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Biotransformation of Bergapten and Xanthotoxin by *Glomerella cingulata*

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The biotransformation of bergapten (**1**) by the fungus *Glomerella cingulata* gave the corresponding reduced acid, 6,7-furano-5-methoxy hydrocoumaric acid (**2**), a new compound. Xanthotoxin (**3**) was also converted to the corresponding reduced acid cnidiol b (**4**) and demethylated metabolite xanthotoxol (**5**) by *G. cingulata*. The structure of the new compound **2** was elucidated by high-resolution mass spectrometry, extensive NMR techniques, including ^{1}H NMR and ^{13}C NMR, ^{1}H−^{1}H correlation spectroscopy, heteronuclear multiple quantum coherence, and heteonuclear multiple bond coherence. The methyl ester or methyl ether or methyl ester and ether derivatives of **2** and **4** were synthesized. All compounds were tested for the β-secretase (BACE1) inhibitory activity in vitro. The methyl ester and ether derivative **8** was shown to possess BACE1 inhibitory activity, and a IC_{50} value was 0.64 ± 0.04 mM.

KEYWORDS: Biotransformation; furanocoumarin; bergapten; xanthotoxin; *Glomerella cingulata*; β-secretase (BACE1) inhibitory activity

INTRODUCTION

Furanocoumarins are a class of potent phytoalexins (*1*−*3*) and allelochemical compounds (*4*) produced by plants of the Apiaceae, Rutaceae, Moraceae, and Fabaceae (*5*). In these plants, the most abundant linear furanocoumarins are psoralen, bergapten (**1**), xanthotoxin (**3**), and isoimpinellin (*5*). Furanocoumarins are well-known to act as phototoxicants in combination with UVA irradiation (wavelength 320−380 nm), exhibiting cytotoxic and mutagenic properties (*6*−*8*). Possible mechanisms leading to adverse effects include binding to cellular constituents (*9*), lysosomal damage (*10*), generation of reactive oxygen species (*11*), and formation of novel antigens through covalent modification of proteins and DNA (*12*). Even in the absence of UVA light, daily doses of 200 and 400 mg of xanthotoxin (**2**)/kg of body weight (given orally over 90 days, 5 days a week) have been reported to exert toxic effects on liver, testes, and epididymis in rat (*13*). Furthermore, a number of furanocoumarins act as inhibitors of drug-metabolizing enzymes. 6′,7′-Dihydroxybergamottin and related furanocoumarins dimers found, for example, in grapefruit juice act as highly potent inhibitors of cytochrome P450 (CYP) 3A and other CYP isozymes (*14*) affecting drug metabolism.

Biotransformation is today considered to be an economically competitive technology by synthetic organic chemists in search of new production routes for fine chemical, pharmaceutical, and agrochemical compounds (*15*). Microorganisms are well-known as efficient and selective catalysts. Previously, we studied the microbial transformation of furanocoumarins isoimperatorin and imperaotirn by *Glomerella cingulata* and evaluated the transformation products on the anti-Alzheimer effects via β-secretase (BACE1) inhibitory (*16*). To obtain new compounds, the present investigation was carried out to continue studying the ability of *G. cingulata* to accomplish structural modifications of furanocoumarins bergapten (**1**) and xanthotoxin (**3**). Consequently, the incubation of **1** and **3** should also permit a comparison of results, determining the influence of the substituent groups of the C_5 and C_8 position in these types of biotransformations. The present work was an attempt to get the microbial transformation products of **1** and **3** by *G. cingulata* and to provide the BACE1 inhibitory activity of these compounds (**Figure 1**).

MATERIALS AND METHODS

General Experimental Procedures. Thin-layer chromatography (TLC) was performed on precoated plates (Si gel 60 F_{254}, 0.25 mm, Merck). The mobile phase was hexane−EtOAc (1:1). Compounds were visualized by spraying plates with 0.5% vanillin in 96% H_2SO_4 followed by brief heating. A Shimazu LC-10A high-performance liquid chromatography (HPLC) system (Shimazu Co., Ltd., Kyoto, Japan) was comprised of a quaternary solvent deliver system, an autosampler, a column temperature controller, and a photodiode array (PDA) coupled with analytical works station. A YMC-Pack ODS-AQ (4.6 mm × 250 mm, 5 μm particle size, YMC Co., Ltd., Japan) with a YMC-Pack ODS-AQ guard column (4.6 mm × 23 mm, 5 μm particle size, YMC Co., Ltd., Japan) were used. The chromatographic parameters were as follows: solvent A, acetonitrile; solvent B, water; both were modified with 0.1% (v/v) acetic acid. The gradient was set as follows: 20% A for 10 min at 1.0 mL/min, 20−70% A in 100 min at 1.0 mL/min, and 70% A for 10 min at 1.0 mL/min. The total runtime was 110 min. The injection volume was 10 μL. Electron ionization mass spectrometry (EIMS), high-resolution electron ionization mass spectrometry (HR-EIMS), fast atom bombardment mass spectrometry (FABMS), and high-resolution fast atom bombardment mass spectrometry (HR-FABMS) were obtained on a JEOL the Tandem Ms station JMS-700 TKM. Nuclear magnetic resonance (NMR) spectra were recorded at 500 MHz for ^{1}H and 125 MHz for ^{13}C on a JEOL ECA-500 spectrometer.

