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Biotransformation of isoimperatorin and imperatorin by *Glomerella cingulata* and β-secretase inhibitory activity

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ABSTRACT

Biotransformation studies conducted on the furanocoumarins isoimperatorin (**1**) and imperatorin (**3**) have revealed that **1** was metabolized by *Glomerella cingulata* to give the corresponding reduced acid, 6,7-furano-5-prenyloxy hydrocoumaric acid (**2**), and **3** was transformed by *G. cingulata* to give the dealkylated metabolite, xanthotoxol (**4**) in high yields (83% and 81%), respectively. The structures of the new compound **2** have been established on the basis of spectral data. The metabolites **2** and **4** were tested for the β-secretase (BACE1) inhibitory activity in vitro, and metabolite **2** slightly inhibited the β-secretase activity with an IC_{50} value of 185.6 ± 6.8 μM. The metabolite **4** was less potent activity than compounds **1–3**. In addition, methyl ester (**2Me**), methyl ether (**2a**) and methyl ester and ether (**2aMe**) of **2** were synthesized, and investigated for the ability to inhibit β-secretase. Compound **2aMe** exhibited the best β-secretase inhibitory activity at the IC_{50} value 16.2 ± 1.2 μM and found to be the **2aMe** showed competitive mode of inhibition against β-secretase with K_i value 11.3 ± 2.8 μM.

1. Introduction

Furanocoumarins are abundant in citrus fruits, umbelliferous vegetables, and certain herbal medicines.[1] Interest in these compounds has long been limited to psoralen, the mainstay of photodynamic therapy, but over the past few years there has been a growing interest in its prenylated derivatives spurred by the potent activity on drug metabolism of dietary compounds such as bergamottin and its dimeric analogs. While bergamottin is mainly contained in citrus plants, its isomers isoimperatorin (**1**) and imperatorin (**3**) are more widespread, occurring not only in lemon and lime oils, but also in the medicinal plant *Angelica dahurica* and its popular culinary herbs such as parsnip, parsley, and fennel.[2] Despite its occurrence in edible plants, isoimperatorin (**1**) and imperatorin (**3**) shows potent pharmacological activity and has been studied for its anti-inflammatory and antitumoral activities.[3] Recently, we reported the β-secretase (BACE1) inhibitory activity of isoimperatorin (**1**) and imperatorin (**3**).[4]

Alzheimer's disease (AD) has become an increasingly severe medical and social problem, due to the rapid growth of aging people populations in industrialized countries, and even in some developing countries. The β-secretase (BACE1) has been recognized as a valuable target for the treatment of AD. The BACE1 inhibitors have promised to be a class of disease disrupting rather than symptom relieving agents.[5] Although many peptides, peptidomimetics and heterocyclic compounds have been designed and evaluated as BACE inhibitors, none of them have been successfully developed as anti-AD agents to date.[5] There are only very few reports on natural products-based BACE inhibitors.[4,6]

Biotransformation is an alternative tool in the structural modification of complex natural products due to its great capabilities to catalyze novel reactions and its regio- and stereo-selectivity. Microorganisms are well known as efficient and selective catalysts. In previous papers, we reported the biotransformation of some complex natural products and obtained a series of new products.[7] Therefore, it is envisioned that biotransformation of isoimperatorin (**1**) and imperatorin (**3**) may provide some analogues which could be utilized for screening for new activities or better activity/side effects profile. By studying the β-secretase inhibitory activities of obtained products, we can explore the structure–activity relationships of such compounds.

2. Results and discussion

2.1. The biotransformation of isoimperatorin (1) with *Glomerella cingulata*

G. cingulata is widely distributed in the world, infecting various plants and causing anthracnose. We investigated the microbial transformation of terpenoids and flavonoids using *G. cingulata*.[8] Then we tried the microbial transformation of furanocoumarins **1** and **3** using *G. cingulata*.

