sample was placed in a tube coated with disodium salt ethylenediamine-*N,N,N',N'*-tetraacetic acid and subsequently centrifuged for 5 min at 6000 rpm at room temperature to obtain the plasma. The brain and small intestine samples were harvested after perfusion of ice-cold phosphate-buffered saline (PBS) from the heart to completely remove the blood. The brain, small intestine, and plasma samples were stored at -80°C until use.

2.2. Tissue Preparation and UPLC–MS/MS

The tissues (small intestine and brain) were homogenized in 50% acetonitrile using a microhomogenizer Phycotron (Microtec, Chiba, Japan) with sonication for 15 s. After centrifugation for 15 min at 17,900 g and 4°C , the supernatant was dried under a stream of nitrogen. The obtained extracts were then dissolved in 120 μL of 40% acetonitrile/ H_2O . For plasma samples, they were mixed with an equal volume of acetonitrile. These mixtures were centrifuged for 10 min (small intestine and brain) or 15 min (plasma) at 17,900 g and 4°C , and the supernatant was filtered through a Millex syringe filter (pore size: 0.2 μm , Millipore, Burlington, MA, USA). UPLC–MS/MS analysis was performed using an Acquity UPLC H-class system coupled with a Xevo G2-S mass spectrometer (Waters, Milford, MA, USA). UPLC separation was performed on an Acquity UPLC BEH C18 column (particle size: 1.7 μm , dimensions: 2.1 mm i.d. x 100 mm, Waters), with the column oven temperature set at 35°C . The gradient program was set as follows:

10%–50% acetonitrile containing 0.1% formic acid for a 10-min linear gradient, followed by 50% acetonitrile containing 0.1% formic acid for a 3-min isocratic flow at a flow rate of 0.2 mL/min. For the samples collected from mice treated with **1** (**1**-treated) and **3** (**3**-treated), the injection volume was set to 2 μ L, whereas for the samples obtained from mice treated with **2** (**2**-treated), the injection volume was set to 4 μ L. The relative distribution of **1** in the brain, small intestine, and plasma was determined based on the UPLC chromatogram at 254 nm using the calibration curve of **1**. Similarly, the relative intensity of each metabolite of **1** in the brain was determined based on the UPLC chromatogram at 254 nm using the calibration curve of **1**. MS analysis was performed with the following parameters: ionization capillary voltage set at 1.5 kV (in negative mode), sampling cone voltage at 25 V, source temperature at 100°C, desolvation temperature at 300°C, cone gas flowrate at 50 L/h, desolvation gas flowrate at 800 L/h, and collision energy ranging from 10 to 40 V. Peak annotations of the observed mass, after subtracting the vehicle control, were conducted based on the UPLC–MS/MS data.

2.3. Preparation of Conjugated Metabolites

General remarks.—The following spectroscopic and analytical instruments were used: ^1H and ^{13}C NMR on Bruker AVANCE III 400 and AVANCE III 500 instruments (Bruker, Billerica, MA, USA); HPLC using Waters Model 600E equipped with a Model 2487 UV detector (Waters); HR–FAB–MS on JMS-MS700V (JEOL, Tokyo, Japan); and HR–ESI–qTOF–MS on XevoG2-S-qTOF (Waters). HPLC was performed on a YMC-Pack ODS-A column (20 mm i.d. $\times$ 150 mm; YMC, Kyoto, Japan). For open or medium-pressure column chromatography, Wako gel C-200 (silica gel; Fujifilm Wako Pure Chemical Corporation) or Kieselgel (Merck, Rahway, NJ, USA) was used. All other chemicals and reagents were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan), NACALAI TESQUE, INC. (Kyoto, Japan), or Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and used as received, unless otherwise specified.

Preparation of 4.—To a solution of **1** (40.1 mg, 0.13 mmol) in DMF (1.5 mL), $\text{NEt}_3 \cdot \text{SO}_3$ (242 mg, 1.34 mmol, 10 equiv.) was added at 0°C. After stirring for 10 min at 0°C, trifluoromethanesulfonic acid (TfOH) (11.7 μ L, 0.13 mmol, 1.0 equiv.) was added dropwise to the mixture at –20°C. The resulting mixture was stirred for 18 h at 0°C, quenched with Et_3N (165 μ L) in MeOH (5.5 mL), and concentrated under vacuum. The residue was purified via column chromatography (silica gel, $\text{CHCl}_3\text{:MeOH:Et}_3\text{N}$ = 80:20:2). Further purification was performed via HPLC using a YMC-Pack ODS-A column (20 mm i.d. $\times$ 150 mm; YMC) with a linear gradient of 30% to 70% MeCN/ H_2O

(0.1% TFA) over 40 min at a flow rate of 8.0 mL/min and UV detection at 254 nm. The second HPLC purification utilized a YMC-Pack ODS-A column (20 mm i.d. $\times$ 150 mm; YMC) with a solvent of 45% MeCN/H₂O (0.1% TFA) at a flow rate of 8.0 mL/min and UV detection at 254 nm. This process afforded **4** (13.4 mg, 29.2 μ mol, 12% yield). For MS analysis and assays, **4** (4.0 mg, 8.7 μ mol) was dissolved in MeCN (0.4 mL) and H₂O (0.1 mL) and the pH was adjusted to 10 with 0.1 M NaOH aq. After stirring for 5 min, the sodium salt (**4a**) of **4** was obtained for further analysis. The R_f value on an ODS plate with MeOH/H₂O (1:1) was found to be 0.27.

¹H NMR (400 MHz, CD₃OD, 0.017 M, 297 K): δ 3.97 (s, 3H, 6'-OCH₃), 3.99 (s, 3H, 4'-OCH₃), 6.28 (s, 1H, 5'-H), 7.40–7.46 (m, 3H, 2-H, 4-H, 6-H), 7.64–7.67 (m, 2H, 3-H, 5-H), 7.74 (d, 1H, α -H, J =15.6 Hz), 7.93 (d, 1H, β -H, J =15.6 Hz) ppm.

¹³C NMR (100 MHz, acetone-*d*₆, 0.044 M, 298 K): δ 56.6 (4'-OCH₃ or 6'-OCH₃), 56.8 (4'-OCH₃ or 6'-OCH₃), 90.1 (5'-C), 111.4 (1'-C), 129.5 (α -C), 129.2 (3-C, 4-C, 5-C), 129.9 (2-C, 6-C), 131.1 (1-C), 135.9 (3'-C), 143.8 (β -C), 152.8 (2'-C), 156.7 (4'-C or 6'-C), 156.9 (4'-C or 6'-C), 193.5 (C=O) ppm.