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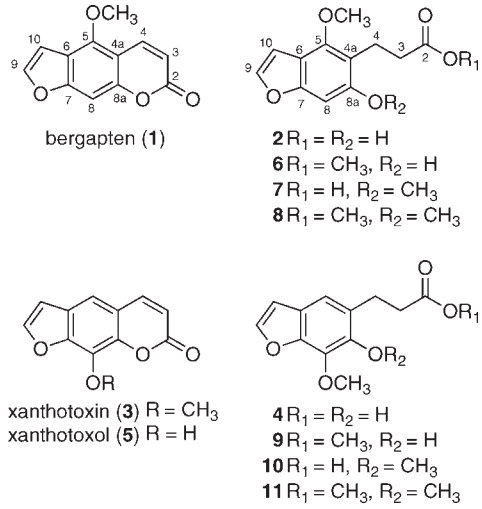

Figure 1. Chemical structures of bergapten and xanthotoxin derivatives.

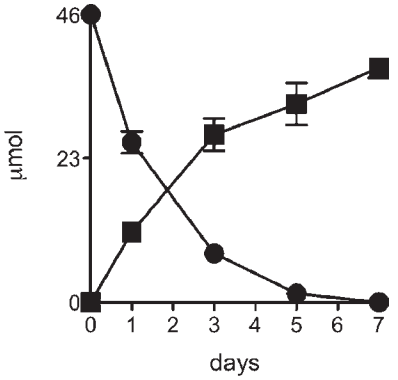

Figure 2. Time course in the biotransformation of **1** by *G. cingulata*: bergapten (**1**) (●) and metabolite **2** (■). The bar is the mean ± SD of three experiments.

IR spectra were determined with a JASCO FT/IR-470 plus Fourier transform infrared spectrometer. A BACE1 (recombinant human BACE1) assay kit was purchased from the PanVera Co. (United States).

Chemicals. Bergapten (**1**) and xanthotoxin (**3**) were purcharsed from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

Preculture of *G. cingulata*. Spores of *G. cingulata* NBRC 5952 (NITE Biological Resource Center, Japan), which had been preserved on potato dextrose agar (PDA) at 4 °C, were inoculated into 200 mL of sterilized culture medium (1.5% saccharose, 1.5% glucose, 0.5% polypeptone, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05%KCl, 0.1% K_2HPO_4, and 0.001% $FeSO_4 \cdot 7H_2O$ in distilled H_2O) in a 500 mL shaking flask, and the flask was shaken (reciprocating shaker, 120 rpm) at 27 °C for 3 days.

Time Course of Biotransformation and Quantification of Metabolite. Precultured *G. cingulata* (3 mL) was transferred into two 300 mL Erlenmeyer flasks containing 100 mL of medium and was stirred (ca. 120 rpm) for 3 days. After the growth of *G. cingulata*, compounds **1** (10 mg, 46 μmol) and **3** (10 mg, 46 μmol) in 0.5 mL of dimethyl sulfoxide (DMSO) were added into the medium, respectively, and cultivated for 7 more days. Every other day, 5 mL of the culture medium was extracted with EtOAc. This extract was analyzed by TLC and HPLC. The mobile phase and detector used were the same as above. The contents of these compounds were calculated by means of the absolute calibration curves. The time course of biotransformation is shown in **Figures 2** and **3**.

Preparative Biotransformation of Bergapten (1). Precultured *G. cingulata* (5 mL) was transferred into a 500 mL Erlenmeyer flask containing 300 mL of medium. Cultivation was carried out at 27 °C with stirring (ca. 120 rpm) for 3 days. After the growth of *G. cingulata*, 50 mg of **1** in 1.0 mL of DMSO was added into the medium and cultivated for an additional 7 days, together with two controls, which contained either mycelia with medium or substrate dissolved in DMSO with medium. No metabolic product was observed in two controls.

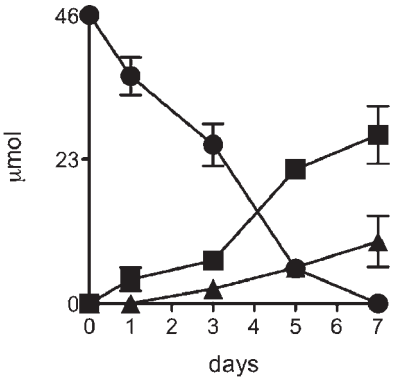

Figure 3. Time course in the biotransformation of **3** by *G. cingulata*: xanthotoxin (**3**) (●), metabolite **4** (■), and metabolite **5** (▲). The bar is the mean ± SD of three experiments.