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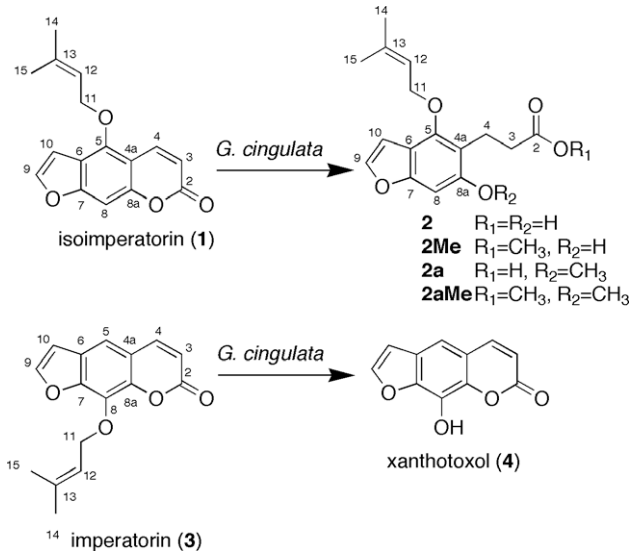

Figure 1. Biotransformation of isoimperatorin (**1**) and imperatorin (**3**), and chemical structures of all the compounds.

To clarify the time course of the microbial transformation of isoimperatorin (**1**) by *G. cingulata*, a small amount of **1** was incubated for 5 days, and one metabolite was detected by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). The time course of metabolite was measured by HPLC. Figure 2 shows the time course of biotransformation of **1** and the pH gradient. The pH value in the biotransformation changed from 4.7 to 6.2. In this system, **1** was transformed to **2** at the conversion rate of 83% for 5 days. To isolate the metabolite, a large-scale incubation of **1** using *G. cingulata* was carried out for 5 days. After the biotransformation, the culture was extracted as described in Materials and methods section and methylated metabolite **2** (**2Me**) was isolated from the EtOAc extract. Metabolite **2** was obtained by the hydrolysis of **2Me**. The structures of these compounds were determined by spectral data.

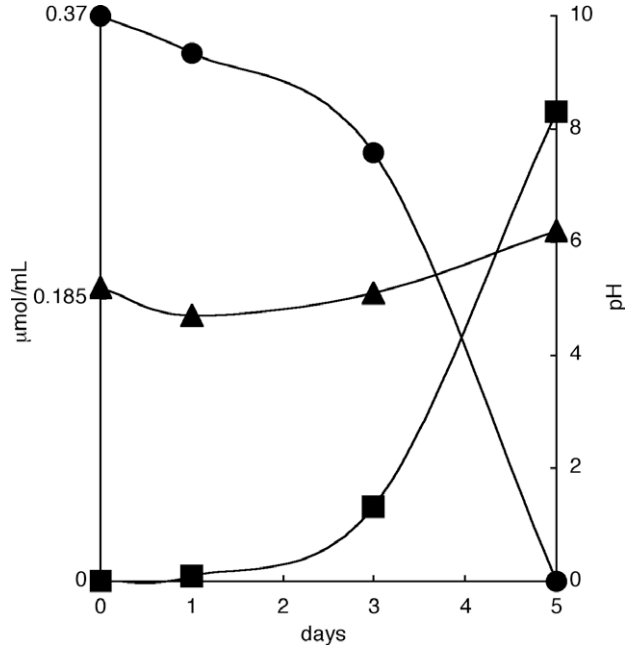

Figure 2. Time course in the biotransformation of **1** by *G. cingulata*: (●) isoimperatorin (**1**); (■) Metabolite **2**; (▲) pH. The obtained sample solution was analyzed under the following conditions: RP-18 GP (4.6 μm*f* × 150 μm), solvent: water and acetonitrile (50:50), detection: UV 254 nm, flow: 0.5 ml/min.

HRFABMS of compound **2** showed $[M+H]^+$ peaks at *m/z* 291.1209 (calcd for *m/z* 291.1231), which established a molecular formula of $C_{16}H_{19}O_5$. The presence of a broad absorption band at 3335 cm^{-1} and a strong absorption band at 1704 cm^{-1} in the IR spectrum suggested the conversion of the original isoimperatorin lactone into phenol and carboxylic acid functionalities. The ^{13}C NMR spectrum showed 16 resonances distributed as two primary, three secondary, four tertiary and seven quaternary carbons. The conversion of original two olefinic doublets (C-3, δ_C 112.5 and C-4, δ_C 139.5) into two aliphatic secondary carbons (C-3, δ_C 34.6 and C-4, δ_C 20.1) indicated the reduction of the C-3,4 double bond. Except for the downfield shift of C-2 and upfield shift of 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 8.16, d, J = 9.8 Hz, H-4) into two multiplets at δ 2.97–3.00 (H-3) and δ 2.52–2.55 (H-4). Thus, chemical structure of metabolite **2** was established as 6,7-furano-5-prenyloxy hydrocoumaric acid, a new compound. The protons and carbons assignments were unambiguously made from the H–H COSY, HMQC and HMBC spectra.