IR (KBr): 3444, 3234, 2991, 1644, 1448, 1197, 1087, 1024, 867, 680, 641, 574 cm⁻¹.

HR-ESI-MS: m/z , 504.9847 ([M+H]⁺, calcd for C₁₇H₁₅O₁₁S₂Na₂ 504.9846).

Preparation of 5.—Compound **7**: To a solution of methyl 1,2,3,4-tetra-*O*-acetyl- β -D-glucuronate (**6**) (1.0 g, 2.68 mmol) in DMF (20 mL), hydrazine acetate (383 mg, 4.16 mmol, 1.6 equiv.) was added. The reaction mixture was stirred for 3 h at room temperature, quenched with water (20 mL), and extracted with EtOAc (20 mL $\times$ 3). The combined organic layers were dried over Na₂SO₄, filtered, and concentrated under vacuum. The resulting residue was purified via column chromatography (silica gel, hexane:EtOAc = 75:25 $\rightarrow$ 50:50), which yielded **7** (658 mg, 1.37 mmol, 74% yield).

R_f (silica, EtOAc/hexane = 1:1): 0.47

¹H NMR (400 MHz, CDCl₃, 0.060 M, 297 K, ref. TMS): δ 2.02 (s, 3H, OCOCH₃), δ 2.03 (s, 3H, OCOCH₃), δ 2.07 (s, 3H, OCOCH₃), 3.73 (s, 3H, CO₂CH₃), 4.61 (d, 1H, 5-H, J =10.1 Hz), 4.90 (dd, 1H, 2-H, J =10.2, 3.5 Hz), 5.16 (dd, 1H, 4-H, J =10.0, 9.4 Hz), 5.49 (d, 1H, 1-H, J =3.5 Hz), 5.61 (t, 1H, 3-H, J =9.8 Hz) ppm. The data coincided with those reported previously.³³

Compound 8: To a solution of **7** (658 mg, 1.37 mmol) in CH₂Cl₂ (6.6 mL), K₂CO₃ (435 mg, 3.15 mmol, 1.6 equiv.) was added. The reaction mixture was stirred for 10 min at room temperature, then trichloroacetonitrile (0.56 mL, 5.51 mmol, 2.8 equiv.) was added. The mixture was stirred for 21 h at room temperature, and the reaction was

quenched with water (10 mL) and extracted with CH₂Cl₂ (10 mL × 3). The combined organic layers were dried over Na₂SO₄, filtered, and concentrated under vacuum. The resulting residue was purified via column chromatography (silica gel, hexane:EtOAc = 75:25), which yielded **8** (222 mg, 0.46 mmol, 24% yield).

R_f (silica, EtOAc/hexane = 1:1): 0.60

¹H NMR (400 MHz, CDCl₃, 0.042 M, 297 K, ref. TMS): δ 2.02 (s, 3H, OCOCH₃), δ 2.05 (s, 3H, OCOCH₃), δ 2.06 (s, 3H, OCOCH₃), 3.76 (s, 3H, CO₂CH₃), 4.50 (d, 1H, 5-H, *J* = 10.2 Hz), 5.15 (dd, 1H, 2-H, *J* = 10.2, 3.6 Hz), 5.27 (dd, 1H, 4-H, *J* = 10.2, 9.7 Hz), 5.63 (t, 1H, 3-H, *J* = 9.8 Hz), 6.64 (d, 1H, 1-H, *J* = 3.6 Hz), 8.75 (s, 1H, NH) ppm. The data coincided with those reported previously.³³

Compound 5: To a solution of **1** (25.2 mg, 0.084 mmol) and **8** (109.2 mg, 0.228 mmol, 2.7 equiv.) in CH₂Cl₂ (0.5 mL), BF₃·Et₂O (4.2 μL, 0.034 mmol, 0.4 equiv.) was added at −40°C. The reaction mixture was stirred at −40°C for 3.5 h, −20°C for 3.5 h, and 0°C for 63 h. The reaction was then quenched with a saturated NaHCO₃ solution (5 mL) and extracted with CH₂Cl₂ (5 mL × 3). The combined organic layers were dried over Na₂SO₄, filtered, and concentrated under vacuum. The resulting crude product (**9**) was used without further purification. To the residue (33.2 mg, 0.054 mmol) in MeCN (0.5 mL), 1 M MeONa (18 μL, 0.018 mmol, 0.33 equiv.) was added. The mixture was stirred at room temperature for 30 min, quenched with H₂O (3 mL), and concentrated under vacuum. The crude material was dissolved in H₂O (300 μL), and a 0.5 M LiOH-H₂O solution (142 μL, 0.071 mmol, 1.3 equiv.) was added. After stirring at room temperature for 5 min, the reaction mixture was filtered through Amberlyst 15 and concentrated under vacuum. The residue was purified via HPLC (column, YMC-Pack ODS-A [20 mm i.d. × 150 mm; YMC]; solvent, 30% MeCN/H₂O (0.1% TFA); flow rate, 8.0 mL/min; UV detector, 254 nm), yielding **5** (9.9 mg, 0.021 mmol, 25% yield, 3 steps from **1**).

R_f (ODS, MeCN:H₂O = 1:1): 0.76

¹H NMR (400 MHz, CD₃CN + D₂O, 0.010 M, 297 K): δ 3.41–3.57 (m, 3H, 2''-H, 3''-H, 4''-H), 3.73 (d, 1H, 5''-H, *J* = 9.7 Hz), 3.90 (s, 3H, 4'-OCH₃ or 6'-OCH₃), 3.94 (s, 3H, 4'-OCH₃ or 6'-OCH₃), 4.86 (d, 1H, 1''-H, *J* = 7.4 Hz), 6.24 (s, 1H, 5'-H), 7.42–7.44 (m, 3H, 2-H, 4-H, 6-H), 7.66–7.69 (m, 2H, 3-H, 5-H), 7.75 (d, 1H, α-H, *J* = 15.4 Hz), 7.79 (d, 1H, β-H, *J* = 15.8 Hz) ppm.