Table 1. ^{13}C NMR Spectroscopic Data of Compounds **1**−**4**

position	**1**[a]	**2**[b]	**3**[a]	**4**[b]
2	161.2 (C)[c]	175.5 (C)	160.4 (C)	174.4 (C)
3	112.5 (CH)	34.4 (CH_2)	114.7 (CH)	34.6 (CH_2)
4	139.2 (CH)	19.9 (CH_2)	144.3 (CH)	27.0 (CH_2)
4a	106.4 (C)	114.3 (C)	116.5 (C)	125.4 (C)
5	149.5 (C)	152.4 (C)	112.9 (CH)	115.7 (CH)
6	112.6 (C)	113.4 (C)	126.1 (C)	122.0 (C)
7	158.4 (C)	156.6 (C)	147.7 (C)	146.2 (C)
8	93.8 (CH)	93.6 (CH)	132.8 (C)	133.1 (C)
8a	152.7 (C)	154.9 (C)	143.0 (C)	144.9 (C)
9	144.8 (CH)	143.2 (CH)	146.6 (CH)	144.8 (CH)
10	105.0 (CH)	105.6 (CH)	106.7 (CH)	107.5 (CH)
OCH_3	60.1 (CH_3)	60.3 (CH_3)	61.3 (CH_3)	60.8 (CH_3)

[a] Measured in $CDCl_3$ at 100 MHz. [b] Measured in acetone-d_6 at 125 MHz. [c] ^{13}C multiplicities were determined by DEPT 135°.

Isolation of Metabolite. After the fermentation, the culture medium and mycelia were separated by filtration. The medium was saturated with NaCl and extracted with EtOAc. The mycelia were also extracted with EtOAc. Each EtOAc extract was combined, the solvent was evaporated, and a crude extract (383 mg) was obtained. The extract was distributed between 5% aqueous $NaHCO_3$ and EtOAc, and the EtOAc phase was evaporated to give the neutral fraction (185 mg). No metabolic compounds were detected from neutral fraction by TLC and HPLC. The alkali phase was acidified to pH 3 with 1 N HCl and distributed between water and EtOAc. The EtOAc phase was evaporated, and the acidic fraction (198 mg) was obtained. The acidic fraction was dissolved in acetone (3 mL), and CH_2N_2 (1 mL) was added to the fraction. The solution was evaporated, and the methylation fraction was obtained. The methylation fraction was subjected to silica gel column chromatography (silica gel 60, 230−400 mesh, Merck) with a *n*-hexane−Et_2O gradient (9:1 to 1:4) to yield compound **6** (45 mg). Compound **6** (20 mg) was dissolved in MeOH (1 mL), 5% NaOH (2 mL) was added to the solution, and the solution was refluxed for 30 min. The solution was acidified to pH 3 with 1 N HCl and distributed between EtOAc and water. The EtOAc phase was evaporated to give **2** (15 mg, substrate **1** was used as an internal standard; relative retention time, $Rt_R = 0.65$).

6,7-Furano-5-methoxy Hydrocoumaric Acid (**2**). White powder. IR (KBr) ν_{max} 3465, 1705, cm^{-1}. The ^{13}C and ^{1}H NMR are shown as **Table 1** and **2**. HR-FABMS (pos) *m*/*z* 237.0746 $[M + H]^+$ (calcd for $C_{12}H_{13}O_5$, 237.0762).

Preparative Biotransformation of Xanthotoxin (3). Precultured *G. cingulata* (5 mL) was transferred into a 500 mL Erlenmeyer flask containing 300 mL of medium. Cultivation was carried out at 27 °C with stirring (ca. 120 rpm) for 3 days. After the growth of *G. cingulata*, 50 mg of **3** in 1.0 mL of DMSO was added into the medium and cultivated for an additional 7 days, together with two controls, which contained either

Table 2. ^{1}H NMR Spectroscopic Data of Compounds **1**−**4**

position	**1**[a]	**2**[b]	**3**[a]	**4**[b]
2				
3	6.27, 1H, d (9.8)[c]	2.52−2.55, 2H, m	6.38, 1H, d (9.8)	2.61−2.64, 2H, m
4	8.15, 1H, d (9.8)	2.96−2.99, 2H, m	7.77, 1H, d (9.8)	2.95−2.98, 2H, m
4a				
5			7.36, 1H, s	7.08, 1H, s
6				
7				
8	7.12, 1H, brs	6.73, 1H, d (1.0)		
8a				
9	7.59, 1H, d (2.4)	7.57, 1H, d (2.3)	7.69, 1H, d (2.4)	7.66, 1H, d (2.0)
10	7.02, 1H, dd (2.4, 1.0)	6.97, 1H, dd (2.3, 1.0)	6.82, 1H, d (2.4)	6.74, 1H, d (2.0)
OCH_3	4.27, 3H, s	4.07, 3H, s	4.30, 3H, s	4.05, 3H, s

[a] Measured in $CDCl_3$ at 400 MHz. [b] Measured in acetone-d_6 at 500 MHz. [c] The *J* values are in Hz in parentheses.

mycelia with medium or substrate dissolved in DMSO with medium. No metabolic product was observed in two controls.