2.2. The biotransformation of imperatorin (3) with *G. cingulata*

To clarify the time course of the microbial transformation of imperatorin (**3**) by *G. cingulata*, a small amount of **3** was incubated for 12 days. One metabolite was detected by TLC and HPLC. The time course of metabolites was measured by HPLC. Figure 3 shows the time course of biotransformation of **3** and the pH gradient. The pH value in the biotransformation changed from 5.1 to 6.9. In this system, **3** was transformed to **4** at the conversion rate of 81% for 12 days. To isolate this metabolite, a large-scale incubation of **3** by *G. cingulata* for 12 days. After the biotransformation, the culture was extracted as described in Experimental section and metabolite **4** was isolated from the EtOAc extract. The structure of **4** was shown to be identical with xanthotoxol (**4**) which is the dealkylated product of **3** by comparison of its spectroscopic data.[9]

The biotransformation pathway is illustrated in Figure 1. Through the previous works,[8] we guessed that prenyloxy side-chain group in **1** could be oxidized or hydrolyzed by *G. cingulata*. Although the expected products were not obtained, *G. cingulata* used in this work did possess ability to reduction of α,β-unsaturated lactone on isoimperatorin (**1**). Fugal reduction of **1** probably proceeds by opening of the lactone ring followed by isomerization and reduction or by hydrogenation of the 3,4-double bond fol-

Table 1
The ^{13}C and ^1H NMR spectroscopic data of compounds **1** and **2**[a]

Atom	1		2	
	δ_C	δ_H	δ_C	δ_H
2	161.3 (C)[b]		175.5 (C)	
3	112.5 (CH)	6.27 1H, d, (9.8)[c]	34.6 (CH_2)	2.97–3.00 2H, m
4	139.5 (CH)	8.16 1H, d, (9.8)	20.1 (CH_2)	2.52–2.55 2H, m
4a	107.5 (C)		115.6 (C)	
5	148.9 (C)		151.5 (C)	
6	114.2 (C)		112.4 (C)	
7	158.5 (C)		156.4 (C)	
8	94.2 (CH)	7.15 1H, s	93.9 (CH)	6.90 1H, d (1.1)
8a	152.6 (C)		155.0 (C)	
9	144.8 (CH)	7.59 1H, d, (2.3)	143.2 (CH)	7.56 1H, d (2.3)
10	105.0 (CH)	6.95 1H, d, (2.3)	105.5 (CH)	6.73 1H, dd (2.3, 1.1)
11	69.7 (CH_2)	4.92 2H, d, (6.9)	69.8 (CH_2)	4.76 2H, d (6.8)
12	119.1 (CH)	5.52–5.54 1H, m	121.6 (CH)	5.54–5.56 1H, m
13	139.8 (C)		138.0 (C)	
14	25.8 (CH_3)	1.80 3H, s	25.7 (CH_3)	1.75 3H, d (0.7)
15	18.2 (CH_3)	1.70 3H, s	18.1 (CH_3)	1.68 3H, br s

[a] Spectra for **1** and **2** were recorded in $CDCl_3$ and acetone-d_6, respectively.
[b] ^{13}C multiplicities were determined by DEPT 135°.
[c] The *J* values are in Hertz in parentheses.