¹³C NMR (100 MHz, CD₃CN-*d*₃, 0.042 M, 297 K): δ 57.4 (4'-OCH₃ or 6'-OCH₃), 58.2 (4'-OCH₃ or 6'-OCH₃), 72.5 (2''-C or 3''-C or 4''-C or 5''-C), 75.0 (2''-C or 3''-C or 4''-C or 5''-C), 76.1 (2''-C or 3''-C or 4''-C or 5''-C), 76.8 (2''-C or 3''-C or 4''-C or 5''-C), 89.6 (5'-C), 105.3 (1''-C), 108.0 (1'-C), 128.1 (4-C), 128.9 (α-C), 129.8 (3-C, 5-C), 130.3 (2-

C, 6-C), 131.7 (1-C), 136.5 (3'-C), 144.0 (β -C), 158.0 (2'-C), 159.8 (4'-C or 6'-C), 160.4 (4'-C or 6'-C), 170.9 (COOH), 194.7 (C=O) ppm.

IR (KBr): 3450, 2935, 1734, 1627, 1570, 1438, 1332, 1278, 1247, 1211, 1168, 1124, 1085, 1019, 974, 789, 765, 687, 577 cm^{-1} .

HR-ESI-MS: m/z , 475.1241 ($[\text{M}-\text{H}]^-$, calcd for $\text{C}_{23}\text{H}_{23}\text{O}_{11}$ 475.1246).

2.4. Thioflavin-T Fluorescence Assay

The aggregation ability of each A β 42 variant was assessed using the thioflavin-T (Th-T; Sigma-Aldrich, St. Louis, MO, USA) fluorescence assay. Synthesized A β 42^{34,35} was used for the experiments. To eliminate any pre-existing aggregation seeds, A β 42 was pretreated with 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) following established protocols.¹⁷ The final concentrations of A β 42 (100 μM in 0.1% NH_4OH as a stock) and Th-T (1 mM in distilled water as a stock) were adjusted to 10 μM and 20 μM , respectively, in PBS (50 mM sodium phosphate and 100 mM NaCl, pH 7.4). Each sample had a final concentration of 50 μM . The solution was distributed into a 96-well black plate (Greiner Bio-One, Monroe, NC, USA), and fluorescence was measured at excitation and emission wavelengths of 430 nm and 485 nm, respectively, using a microplate reader (Fluoroskan Ascent; Thermo Scientific) at 25°C with agitation at 10-min intervals. The Th-T relative fluorescence was determined by subtracting the fluorescence of the vehicle control without A β 42.

In an interference test to assess the binding competition between each compound and Th-T for A β aggregates, Th-T fluorescence was measured immediately after adding each compound (50 μM) following a 24-hour incubation of the A β 42 solution (10 μM) alone (without compound) at 37°C. Another interference test was conducted to examine whether each compound competed with Th-T itself in terms of fluorescence. In this test, the fluorescence was measured immediately after mixing Th-T with each compound (50 μM).

2.5. TEM

The aggregates obtained from each A β solution after the Th-T assay were analyzed via TEM using a JEM-1400 microscope (JEOL, Tokyo, Japan). Following centrifugation of each A β solution (4°C, 17,900 g, 10 min), the supernatant was discarded, and the pellet was gently resuspended in water by vortexing for 1 min just before TEM analysis. A 15 μL suspension of the sample was applied to a 200-mesh carbon-coated copper grid (Nisshin EM, Tokyo, Japan) and incubated for 5 min. The samples were then negatively stained twice with 2% uranyl acetate. TEM imaging was performed to visualize the

samples. The width of the fibrils was measured in triplicate using ImageJ software (Wayne Rasband, NIH, MD, USA).

2.6. Dot Blotting

A β solution (10 μ M), prepared from HFIP-treated A β 42, was incubated with or without each compound (10 or 50 μ M) at 37°C for 1 h. A volume of 1 μ L (45 ng) from each A β sample was applied to a nitrocellulose membrane (0.2 μ m pore size, Biorad), and the membrane was then blocked using Blocking Reagent for Can Get Signal (Toyobo, Tokyo, Japan). Next, the membrane was probed with the A11 antibody³⁶ (Invitrogen, Carlsbad, CA, USA) at a concentration of 1 μ g/mL, followed by treatment with a secondary antibody (anti-rabbit IgG). Chemiluminescent development was performed using ECL (GE Healthcare) and quantified as described previously.³⁷ The dilution of the first and second antibodies was performed using Can Get Signal Solutions 1 and 2 (Toyobo), respectively. A washing buffer containing 10 mM sodium phosphate, 140 mM NaCl, pH 7.4, and 0.5% Tween-20 was used for the washing steps.

2.7. Statistical Analysis

The statistical significance of the differences was analyzed using one-way analysis of variance, followed by *post hoc* multiple comparisons using Tukey's test. A significance level of $P < 0.05$ was used to determine statistical significance.

3. RESULTS

3.1. UPLC–MS/MS Analysis of the Tissues of Mice Administered with 1–3

The blood–brain barrier (BBB) is a layer of endothelial cells in the brain that offers protection so that the brain can let in only the beneficial and necessary substances. To investigate the ability of small compounds with antiaggregation activity to cross the BBB and their metabolism in the brain, **1** and **2** were orally administered to C57/BL6 mice. The pharmacokinetics and metabolism of **3**, also known as Apolyn[®], have been extensively studied and reviewed by LeWitt.³⁸ For reference, **3** was subcutaneously administered to the mice. Plasma, small intestine, and brain tissues were collected from each group and analyzed using LC/Q ToF–MS/MS. Table 1 provides the peak annotations of the observed mass for the metabolites of **1–3** based on the UPLC–MS/MS data (Figure S1). Figures 2–4 and Figures S2–S3 depict representative UV chromatograms of the tissues/plasma in the experimental setup for each small compound (**1–3**). These analyses revealed the presence of the monosulfate ($M + 80$) and monoglucuronide forms ($M + 176$) of **3**, in addition to the unreacted form (**3**, M), in the plasma (Figure 4A), small

intestine (Figure 4B), and brain (Figure 4C) tissues of the mice treated with **3**; this finding was consistent with that reported in a previous study.³⁸ Previous findings from a review³⁸ summarizing the distribution of **3** in humans indicate that the blood levels of **3** increase rapidly when administered subcutaneously, and it can be distributed to the small intestine and brain via the bloodstream. The detected conjugates of **3** are likely to follow a similar distribution pathway.