Isolation of Metabolite. After the fermentation, the culture medium and mycelia were separated by filtration. The medium was saturated with NaCl and extracted with EtOAc. The mycelia were also extracted with EtOAc. Each EtOAc extract was combined, the solvent was evaporated, and a crude extract (340 mg) was obtained. The extract was distributed between 5% aqeous $NaHCO_3$ and EtOAc, and the EtOAc phase was evaporated to give the neutral fraction (183 mg). The alkali phase was acidified to pH 3 with 1 N HCl and distributed between water and EtOAc. The EtOAc phase was evaporated, and the acidic fraction (157 mg) was obtained. The acidic fraction was dissolved in acetone (3 mL), and CH_2N_2 (1 mL) was added to the fraction. The solution was evaporated, and the methylation fraction was obtained. The methylation fraction was subjected to silica gel column chromatography (silica gel 60, 230−400 mesh, Merck) with a *n*-hexane−Et_2O gradient (9:1 to 1:4) to yield compound **9** (29 mg). Compound **9** (20 mg) was dissolved in MeOH (1 mL), 5% NaOH (2 mL) was added to the solution, and the solution was refluxed for 30 min. The solution was acidified to pH 3 with 1 N HCl and distributed between EtOAc and water. The EtOAc phase was evaporated to give **4** (13 mg, substrate **3** was used as an internal standard; Rt_R = 0.78). The neutral fraction was subjected to silica gel column chromatography (silica gel 60, 230−400 mesh, Merck) with a *n*-hexane−EtOAc gradient (9:1 to 1:9) to yield compound **5** (9 mg).

6,7-Furano-8-methoxy Hydrocoumaric Acid (**4**). White powder. IR (KBr) ν_{max} 3393, 1696, cm^{-1}. The ^{13}C and ^{1}H NMR are shown as **Table 1** and **2**. HR-FABMS (pos) *m*/*z* 237.0746 $[M + H]^+$ (calcd for $C_{12}H_{13}O_5$, 237.0762).

Xanthoxol (**5**). White needles. IR (KBr) ν_{max} 3324, 1705, 1594 cm^{-1}. ^{1}H NMR data ($CDCl_3$, 500 MHz): δ 8.10 (1H, d, *J* = 9.8 Hz, H-4), 8.06 (1H, d, *J* = 2.3 Hz, H-9), 7.44 (1H, s, H-5), 7.03 (1H, d, *J* = 2.3 Hz, H-10), 6.39 (1H, d, *J* = 9.8 Hz, H-3). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 160.3 (C, C-2) 147.6 (CH, C-9), 145.8 (CH, C-4), 145.6 (C, C-7), 140.0 (C, C-8a), 130.4 (C, C-8), 125.4 (C, C-6), 116.4 (C, C-4a), 114.0 (CH, C-3), 110.3 (CH, C-5), 107.3 (CH, C-10). EIMS *m*/*z* 202 $[M]^+$ (100), 174 (63), 149 (10), 146 (9), 89 (15). HR-EIMS *m*/*z* 202.0262 $[M]^+$ (calcd for $C_{11}H_6O_4$, 202.0325).

General Procedure for the Preparation of Derivatives of Metabolite. *Methylation of Phenol.* A methyl iodide (CH_3I, 1.5 equiv) and sodium hydride (NaH, 1.5 equiv) were added to a solution of **6** or **9** (1 equiv) in dry *N*,*N*-dimethylformamide (DMF), respectively. The mixture was stirred at room temperature for 3 h. The reaction mixture was poured into ice water, and the whole was extracted with CH_2Cl_2. The CH_2Cl_2 extract was successively washed brine and then dried over Na_2SO_4, and the filtrate under reduced pressure furnished a residue, which was purified by silica gel column chromatography, gave compound **8** or **11**, respectively.

Hydrolysis of Methyl Ester. Compound **8** or **11** was dissolved in MeOH (1.0 mL), 5% NaOH (2.0 mL) was added to the solution, and the solution was refluxed for 30 min, respectively. The solution was acidified with 1 N HCl and distributed between EtOAc and water. The EtOAc phase was evaporated and purified by silica gel column chromatography and gave compound **7** or **10**, respectively.

