Tetrahedron 67 (2011) 495–500

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Microbial reduction of coumarin, psoralen, and xanthyletin by *Glomerella cingulata*

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ARTICLE INFO

Article history:
Received 24 August 2010
Received in revised form 27 October 2010
Accepted 29 October 2010
Available online 5 November 2010

Keywords:
Biotransformation
Coumarin
Psoralen
Xanthyletin
Glomerella cingulata

ABSTRACT

Microbial transformation of coumarin, psoralen, and xanthyletin was performed with the fungus *Glomerella cingulata*. The main reaction pathways involved reduction at α,β-unsaturated δ-lactone ring on coumarin analogue. Coumarin was metabolized by *G. cingulata* to give the corresponding reduced acid, hydrocoumaric acid. In the biotransformation of psoralen, two reduced metabolites, 6,7-furano-hydrocoumaric acid, and 6,7-furano-*o*-hydrocoumaryl alcohol were isolated from the incubation of psoralen. Xanthyletin was converted to reduced products 9,9-dimethyl-6,7-pyrano-hydrocoumaric acid and 9,9-dimethyl-6,7-pyrano-o-hydrocoumaryl alcohol by *G. cingulata*. The structures of the new compounds were characterized using spectroscopic techniques. In addition, all of compounds including methyl ester derivatives of the metabolites were tested for the β-secretase (BACE1) inhibitory activity in vitro. 6,7-Furano-hydrocoumaric acid methyl ester was shown to possess BACE1 inhibitory activity, and an IC_{50} value was 0.84 ± 0.06 mM.

1. Introduction

Coumarins are widely distributed in nature and are found in all parts of plants.[1] These compounds are especially common in grasses, orchids, citrus fruits, and legumes.[1,2] Being so abundant in nature, coumarins make up an important part of human diet. Based on chemical structure (Fig. 1), they can be broadly classified as (a) simple coumarins (e.g., coumarin, **1**), (b) furanocoumarins of the linear (e.g., psoralen, **3**) or angular (e.g., angelicine) type, and (c) pyranocoumarins of the linear (e.g., xanthyletin, **6**) or angular (e.g., seselin) type.[1] Simple coumarins are very widely distributed in the plant kingdom.[1] Interestingly, citrus oils, in particular, contain abundant amounts of both simple as well as furanocoumarins.[3] Humans are also exposed to furanocoumarins (e.g., bergapten and xanthotoxin) in umbelliferous vegetables, such as parsnips, celery, and parsley in substantial amounts.[4] A number of furanocoumarins act as inhibitors of drug metabolizing enzymes. 6′,7′-Dihydroxybergamottin and related furanocoumarins dimmers found, e.g., in grapefruit juice act as highly potent inhibitors of cytochrome P450 (CYP) 3A and other CYP isozymes affecting drug metabolism.[5] Furthermore, furanocoumarins were the inhibitors of the β-secretase (BACE1).[6]

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.[7] Microorganisms are well known as efficient and selective catalysts. It is anticipated that the microbial metabolism of coumarins would produce significant quantities of metabolites that would be difficult to obtain from chemical synthesis or either animal systems. Moreover, this may provide some novel metabolites that may serve as starting compounds for semi-synthesis of other derivative, or as analytical standards for mammalian metabolic studies. Previously, we studied the microbial transformation of furanocoumarins by *Glomerella cingulata* and

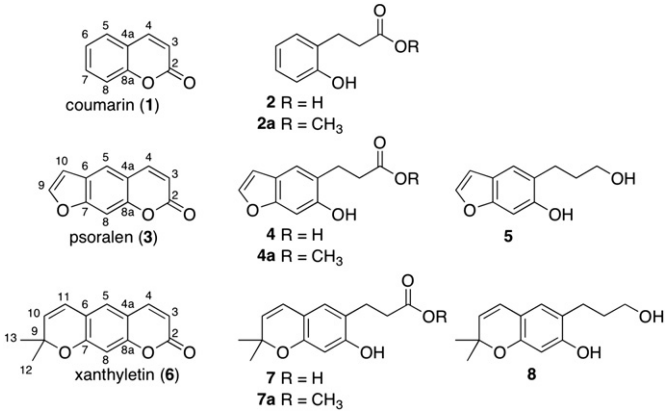

Fig. 1. Chemical structures of coumarin, psoralen, and xanthyletin derivatives.

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0040-4020/$ – see front matter © 2010 Published by Elsevier Ltd.
doi:10.1016/j.tet.2010.10.089

evaluated the transformation products on the BACE1 inhibitory activity.[8,9] Therefore, it is envisioned that biotransformation of coumarin (**1**), psoralen (**3**), and xanthyletin (**6**) may provide analogues, which could be tested for new and improved activities. The goal of the present work was the transformation of coumarins **1**, **3**, and **6** by *G. cingulata* and the evaluation of these compounds as BACE1 inhibitors (Fig. 1).