In mice treated with **1**, the sulfate ($M + 80$), monoglucuronide ($M + 176$), and diglucuronide forms ($M + 352$) of **1**, along with the unreacted form (**1**), were identified in the plasma (Figure 2A), small intestine (Figure 2B), and brain (Figure 2C) tissues based on mass spectrometry (Figure S1). The UPLC chromatogram, using a calibration curve, revealed that 0.79%, 27%, and 11% of the administered **1** was distributed to the brain, small intestine, and plasma, respectively. Moreover, relative intensity analysis, based on the total ion chromatogram of **1** in the brain, indicated the presence of the sulfate form (2.8), monoglucuronide form (10), diglucuronide form (1.3), and intact form (1). A previous *in vitro* study showed that **1** can undergo conversion to oxidized forms, such as flavonoids, after incubation at 37°C for 4 hours, which accounts for its inhibitory activity against A β 42 aggregation.¹⁵ Therefore, it is possible that **1** takes a longer time to reach the brain than it does to reach the plasma and small intestine. Alternatively, metabolic modification may prevent the oxidation or degradation of **1**. Interestingly, the converted flavonoids from **1** were not prominently observed in the UV spectra of the plasma, small intestine, or brain. Considering our previous study, where the adduct of converted flavonoids from **1** with A β was detected in the brain of Tg mice with overexpressed A β ,¹⁵ it is plausible that the labile flavonoids derived from **1** are stabilized by forming adducts with brain A β proteins, thereby preventing further aggregation of A β in the brain.

In addition, the sulfate form ($M + 80$), monoglucuronide form ($M + 176$), and methylated form ($M + 14$) of compound **2**, along with the unreacted form (**2**), were observed in the plasma (Figure 3A), small intestine (Figure 3B), and brain (Figure 3C) tissues based on mass spectrometry. Furthermore, combination metabolites of **2**, such as the sulfate and methylated form ($M + 94$) and the monoglucuronide and methylated form ($M + 190$), were detected. These findings indicated that all tested compounds could successfully cross the BBB and reach the brain (Figures 2–4). The UV signal intensities of **3** were the highest among all detected signals in the plasma, small intestine, and brain. Conversely, the signal intensities of the unreacted forms of both **1** and **2** were relatively low. Compared with subcutaneously administered **3**, orally administered **1** and **2** may have undergone passage through the stomach and small intestine. Notably, diglucuronidation was only observed for **1** among the tested compounds.

These analyses were conducted to determine the presence of administered compounds and/or their metabolites in the brain at UV-detectable levels. The observed large background wobble in the spectra at early retention time (approximately 4 minutes) is likely attributed to injection shock and/or the presence of components that were not adsorbed on the column. Consequently, the peaks of the target compounds may have appeared to be obscured within the baseline, and baseline correction did not effectively enhance the visualization of these peaks. Most compounds with high peak intensities were degradation products of the administered compounds themselves (i.e., phase I reactants, such as air-oxidated forms and hydrolysis reduced forms) but not metabolites from phase II reactions.

3.2. Evaluation of the Inhibitory Activities of the DDC Metabolites Against A β 42 Aggregation

Green perilla is an annual herbaceous plant native to Asia. In this study, we specifically focused on **1** derived from green perilla due to its traditional use as a food ingredient and its potential impact on the biological mechanisms underlying AD etiology. To understand how the bioavailable metabolites of **1** influence AD, we investigated the role of phase II metabolism and its conjugation in the inhibitory activity of **1**. Two conjugated forms of **1**, namely the sulfate form (**4**; 3',4'-*O*-disulfate) and the glucuronide form (**5**; 3'-*O*- β -D-glucuronide), were synthesized using conventional methods (Figures 5A and B). Subsequently, the inhibitory activity of **1**, **4a** (the sodium salt form of **4**), and **5** against A β 42 aggregation was investigated using the Th-T assay, a commonly used method for monitoring the formation of cross- β structured aggregates (fibrillization) in amyloidogenic proteins.³⁹ The aggregation of A β 42 (10 μ M) was monitored in the presence of each compound (20 or 50 μ M) and the Th-T reagent (20 μ M). It is important to note that the Th-T assay may introduce some bias when evaluating the antiamyloidogenic properties of compounds, as it can competitively bind with Th-T, as seen with compounds such as (–)-epigallocatechin-3-gallate,⁴⁰ curcumin⁴¹, and resveratrol.⁴¹ Despite the abovementioned problems, Th-T is one of the most widely used methods for evaluating amyloid aggregation, considering its simplicity and high throughput.³⁹ To dispel these problems, we performed a competitive assay to examine the inherent fluorescence of **1**, **4a**, and **5** and their potential interference with Th-T fluorescence upon binding to A β 42 aggregates. The results showed no significant interference with Th-T fluorescence by any of the compounds (Figure S4A). Furthermore, the inherent fluorescence of **1**, **4a**, and **5** was not significant compared with the Th-T fluorescence (Figure S4B). As shown in Figure 6A, A β 42 required approximately 6 h for

nucleation and reached saturation at approximately 16 h after elongation. As reported previously, **1** prevented both the nucleation and elongation phases of A β 42.¹⁵ At a concentration of 50 μ M, **4a** showed a decrease in the amount of aggregation compared with A β 42 alone (Figure 6A). Compound **5**, at both 20 and 50 μ M, preferably inhibited the elongation phase during A β 42 aggregation.

In our previous study, we observed that A β 42 treated with **1** resulted in sparse bundles of amyloid fibrils, and short fibrils were detected by TEM.¹⁵ Similarly, the aggregates formed upon treatment with **4a** and **5** exhibited sparse morphologies (Figure 6B), and their widths were considerably shortened (Figure S5). These results suggest that **1** retains its antiaggregation activity even after undergoing metabolism, and the metabolites **4a** and **5** do not exhibit stronger antiaggregation ability than the aglycone form (**1**).

3.3. Evaluation of DDC Metabolites for Their Inhibitory Activities Against A β 42 Oligomerization

Given the moderate modulation of the nucleation phase by **4a** and **5** (Figure 6A), dot blotting using an oligomer-specific antibody (A11),³⁶ whose selectivity for oligomers against monomers or fibrils was previously confirmed,⁴² was performed in an additional attempt to characterize whether the metabolites delayed A β 42 nucleation, which is relevant to oligomerization.⁴³ As depicted in Figure 6C, A11-positive oligomers were readily generated in A β 42 alone (10 μ M) after incubation for 1 h, whereas the signal intensity of treatment with **1** for oligomers was significantly downregulated at the concentration of 50 μ M, as reported in a previous study.¹⁵ The oligomer signal for A β 42 treated with **4a** and **5** also significantly decreased at the doses of 20 and 50 μ M. The difference in the inhibitory activities against A β 42 oligomerization between **4a** and **5** might originate from the presence of the carboxylic group. These results indicated that both metabolites could modulate A β 42 nucleation, but their potency did not surpass that of the aglycone form (**1**).