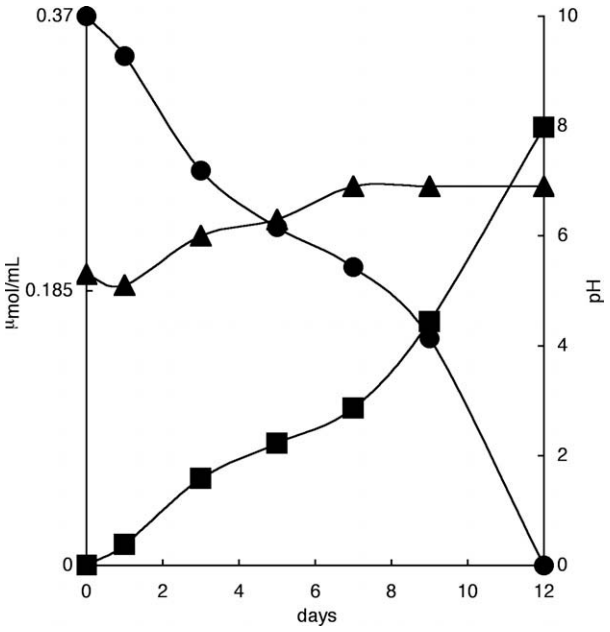

Figure 3. Time course in the biotransformation of **3** by *G. cingulata*: (●) imperatorin (**3**); (■) metabolite **4**; (▲) pH. The obtained sample solution was analyzed under the following conditions: RP-18 GP (4.6 μm*f* × 150 μm), solvent: water and acetonitrile (50:50), detection: UV 254 nm, flow: 0.5 ml/min.

lowed by, or concomitant with, opening of the lactone ring. However, intermediary metabolites in each pathway were not observed during the time course experimental. On the other hand, biotransformation of **3** by *G. cingulata* produced metabolite hydrolyzed at prenyloxy side-chain. In previously report,[10] imperatorin (**3**) was metabolized by *Aspergillus flavus* to give five products, which main compound was specifically oxidized at the C-14 and C-15 positions, in addition on the cleavage of the prenyloxy side-chain, and xanthotoxol was produced as minor metabolite. Surprisingly, biotransformation of imperatorin (**3**) by *G. cingulata* was selectivity offered the one metabolite, xanthotoxol (**4**), in high yield. In conclusion, the incubation of isoimperatorin (**1**) and imperatorin (**3**) with *G. cingulata* were produced the one metabolic product in high yields (83% and 81%), respectively. In the bioconversion of isoimperatorin (**1**) and imperatorin (**3**) which are different the position of prenyloxy side-chain combined to C-5 or C-8 at furanocoumarin skeleton by *G. cingulata*, there was a difference in the metabolic pathway.

All compounds inhibited β-secretase (BACE1) in a concentration-dependent manner (Fig. 4). The BACE1 inhibitory activities of compounds **1**–**4** in addition methyl ester (**2Me**), methyl ether (**2a**) and methyl ester and ether (**2aMe**) of **2** were determined the IC_{50} values, respectively. Among them, compound **2aMe** showed the best β-secretase inhibitory activity at IC_{50} value 16.2 ± 1.2 μM. Compounds **1**, **2**, **3**, **2Me** and **2a** showed inhibitory activity against β-secretase with IC_{50} values 244.2 ± 8.2, 185.6 ± 6.8, 91.8 ± 7.5, 30.5 ± 2.7 and 203.3 ± 4.1 μM, respectively. Metabolite **4** was found to be inactive. The inhibitory activity of metabolite **2**, with corresponding reduced acid of **1**, was slightly stronger than that of substrate **1**. The activities of compounds **2Me** and **2aMe**, with a carboxylic acid methyl ester, showed the increased inhibitory effects, whereas in the latter, an additional methyl ether group seems to have no significant influence. This evidence was confirmed by comparing the structure and activities of **2** and **2a**. From the kinetics analysis (Fig. 5), compound **2aMe** was estimated as being a competitive-type inhibitor against β-secretase with K_i value 11.3 ± 2.8 μM.

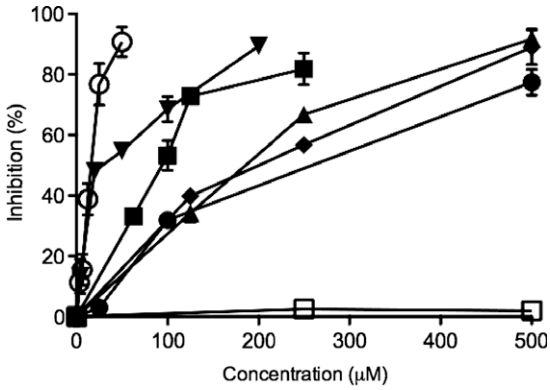

Figure 4. Inhibition of β-secretase activity by compounds **1**–**4**. -●- Isoimperatorin (**1**); -▲- 6,7-furano-5-prenyloxy hydrocoumaric acid (**2**); -▼- 6,7-furano-5-prenyloxy hydrocoumaric acid methyl ester (**2Me**); -♦- 6,7-furano-8a-methoxy-5-prenyloxy hydrocoumaric acid methyl ester (**2a**); -○- 6,7-furano-8a-methoxy-5-prenyloxy hydrocoumaric acid methyl ester (**2aMe**); -■- imperatorin (**3**); -□- xanthotoxol (**4**).