6,7-Furano-5-methoxy Hydrocoumaric Acid Methyl Ester (**6**). White powder. IR (KBr) ν_{max} 1732 cm^{-1}. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.42 (1H, d, *J* = 2.2 Hz, H-9), 6.83 (1H, dd, *J* = 2.2, 01.0 Hz, H-10), 6.82 (1H, d, *J* = 1.0 Hz, H-8), 4.10 (3H, s, OCH_3), 3.67 (3H, s, $COO\underline{CH_3}$), 2.96−2.94 (2H, m, H-4), 2.75−2.73 (2H, m, H-3). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 177.3 (C, C-2) 156.1 (C, C-7), 153.2 (C, C-8a), 151.2 (C, C-5), 142.5 (CH, C-9), 113.4 (C, C-4a), 110.7 (C, C-6), 104.6 (CH, C-10), 94.9 (CH, C-8), 59.6 (CH_3, OCH_3), 52.2 (CH_3, $COO\underline{CH_3}$), 34.1 (CH_2, C-3), 18.5 (CH_2, C-4). EIMS *m*/*z* 250 $[M]^+$ (23), 218 (95), 177 (33), 176 (100), 161 (23), 147 (24), 146 (19), 133 (20). HR-EIMS *m*/*z* 250.0843 $[M]^+$ (calcd for $C_{13}H_{14}O_5$, 250.0841).

6,7-Furano-5,8a-dimethoxy Hydrocoumaric Acid (**7**). Colorless powder. IR (KBr) ν_{max} 1697, 1620, 1590 cm^{-1}. ^{1}H NMR data ($CDCl_3$, 700 MHz): δ 7.46 (1H, d, *J* = 2.2 Hz, H-9), 6.84 (1H, dd, *J* = 2.2, 0.8 Hz, H-10), 6.77 (1H, brs, H-8), 4.06 (3H, s, 5-OCH_3), 3.84 (3H, s, 8a-OCH_3), 3.05 (2H, m, H-4), 2.57 (2H, m, H-3). ^{13}C NMR ($CDCl_3$, 175 MHz): δ 179.2 (C, C-2) 156.6 (C, C-8a), 155.8 (C, C-7), 151.2 (C, C-5), 142.5 (CH, C-9), 114.2 (C, C-4a), 111.2 (C, C-6), 104.6 (CH, C-10), 89.2 (CH, C-8), 60.1 (CH_3, 5-OCH_3), 55.8 (CH_3, 8a-OCH_3), 33.9 (CH_2, C-3), 19.0 (CH_2, C-4). EIMS *m*/*z* 304 $[M]^+$ (5), 286 (86), 271 (47), 218 (60), 176 (100), 69 (24), 41 (18). HR-FABMS (pos) *m*/*z* 251.0912 $[M + H]^+$ (calcd for $C_{13}H_{15}O_5$, 251.0920).

6,7-Furano-5,8a-dimethoxy Hydrocoumaric Acid Methyl Ester (**8**). Pale yellow oil. IR (KBr) ν_{max} 1727, 1619, 1587 cm^{-1}. ^{1}H NMR data ($CDCl_3$, 500 MHz): δ 7.46 (1H, d, *J* = 2.3 Hz, H-9), 6.83 (1H, dd, *J* = 2.3, 0.9 Hz, H-10), 6.76 (1H, brs, H-8), 4.05 (3H, s, 5-OCH_3), 3.83 (3H, s, 8a-OCH_3), 3.68 ($COO\underline{CH_3}$), 3.03 (2H, m, H-4), 2.51 (2H, m, H-3). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 174.2 (C, C-2) 156.6 (C, C-8a), 155.8 (C, C-7), 151.4 (C, C-5), 142.4 (CH, C-9), 114.5 (C, C-4a), 111.3 (C, C-6), 104.6 (CH, C-10), 89.2 (CH, C-8), 60.1 (CH_3, 5-OCH_3), 55.8 (CH_3, 8a-OCH_3), 51.5 (CH_3, $COO\underline{CH_3}$), 34.0 (CH_2, C-3), 19.2 (CH_2, C-4). EIMS *m*/*z* 264 $[M]^+$ (36), 192 (12), 191 (100), 176 (7), 161 (9), 131 (18). HR-EIMS *m*/*z* 264.1009 (calcd for $C_{14}H_{16}O_5$, 264.0998).

6,7-Furano-8-methoxy Hydrocoumaric Acid Methyl Ester (**9**). Pale yellow oil. IR (film) ν_{max} 3439, 1731 cm^{-1}. ^{1}H NMR data ($CDCl_3$, 500 MHz): δ 7.48 (1H, d, *J* = 2.3 Hz, H-9), 7.01 (1H, s, H-5), 6.64 (1H, d, *J* = 2.3 Hz, H-10), 3.03 (2H, t, *J* = 7.7 Hz, H-4), 2.69 (2H, t, *J* = 7.7 Hz, H-3). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 173.9 (C, C-2) 144.3 (C, C-7), 143.7 (CH, C-9), 142.8 (C, C-8a), 131.6 (C, C-8), 123.4 (C, C-4a), 121.6 (C, C-6), 114.6 (C, C-5), 106.6 (CH, C-10), 60.5 (CH_3, OCH_3), 51.6 (CH_3, $COO\underline{CH_3}$), 34.3 (CH_2, C-3), 26.0 (CH_2, C-4). EIMS *m*/*z* 250 $[M]^+$ (43), 219 (16), 218 (81), 190 (23), 177 (38), 176 (100), 175 (20), 147 (33). HR-EIMS *m*/*z* 250.0831 $[M]^+$ (calcd for $C_{13}H_{14}O_5$, 250.0842).