4. DISCUSSION

This study comprehensively identified 10 metabolites of orally administered antiaggregation compounds in the plasma, small intestine, and brain of mice. To the best of our knowledge, this is the first study to employ UPLC–MS/MS to examine the pharmacokinetics of antiaggregation metabolites in mice. The majority of the research on the brain metabolism of antiaggregative dietary ingredients has been conducted on rats. Cole et al. reported a relationship between the brain intake of curcumin and its amount in

the plasma, although only nonquantitative immunohistochemical data on brain curcumin were provided.⁴⁴ Because mice are simpler to manage for genetic recombination studies than rats, the experimental methodologies described in this study may be applied to AD model animals, such as transgenic mice or knock-in mice. In addition, preparation of two metabolites of **1**, including the sulfate and glucuronide forms, and their ability to inhibit A β 42 aggregation were demonstrated in the present study. Further studies will be required to reveal the half-life and dose effect of these metabolites in the brain.

Our group has elucidated the role of carboxylic acid in the antiaggregation properties of triterpenoids and anthraquinoids by forming a salt bridge with the side chains of Lys16 and 28 in A β 42.¹⁷ Furthermore, considering the role of carboxylic acid in the glucuronide form in antiaggregation, we reported that there was almost no difference in inhibitory activities between the glucuronide and glucoside forms (e.g., quercetin-3-*O*-glucuronide vs. quercetin-3-*O*-glucoside, kaempferol-3-*O*-glucuronide vs. kaempferol-3-*O*-glucoside).⁴⁵ These findings imply that the contribution of carboxylic acid in the glucuronide form to antiaggregation may be limited, unlike that of triterpenoids and anthraquinoids (e.g., the antiaggregative ability of uncarinic acid C was reported to originate from the carboxylic acid moiety⁴⁶), possibly due to steric hindrance or hydrophilicity around the sugar structure. Indeed, X-ray analysis revealed some involvement of the glucuronide moiety of several flavonoid metabolites in complex formation with transthyretin, an amyloidogenic protein.⁴⁷ In addition, the sulfonate group of **1** is one of the important structural factors responsible for the inhibition of aggregation because the polar functionality can contribute to electrostatic interactions targeting amino acid residues (e.g., lysine and arginine), as previously reported in other studies.^{48,49} Alternatively, an α,β -unsaturated carbonyl structure conjugated with the aromatic ring in **1** could in part participate in the antiaggregation property.

To date, no study has compared the inhibitory activities between conjugated forms after phase II metabolism and the aglycone form against A β 42 aggregation. However, antiaggregation properties of specific glucuronide forms of flavonoids, such as quercetin-3-*O*- β -D-glucuronide²³ and 3-*O*-methyl-epicatechin-5-*O*- β -D-glucuronide,²⁴ have been individually investigated. It is worth noting that the conjugation through phase II metabolism weakened the antiaggregation properties of the aglycone form (Figure 6). These findings are consistent with those of our previous study, which demonstrated that glycosylation and glucuronidation reduced the inhibitory activities of quercetin.⁴⁵ The conjugated forms of **1**, following phase II metabolism in the liver, may enter the brain. These metabolites were also detected in the small intestine and plasma of all mice treated with **1–3** (Figures 2–4, Table 1). Under healthy conditions, P-glycoprotein was reported

to be involved in the permeability across the BBB.⁵⁰ Recently, it has been suggested that flavonoids circulate in the body via the lymphatic system,^{51,52} implying a possible route of entry into the brain other than the BBB. BBB breakdown is considered an early biomarker of human cognitive dysfunction.⁵³ Alternatively, there is a possibility that **1** may be affected by sulfatase or glucuronidase after crossing the BBB. This knowledge may inspire development of strategies for disease-modifying therapeutic interventions focused on functional food ingredients and promote advancements in the field of food science.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at XXX.

The ¹H and ¹³C NMR spectra of **4** and **5**, along with representative mass spectra in UPLC–MS analysis (Figure S1), are shown. The UPLC spectra of vehicle-treated mice are presented in Figure S2 for polypropylene glycol and Figure S3 for 0.9% NaCl. The Th-T interference test, which includes the inherent fluorescence of the compounds, is depicted in Figure S4. Additionally, the quantitative analysis of transmission electron microscopy can be found in Figure S5. All these figures are available in PDF format.

AUTHOR INFORMATION

K.T.: Investigation, Writing-Original draft preparation; K.M.: Conceptualization, Supervision, Investigation, Writing-Original draft preparation; Y.S.: Data curation; N.I.: Investigation, Writing-Original draft preparation; M.H.: Investigation; N.S.: Investigation; T.K.: Resources; T.S.: Resources; K.I.: Supervision, Writing-Reviewing and Editing.

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NOTES

The authors declare no competing financial interest.

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ABBREVIATIONS

AD, Alzheimer's disease; A β , amyloid β -protein; BBB, blood–brain barrier; DDC, 2',3'-dihydroxy-4',6'-dimethoxychalcone; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; TEM, transmission electron microscopy; Th-T, thioflavin-T; UPLC–MS/MS, ultraperformance liquid chromatography–mass spectrometry/mass spectrometry.

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Figure captions

Figure 1. Structures of DDC (**1**), taxifolin (**2**), apomorphine (**3**), and their metabolites: DDC 2',3'-*O*-disulfate (**4**) and DDC 3'-*O*- β -D-glucuronide (**5**).

Figure 2. UPLC chromatogram of (A) plasma, (B) small intestine, and (C) brain samples obtained from mice orally administered with **1**. The chromatographic conditions were as follows: solvent: 10%–50% CH₃CN, 0.1% formic acid (0–10 min, linear gradient); flow rate: 0.2 mL/min; column temperature: 35°C; sample temperature: 5°C; ion mode: ESI[−]. GlcA = glucuronide.

Figure 3. UPLC chromatogram of (A) plasma, (B) small intestine, and (C) brain samples obtained from mice orally administered with **2**. The chromatographic conditions were as follows: solvent: 10%–50% CH₃CN, 0.1% formic acid (0–10 min, linear gradient); flow rate: 0.2 mL/min; column temperature: 35°C; sample temperature: 5°C; ion mode: ESI[−]. GlcA = glucuronide.

Figure 4. UPLC chromatogram of (A) plasma, (B) small intestine, and (C) brain samples obtained from mice subcutaneously administered with **3**. The chromatographic conditions were as follows: solvent: 10%–50% CH₃CN, 0.1% formic acid (0–10 min, linear gradient); flow rate: 0.2 mL/min; column temperature: 35°C; sample temperature: 5°C; ion mode: ESI[−]. GlcA = glucuronide.