3. Materials and methods

3.1. 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 (3:7). Compounds were visualized by spraying plates with 1% vanillin in 96% H_2SO_4 followed by brief heating. High performance liquid chromatography (HPLC) analysis with a LC-CCPS system (Tosoh, Tokyo) with a spectrometer UV-8020 (Tosoh, Tokyo). Separation was done with a C18 5-μm analytical column (Mightsil RP-18, 150 × 4.6 μm, Kanto Chemical, Tokyo) equipped with a C18 5-μm guard column (Mightsil RP-18, 5–4.6 μm, Kanto Chemical), mobile phase was 50% acetonitrile. EIMS, HREIMS, FAB-MS and HRFABMS were obtained on a JEOL the Tandem Ms station JMS-700 TKM. NMR spectra were recorded at 500 MHz for ^1H and 125 MHz for ^{13}C on a JEOL ECA-500 spectrometer. Tetramethylsilane (TMS) was used as the internal standard reference (δ, 0.00) for ^1H NMR spectra measured in $CDCl_3$. IR spectra were determined with a JASCO FT/ IR-470 plus Fourier transform infrared spectrometer. BACE1 (recombinant human BACE1) assay kit was purchased from the PanVera Co., USA.

3.2. Chemicals

Isoimperatorin (**1**) and imperatorin (**3**) were isolated from *A. dahurica* as the β-secretase inhibitors.[4]

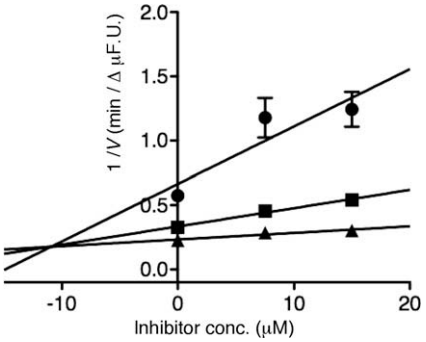

Figure 5. Dixon plots for inhibition of compound **2aMe** on β-secretase for the proteolysis of substrate. In the presence of different concentration of substrate: 750 nM (●), 500 nM (■) and 250 nM (▲).

3.3. Preculture of *G. cingulata*

Spores of *G. cingulata* (the strain isolated from diseased grape was a gift from Dr. M. Hyakumachi, Gifu University, Gifu, 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, 100 rpm) at 27 °C for 3 days.

3.4. 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. 100 rpm) for 3 days. After the growth of *G. cingulata*, **1** (10 mg, 37 μmol) and **3** (10 mg, 37 μmol) in 0.5 mL of dimethyl sulfoxide (DMSO) was added into the medium, respectively, and cultivated for 5 (for **1**) or 12 (for **3**) 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.

3.5. Preparative biotransformation of isoimperatorin (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. 100 rpm) for 3 days. After the growth of *G. cingulata*, 50 mg of **1** in 1.0 mL of dimethyl sulfoxide (DMSO) was added into the medium and cultivated for an additional 5 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.

3.6. Isolation of metabolite in 1

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 (320 mg) was obtained. The extract was distributed between 5% $NaHCO_3$ aq and EtOAc, and EtOAc phase was evaporated to give the neutral fraction (112 mg). No metabolic compounds were detected from neutral fraction by TLC and HPLC. The alkali phase was acidified to pH 3 with 2 N HCl and distributed between water and EtOAc. The EtOAc phase was evaporated, and the acidic fraction (268 mg) was obtained. The acidic fraction was dissolved in CH_2Cl_2 (1 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 open-column chromatography (Silica Gel 60, 230–400 mesh, Merck) with a hexane–EtOAc gradient (9:1–1:9) to yield compound **2Me** (41 mg, 73%). Compound **2Me** (20 mg) was dissolved in MeOH (0.3 mL), 5% NaOH (1 mL) added to the solution, and the solution was refluxed for 30 min. The solution was acidified to pH 3 with 2 N HCl and distributed between EtOAc and water. The EtOAc phase was evaporated to give **2** (16 mg).