6,7-Furano-8,8a-dimethoxy Hydrocoumaric Acid (**10**). Pale yellow oil. IR (KBr) ν_{max} 1712 cm^{-1}. ^{1}H NMR data ($CDCl_3$, 500 MHz): δ 7.56 (1H, d, *J* = 2.0 Hz, H-9), 7.06 (1H, s, H-5), 6.67 (1H, d, *J* = 2.0 Hz, H-10), 4.18 (3H, s, 8-OCH_3), 3.91 (3H, s, 8a-OCH_3), 3.01 (2H, m, H-4), 2.70 (2H, m, H-3). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 178.1 (C, C-2), 146.8 (C, C-8a), 145.9 (C, C-7), 144.9 (CH, C-9), 138.4 (C, C-8), 129.6 (C, C-4a), 124.9 (C, C-6), 114.3 (CH, C-5), 106.6 (CH, C-10), 61.3 (CH_3, 8a-OCH_3), 60.6 (CH_3, 8-OCH_3), 34.8 (CH_2, C-3), 26.0 (CH_2, C-4). HR-FABMS (pos) *m*/*z* 251.0991 $[M + H]^+$ (calcd for $C_{13}H_{15}O_5$, 251.0920).

6,7-Furano-8,8a-dimethoxy Hydrocoumaric Acid Methyl Ester (**11**). Pale yellow oil. IR (film) ν_{max} 1737 cm^{-1}. ^{1}H NMR data ($CDCl_3$, 700 MHz): δ 7.56 (1H, d, *J* = 2.2 Hz, H-9), 7.05 (1H, s, H-5), 6.66 (1H, d, *J* = 2.2 Hz, H-10), 3.00 (2H, m, H-4), 2.65 (2H, m, H-3). ^{13}C NMR ($CDCl_3$, 175 MHz): δ 173.7 (C, C-2) 146.9 (C, C-8a), 145.8 (C, C-7), 144.9 (CH, C-7), 138.4 (C, C-8), 130.0 (C, C-4a), 124.9 (C, C-6), 114.3 (CH, C-5), 106.6 (CH, C-10), 61.3 (CH_3, 8a-OCH_3), 60.6 (CH_3, 8-OCH_3), 51.6 (CH_3, $COO\underline{CH_3}$), 35.1 (CH_2, C-3), 26.2 (CH_2, C-4). EIMS *m*/*z* 264 $[M]^+$ (100), 207 (53), 191 (26), 190 (23), 176 (19), 161 (11), 147 (16). HR-EIMS *m*/*z* 264.0999 $[M]^+$ (calcd for $C_{14}H_{16}O_5$, 264.0998).

BACE1 Enzyme Assay. The assay was carried out according to the supplied manual with modifications (*16*). Briefly, a mixture of 10 μL of assay buffer (50 mM sodium acetate, pH 4.5), 10 μL of BACE1 (1.0 U/mL), 10 μL of the substrate (750 nM Rh-EVNLDAEFK-Quencher in 50 mM ammonium bicarbonate), and 10 μL of sample dissolved in 30% DMSO was incubated for 60 min at room temperature in the dark. The mixture was irradiated at 550 nm, and the emission intensity at 590 nm was recorded. The inhibition ratio was obtained by the following equation:

$$\text{inhibition}\ (\%) = [1 - \{(S - S_0)/(C - C_0)\}] \times 100$$

where C was the fluorescence of the control (enzyme, buffer, and substrate) after 60 min of incubation, C_0 was the fluorescence of control at zero time, S was the fluorescence of the tested samples (enzyme, sample solution, and substrate) after incubation, and S_0 was the fluorescence of the tested samples at zero time. To allow for the quenching effect of the samples, the sample solution was added to the reaction mixture C, and any reduction in fluorescence by the sample was then investigated. All data are the means of three experiments. 6,7-Furano-8a-methoxy-5-prenyloxy hydrocoumaric acid methyl ester was used as a positive control (*16*).

RESULTS AND DISCUSSION

Biotransformation of Bergapten (1) with *G. cingulata*. To clarify the time course of the microbial transformation of bergapten (**1**) by *G. cingulata*, a small amount of **1** was incubated for 7 days, and one metabolite was detected by TLC and HPLC. The time course of metabolite was measured by HPLC. The time course of biotransformation of **1** is shown in **Figure 2**. In this system, **1** was converted to **2** in 83% yield for 7 days (**Figure 2**). To isolate the metabolite, a large-scale incubation of **1** using *G. cingulata* was carried out for 7 days. After the biotransformation, the culture was extracted as described in the Materials and Methods, and methylated metabolite **2** (compound **6**) was isolated from the EtOAc extract. Metabolite **2** was obtained by the hydrolysis of **6**. The structures of these compounds were determined by spectral data.