Figure 5. Preparative scheme of (A) **4** and (B) **5**.

Figure 6. Inhibitory activities of **1**, **4a**, and **5** against the aggregation of A β 42. (A) Time–response curves of A β 42 aggregation (10 μ M) during incubation for 24 h at 25°C with each compound (20 or 50 μ M). Data are presented as mean $\pm$ s.d. ($n = 3$). Green shadows indicate the nucleation phase, where the relative A β 42 aggregation was 50%. (B) TEM analysis of the A β 42 aggregates incubated with each compound (50 μ M) after the Th-T test. Scale bar = 200 nm. (C) Dot blotting test using an oligomer-specific antibody (A11). After A β 42 (10 μ M) was incubated with each compound (20 or 50 μ M) for 1 h at 37°C, an aliquot (1 μ L, 45 ng) was used for the analysis. Relative intensity to A β 42 of dot blots in the upper panel for 20 μ M (*light gray circle*) and 50 μ M (*dark gray circle*) of each compound are indicated in the lower panel. Data are presented as mean $\pm$ s.d. ($n = 3$): iii, $p < 0.001$; iv, $p < 0.0001$ versus A β 42 alone.

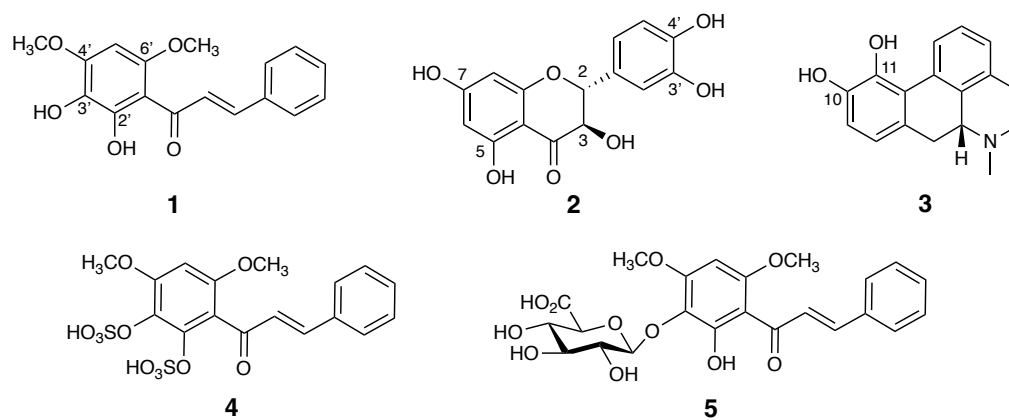
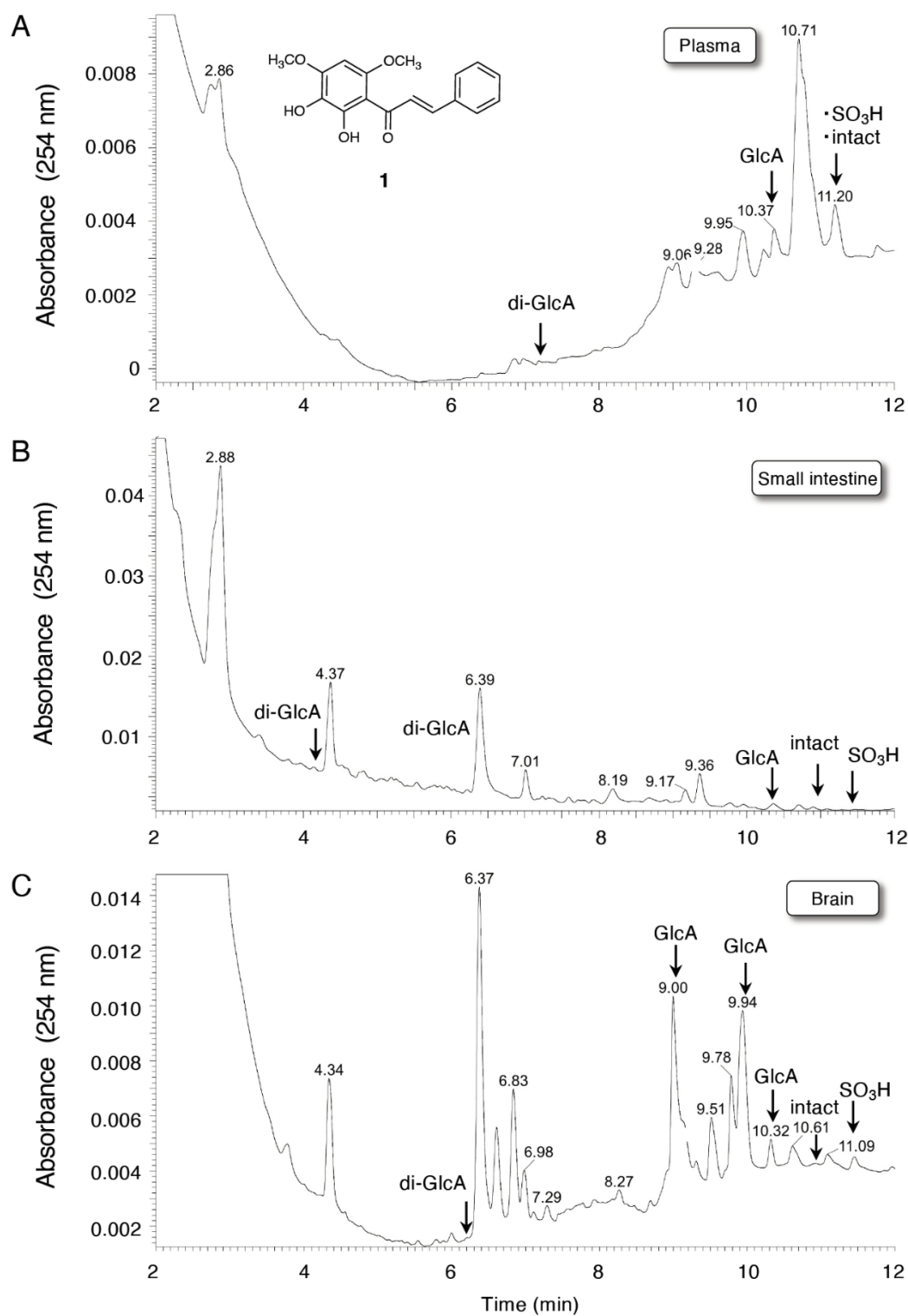


Figure 1.