3.6.1. 6,7-Furano-5-prenyloxy hydrocoumaric acid (2)

White powder; IR (KBr) ν_{max} 3335, 1704, 1622, 1599 cm^{-1}; ^1H and ^{13}C NMR shown as Table 1; HRFABMS (pos) *m*/*z* 291.1209 $[M+H]^+$ (calcd for $C_{16}H_{19}O_5$, 291.1231).

3.6.2. 6,7-Furano-5-prenyloxy hydrocoumaric acid methyl ester (2Me)

Pale yellow oil; IR (film) ν_{max} 3367, 1735, 1622, 1599 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 7.78 (1H, br s, OH), 7.41 (1H, d, J = 2.3 Hz, H-9), 6.82 (1H, d, J = 0.8 Hz, H-8), 6.77 (1H, dd, J = 2.3, 0.8 Hz, H-10), 5.50–5.54 (1H, m, H-12), 4.77 (2H, d, J = 6.9 Hz, H-11), 3.66 (3H, s, $COOCH_3$), 2.98–2.95 (2H, m, H-4), 2.75–2.73 (2H, m, H-3), 1.79 (3H, d, J = 0.85 Hz, H-14), 1.70 (3H, br s, H-15); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 177.4 (C, C-2) 155.9 (C, C-7), 153.2 (C, C-8a), 150.5 (C, C-5), 142.4 (CH, C-9), 137.8 (C, C-13), 120.3 (CH, C-12), 114.3 (C, C-4a), 111.7 (C, C-6), 104.6 (CH, C-10), 95.1 (CH, C-8), 68.9 (CH_2, C-11), 52.2 (CH_3, $COOCH_3$), 34.1 (CH_2, C-3), 25.9 (CH_3, C-14), 18.6 (CH_2, C-4), 18.1 (CH_3, C-15); EIMS *m*/*z* 304 $[M]^+$(4), 236 (28), 204 (100), 176 (12), 162 (71), 64 (46), 41 (19); HREIMS *m*/*z* 304.1293 $[M]^+$ (calcd for $C_{17}H_{20}O_5$, 304.1311).

3.6.3. 6,7-Furano-8a-methoxy-5-prenyloxy hydrocoumaric acid (2a)

Compound **2aMe** (20 mg, 0.06 mmol) was dissolved in MeOH (0.5 mL), 5% NaOH (1 mL) added to the solution, and the solution was refluxed for 30 min. The solution was acidified with 2 N HCl and distributed between EtOAc and water, The EtOAc phase was evaporated and purified by silica-gel open-column chromatography (Silica Gel 60, 230–400 mesh, Merck) with a hexane–EtOAc gradient (9:1–1:1) to yield compound **2a** (15 mg, 74%) obtained as colorless powder; IR (KBr) ν_{max} 1706, 1620, 1590 cm^{-1}; ^1H NMR data ($CDCl_3$, 500 MHz) δ 7.45 (1H, d, J = 2.3 Hz, H-9), 6.79 (1H, dd, J = 2.3, 0.8 Hz, H-10), 6.78 (1H, br s, H-8), 5.54–5.57 (1H, m, H-12), 4.70 (2H, d, J = 6.8 Hz, H-11), 3.84 (3H, s, OCH_3), 3.08–3.05 (2H, m, H-4), 2.60–2.56 (2H, m, H-3), 1.78 (3H, br s, H-14), 1.69 (3H, br s, H-15); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 177.8 (C, C-2) 156.6 (C, C-7), 155.6 (C, C-8a), 150.3 (C, C-5), 142.5 (CH, C-9), 138.1 (C, C-13), 120.3 (CH, C-12), 115.3 (C, C-4a), 112.4 (C, C-6), 104.6 (CH, C-10), 89.5 (CH, C-8), 69.5 (CH_2, C-11), 55.8 (CH_3, OCH_3), 33.7 (CH_2, C-3), 25.7 (CH_3, C-14), 19.2 (CH_2, C-4), 18.1 (CH_3, C-15); EIMS *m*/*z* 304 $[M]^+$(5), 286 (86), 271 (47), 218 (60), 176 (100), 69 (24), 41 (18); HREIMS *m*/*z* 304.1295 (calcd for $C_{17}H_{20}O_5$, 304.1310).