HR-FABMS of compound **2** showed $[M + H]^+$ peaks at m/z 237.0746 (calcd for $C_{12}H_{13}O_5$, 237.0762), which established a molecular formula of $C_{12}H_{12}O_5$. The presence of a broad absorption band at 3465 cm^{-1} and a strong absorption band at 1705 cm^{-1} in the IR spectrum suggested the conversion of the original bergpten lactone into phenol and carboxylic acid functionalities. The ^{13}C NMR spectrum showed 12 resonances distributed as one primary, two secondary, three tertiary, and six quaternary carbons. The conversion of the original two olefinic doublets (C-3, δ_C 112.5, and C-4, δ_C 139.2) into two aliphatic secondary carbons (C-3, δ_C 34.4, and C-4, δ_C 19.9) indicated the reduction of the C-3,4 double bond. Except for the downfield shift of C-2 and C-4a, the ^{13}C resonances remained the same as those of the starting material (**Table 1**). Furthermore, the ^1H NMR spectrum showed the conversion of two doublets (δ 6.27, d, J = 9.8 Hz, H-3, and δ 8.15, d, J = 9.8 Hz, H-4) into two multiplets at δ 2.52−2.55 (H-3) and δ 2.96−2.99 (H-4) (**Table 2**). Thus, the chemical structure of metabolite **2** was established as 6,7-furano-5-methoxy hydrocoumaric acid, a new compound. The protons and carbon assignments were unambiguously made from the H−H correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), and heteonuclear multiple bond coherence (HMBC) spectra.

Biotransformation of Xanthotoxin (3) with *G. cingulata*. To clarify the time course of the microbial transformation of xanthotoxin (**3**) by *G. cingulata*, a small amount of **3** was incubated for 7 days. Two metabolites were detected by TLC and HPLC. The time course of metabolites was measured by HPLC. In this system, **3** was converted to **4** and **5** in 61 and 26% yields for 7 days, respectively (**Figure 3**). To isolate this metabolite, a large-scale incubation of **3** by *G. cingulata* was done for 7 days. After the biotransformation, the culture was extracted as described in the Materials and Methods, and metabolites **4** and **5** were isolated from the EtOAc extract.

HR-FABMS of compound **4** showed $[M + H]^+$ peaks at m/z 237.0746 (calcd for $C_{12}H_{13}O_5$, 237.0762), which established a molecular formula of $C_{12}H_{12}O_5$. The presence of a broad absorption band at 3393 cm^{-1} and a strong absorption band at 1696 cm^{-1} in the IR spectrum suggested the conversion of the original xanthotoxin lactone into phenol and carboxylic acid

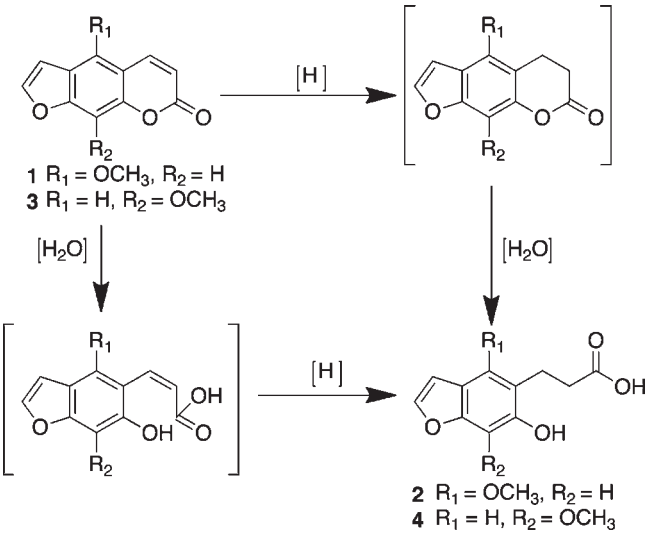

Figure 4. Possible metabolic pathways of corresponding reduced acid from bergapten (**1**) and xanthotoxin (**3**) by *G. cingulata*, respectively.

functionalities. The ^{13}C NMR spectrum showed 12 resonances distributed as one primary, two secondary, three tertiary, and six quaternary carbons. The conversion of original two olefinic doublets (C-3, δ_C 114.7, and C-4, δ_C 144.3) into two aliphatic secondary carbons (C-3, δ_C 34.6, and C-4, δ_C 27.0) indicated the reduction of the C-3,4 double bond. Except for the downfield shift of C-2 and C-4a, the ^{13}C resonances remained the same as those of the starting material (**Table 1**). Furthermore, the ^1H NMR spectrum showed the conversion of two doublets (δ 6.38, d, J = 9.8 Hz, H-3, and δ 7.77, d, J = 9.8 Hz, H-4) into two multiplets at δ 2.61−2.64 (H-3) and δ 2.95−2.98 (H-4) (**Table 2**). Thus, the chemical structure of metabolite **4** was established as cnidiol b, 6,7-furano-8-methoxy hydrocoumaric acid (*17*). The structure of **5** was shown to be identical with xanthotoxol (**5**), which is the demethylated product of **3** by comparison of its spectroscopic data (*18*, *19*).