**Figure 2.**

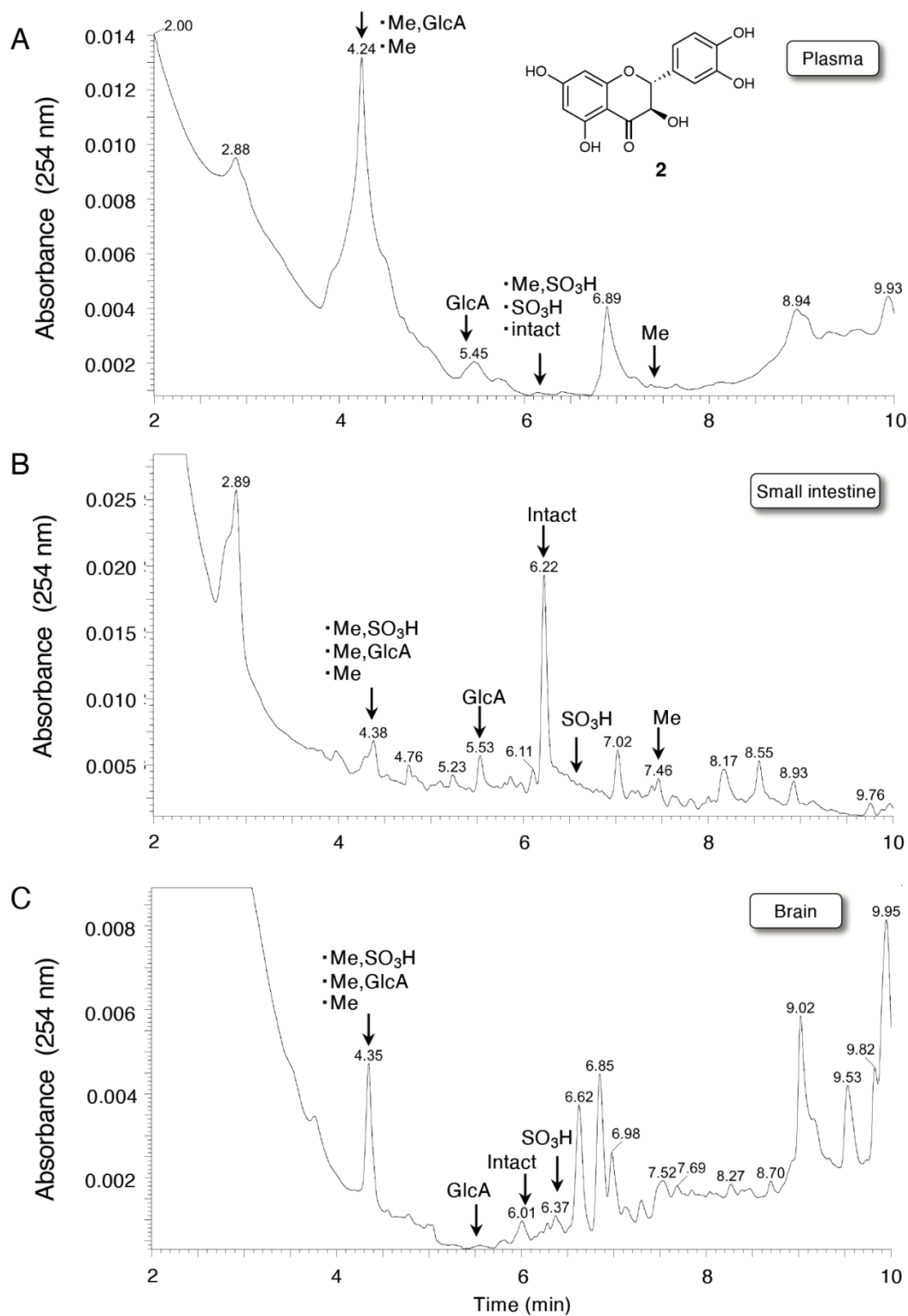


Figure 3.