3.6.4. 6,7-Furano-8a-methoxy-5-prenyloxy hydrocoumaric acid methyl ester (2aMe)

A solution of compound **2Me** (50 mg, 0.16 mmol) in *N*,*N*-dimethylformamide (DMF, 0.5 mL) was treated with methyl iodide (0.3 mmol) in the presence of sodium hydride (0.3 mmol) and mixture was stirred at room temperature for 12 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, then dried over Na_2SO_4 and the filtrate under reduced pressure furnished a residue, which was purified by silica-gel open-column chromatography (Silica Gel 60, 230–400 mesh, Merck) with a hexane–Et_2O gradient (1:0–7:3) to yield compound **2aMe** (43 mg, 82%) was obtained as pale yellow powder; IR (KBr) ν_{max} 1736, 1682, 1552 cm^{-1}; ^1H NMR data ($CDCl_3$, 500 MHz) δ 7.45 (1H, d, J = 2.2 Hz, H-9), 6.78 (1H, dd, J = 2.2, 0.8 Hz, H-10), 6.77 (1H, br s, H-8), 5.53–5.56 (1H, m, H-12), 4.68 (2H, d, J = 6.8 Hz, H-11), 3.83 (3H, s, OCH_3), 3.68 ($OOCH_3$), 3.06–3.03 (2H, m, H-4), 2.54–2.50 (2H, m, H-3), 1.78 (3H, br s, H-14), 1.68 (3H, br s, H-15); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 174.1 (C, C-2) 156.6 (C, C-7), 155.5 (C, C-8a), 150.3 (C, C-5), 142.4 (CH, C-9), 137.9 (C, C-13), 120.4 (CH, C-12), 115.6 (C, C-4a), 112.5 (C, C-6), 104.6 (CH, C-10), 89.5 (CH, C-8), 69.5 (CH2, C-11), 55.8 (CH_3, OCH_3), 51.3 (CH_3, $COOCH_3$), 34.0 (CH_2, C-3), 25.7 (CH_3, C-14), 19.4 (CH_2, C-4), 18.0 (CH_3, C-15); EIMS *m*/*z* 318 $[M]^+$(3), 286 (7), 250(63), 218 (82), 176 (100), 69 (21), 41 (16); HREIMS *m*/*z* 318.1482 (calcd for $C_{18}H_{22}O_5$, 318.1467).

3.7. Preparative biotransformation of imperatorin (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. 100 rpm) for 3 days. After the growth of *G. cingulata*, 50 mg of **3** in 1.0 mL of dimethyl sulfoxide (DMSO) was added into the medium and cultivated for an additional 12 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.

3.8. Isolation of metabolite in 3

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 (375 mg) was obtained. The extract was subjected to silica-gel open-column chromatography (Silica Gel 60, 230–400 mesh, Merck) with a hexane–Et_2O gradient (9:1–3:7) to yield metabolite **4** (24 mg, 64%).

3.8.1. Xanthoxol (4)

yellowish needles: IR (KBr) ν_{max} 3324, 1705, 1594 cm^{-1}; ^1H 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, H10), 3.35 (1H, br s, OH); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 160.2 (C, C-2) 147.6 (CH_2, C-9), 145.7 (CH_2, C-4), 145.1 (C, C-7), 139.9 (C, C-8a), 130.3 (C, C-8), 125.4 (C, C-6), 116.4 (C, C-4a), 114.0 (CH_2, C-3), 110.2 (CH_2, C-5), 107.2 (CH_2, C-3′); EIMS m/z 202 $[M]^+$(100), 174 (63), 149 (10), 146 (9), 89 (15); HREIMS m/z 202.0262 $[M]^+$ (calcd for $C_{11}H_6O_4$, 202.0325).

3.9. β-Secretase (BACE1) enzyme assay

The assay was carried out according to the supplied manual with modifications.[4,10] 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 mean of three experiments.

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