Previously, we had incubated furanocoumarins isoimperatorin, with an prenyloxy grup at C_5 position, and imperatorin, with an prenyloxy group at the C_8 position, by *G. cingulata* (*16*). Isoimperatorin was metabolized by *G. cingulata* to give the corresponding reduced acid in high yield. By contrast, biotransformation of imperatorin by *G. cingulata* offered the dealkylated metabolite, xanthotoxol (**5**), in high yield. In the biotransformation of bergapten (**1**) and xanthotoxin (**3**), fungal reduction progressed not only bergapten (**1**) but also xanthotoxin (**3**). In addition, dealkylated product was produced as a minor metabolite in the biotransformation of **3**. The reduction or dealkylated products of **3** identified in this study were also isolated in the previous biotransformation of **3** by the fungus *Gibberella pulicars* (*20*). The *G. pulicars*, along with the goat (*21*), are apparently the only organisms thus far known to metabolize xanthotoxin (**3**) by a reductive mechanism, leading to the corresponding reduced acid. Metabolites **2** and **4** were probably formed in a manner analogous to that of melilotic acid, a metabolite of coumarin. The work of Häser (*22*) indicated that due to the occurrence of the intermediates dihydrocoumarin and *o*-coumaric acid, two metabolic pathways operate in the biotransformation of coumarin by the fungus *Pseudomonas orientalis* and *Bacillus cereus*; that is, coumarin converted to dihydrocoumarin by hydrogenation or *o*-coumaric acid by opening of the lactone ring, which is then converted to melolitic acid by hydrolysis or hydrogenation, respectively. Therefore, the fungal reduction of **1** and **2** presumably

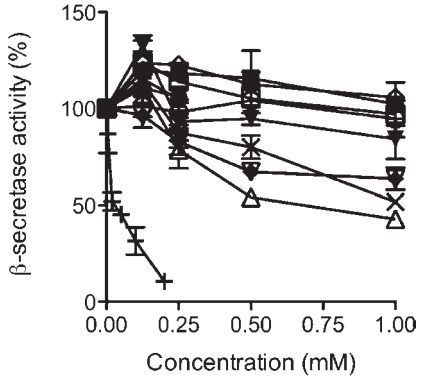

Figure 5. β-Secretase inhibitory activity of bergapten and xanthotoxin derivatives **1**−**11**. The bar is the mean ± SD of three experiments. Key: **1** (●), **2** (■), **3** (▲), **4** (▼), **5** (♦), **6** (○), **7** (□), **8** (△), **9** (▽), **10** (◇), **11** (×), and 6,7-furano-8a-methoxy-5-prenyloxy hydrocoumaric acid methyl ester as positive control (+).

proceeds by hydrogenation of the 3,4-double bond followed by opening of the lactone ring or opening of the lactone ring followed by, or concomitant with, reduction (**Figure 4**). However, intermediate metabolites in each metabolic pathways were not detected in our biotransformation experiments. As the results show, *G. cingulata* have the abilities to reduction of α,β-unsaturated lactone on furanocoumarin with an alkoxy group at the C_5 position, and dealkylation or reduction of 8-alkoxyfuranocoumarin, depending on the kind of alkoxy group at the C_8 position. Because there are no reports on microbial transformation of begapten (**1**), the data on the microbial transformation product metabolite may be used for further pharmacological evaluation of **1**.

Subsequently, the BACE1 inhibitory effects of all of these compounds were evaluated to search for potential novel anti-Alzheimer agents. Results of evaluation of BACE1 inhibitory activity established that compounds **8**, **9**, and **11** inhibited 57.2 ± 0.4, 36.4 ± 3.3, and 48.3 ± 1.2% of the BACE1 activity at a concentration of 1 mM (**Figure 5**), and the IC_{50} value of **8** was 0.64 ± 0.04 mM. The BACE1 inhibitory effects of compounds **8** and **11** were stronger than those of **2**, **6**, **4**, and **9**, indicating the presence of methyl ether and ester, which are important for the inhibitory activity. Similar effects of modification at 8a-OH and carboxylic acid were reported previously (*16*).

In conclusion, one metabolite **2** was obtained from the incubation of **1** with *G. cingulata*, and two metabolites were obtained from the incubation of **3**. Compound **2** has not previously been reported. The results indicated that *G. cingulata* possesses the characteristics of reaction on the furanocoumarin skeleton as we previously reported (*16*). The reactions involved the reduction of the α,β-unsaturated lactone ring on furanocoumarin. In addition, results showed that methyl ester and ether derivatives of metabolites **8** and **11** exhibited BACE1 inhibitory activity. This investigation also demonstrates that biotransformation is a powerful tool for the structural modification of natural products.

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