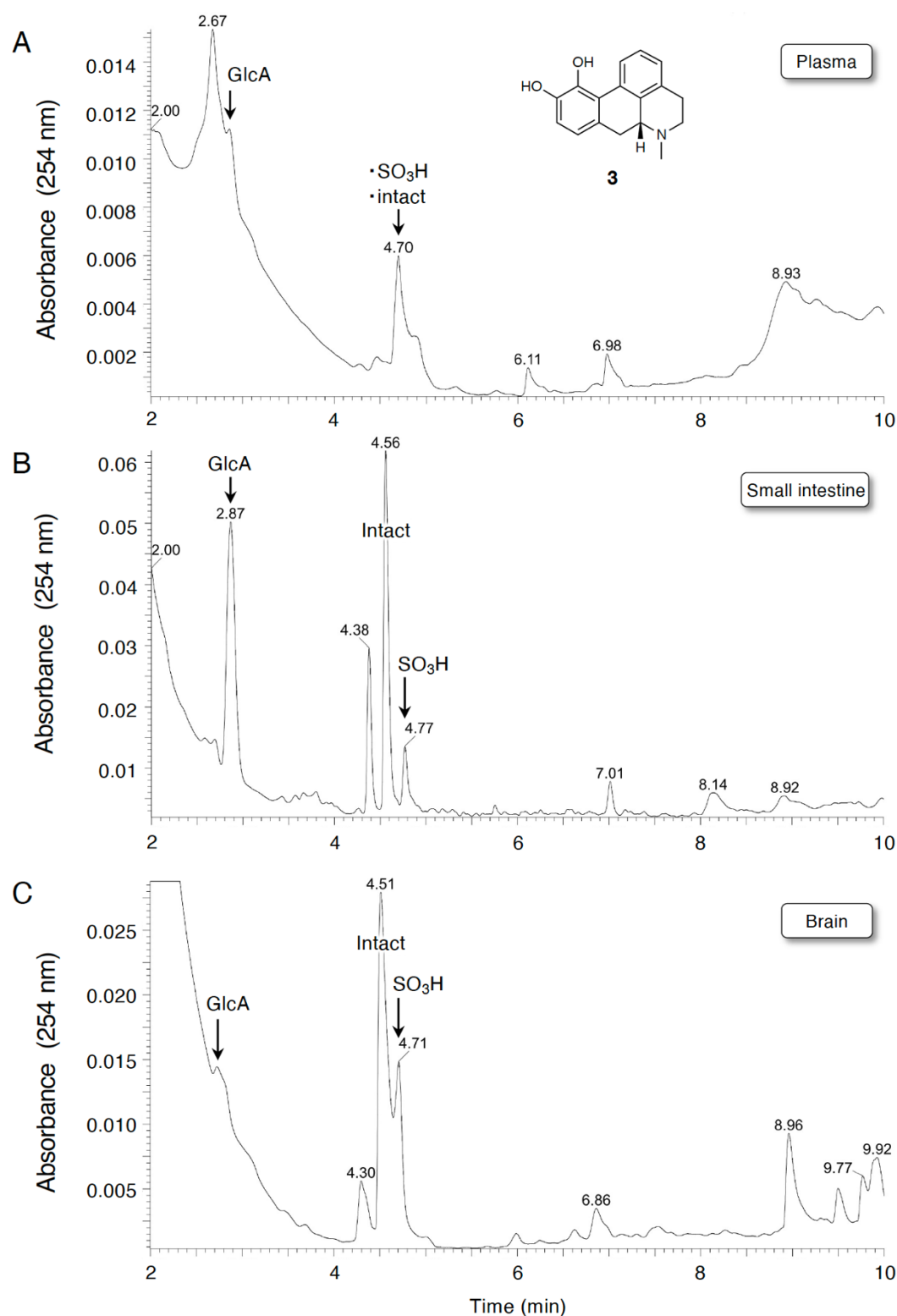


Figure 4.

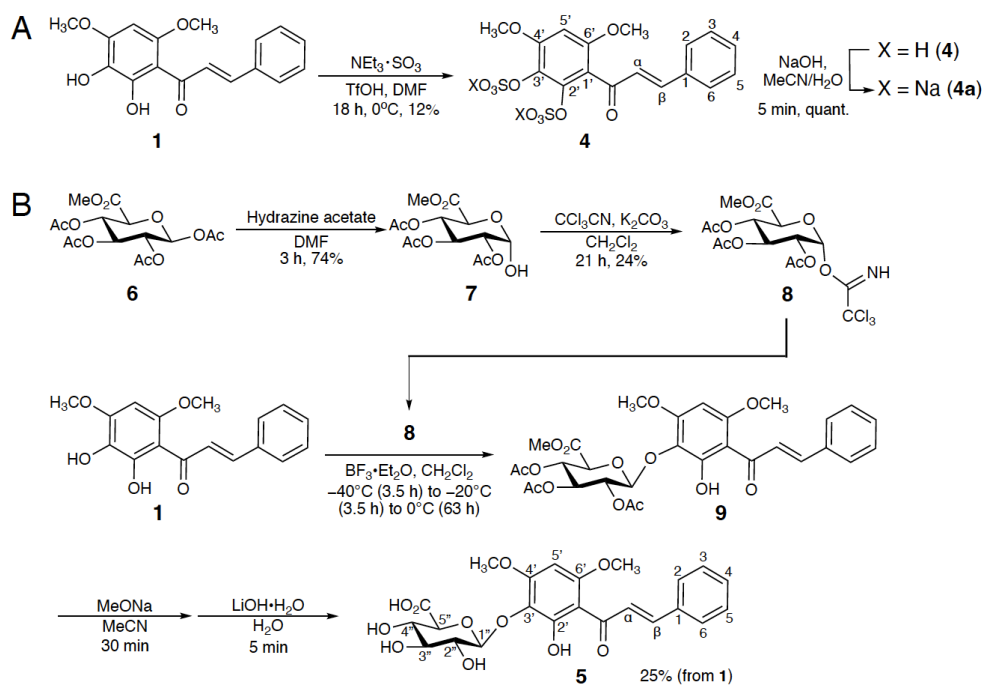


Figure 5.

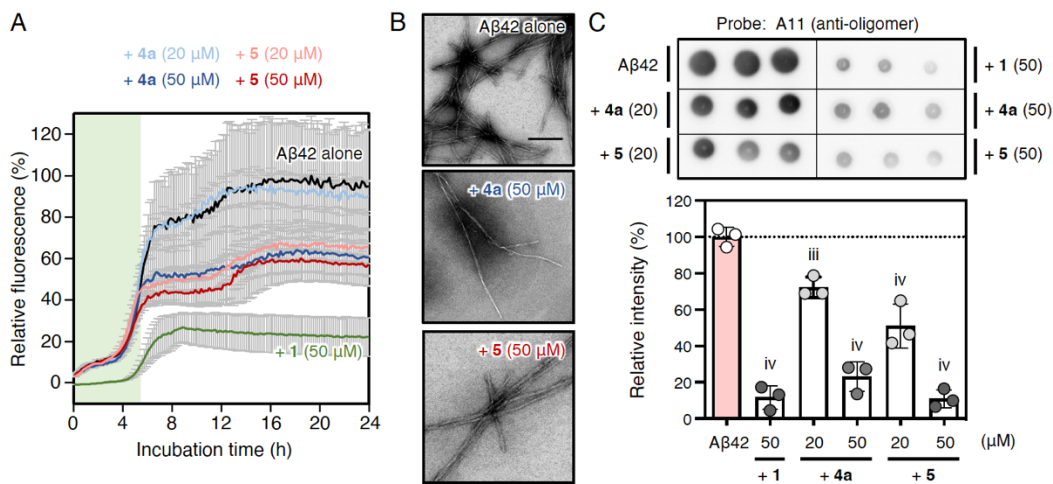


Figure 6.

Table 1. Peak annotation of the observed mass of the metabolites of **1–3**

Metabolite	Calculated [M–H] [–]	Observed [M–H] [–]		
		Plasma	Small	Brain
DDC (1)				
Intact (M)	299.0919	299.0936	299.0900	299.0936
Sulfate (M+80)	379.0488	379.0478	379.0478	379.0478
Monoglucuronide (M+176)	475.1224	475.1224	475.1224	475.1269
Diglucuronide (M+352)	651.1561	651.1574	651.1574	651.1574
Taxifolin (2)				
Intact (M)	303.0505	303.0504	303.0504	303.0504
Sulfate (M+80)	383.0073	383.0077	383.0077	383.0077
Glucuronide (M+176)	479.0826	479.0841	479.0796	479.0841
Methylated (M+14)	317.0661	317.0679	317.0643	317.0679
Methyl, sulfate (M+94)	397.0229	397.0234	397.0234	397.0234
Methyl, glucuronide	493.0982	493.0976	493.0976	493.0976
Apomorphine (3)				
Intact (M)	266.1181	266.1189	266.1189	266.1189
Sulfate (M + 80)	346.0749	346.0764	346.0764	346.0764
Glucuronide (M + 176)	442.1502	442.1513	442.1513	442.1513

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