2. Results and discussion

Coumarin (**1**), psoralen (**3**), and xanthyletin (**6**) were administered to fungal cultures of *G. cingulata*, the cultures were incubated for 7 days. HPLC chromatograms of extracted of cultures incubated with coumarin (**1**) indicated that **1** was metabolized to **2** in 30% yield for 7 days (Fig. 2). Subsequently, to obtain sufficient quantities of each product for broth chemical characterization and bioassay, larger scale mycelia incubated with **2** were performed, the culture was extracted as described in Experimental section, and methylated metabolite **2** (compound **2a**) was isolated. Metabolite **2** was obtained by the hydrolysis of **2a**. The structure of these compounds were determined through use of HR-FABMS, ^{1}H and ^{13}C NMR (Table 1), H–H COSY, HSQC, and HMBC spectroscopic analyses, and the metabolite **2** was identified as hydrocoumaric acid.[10]

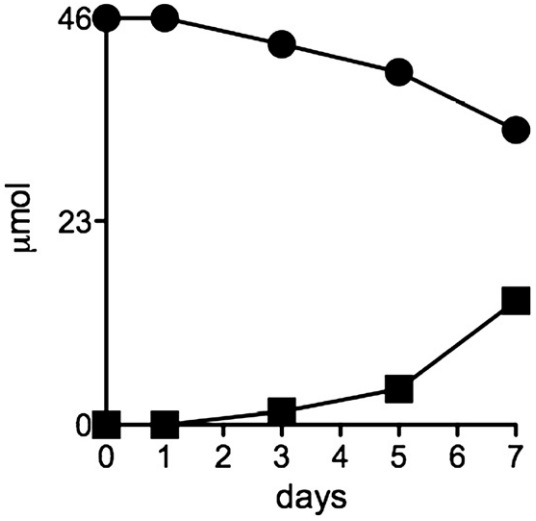

Fig. 2. Time course in the biotransformation of **1** by *G. cingulata*: (●) coumarin (**1**); (■) metabolite **2**.

HPLC and TLC analysis of the broth extracts of cultures incubated with paoralen (**3**) indicated it to be completely metabolized to two products **4** and **5** at the conversion rate of 47 and 38% yields for 7 days (Fig. 3). To isolate the metabolites, larger scale culture of *G. cingulta* was incubated with **3** were performed, the culture was extracted as described in Experimental section, and metabolites **4** and **5** were obtained.

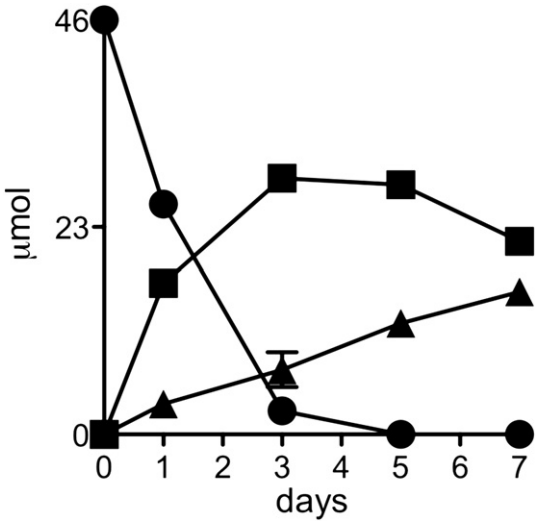

Fig. 3. Time course in the biotransformation of **3** by *G. cingulata*: (●) psoralen (**3**); (■) metabolite **4**; (▲) metabolite **5**.

HR-FABMS of compound **4** showed $[M+H]^{+}$ peaks at *m/z* 207.0685 (calcd for $C_{11}H_{11}O_4$, 207.0658), which established a molecular formula of $C_{11}H_{10}O_4$. The presence of a broad absorption band at 3264 cm^{-1} and a strong absorption band at 1698 cm^{-1} in the IR spectrum suggested the conversion of the original psoralen lactone into phenol and carboxylic acid functionalities. The ^{13}C NMR spectrum showed 11 resonances distributed as two secondary, four tertiary and five quaternary carbons. The conversion of original two olefinic carbons (C-3, δ_C 114.7 ppm and C-4, δ_C 144.0 ppm) into two aliphatic secondary carbons (C-3, δ_C 34.8 ppm and C-4, δ_C 26.8 ppm) 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 ^{1}H NMR spectrum showed the conversion of two doublets (δ_H 6.38 ppm, d, *J*=9.8 Hz, H-3 and δ_H 7.81 ppm, d, *J*=9.8 Hz, H-4) into two triplets at δ_H 2.65 (H-3) and δ_H 2.98 (H-4). Thus, chemical

Table 1
The ^{13}C NMR spectroscopic data of compounds **1**–**8**

Position	**1**[a]	**2**[b]	**3**[c]	**4**[a]	**5**[c]	**6**[b]	**7**[c]	**8**[c]
2	160.8 (C)[d]	174.7 (C)	161.0 (C)	174.7 (C)	60.7 (CH_2)	161.2 (C)	174.7 (C)	60.7 (CH_2)
3	116.7 (CH)	34.4 (CH_2)	114.7 (CH)	34.8 (CH_2)	32.6 (CH_2)	113.0 (CH)	34.6 (CH_2)	32.3 (CH_2)
4	143.4 (CH)	26.4 (CH_2)	144.0 (CH)	26.8 (CH_2)	25.3 (CH_2)	143.3 (CH)	25.8 (CH_2)	24.4 (CH_2)
4a	118.8 (C)	128.1 (C)	115.4 (C)	124.9 (C)	123.6 (C)	112.7 (C)	120.3 (C)	119.1 (C)
5	127.8 (CH)	130.9 (CH)	119.8 (CH)	122.3 (CH)	121.6 (CH)	124.7 (CH)	128.6 (CH)	128.0 (CH)
6	124.4 (CH)	120.4 (CH)	124.9 (C)	120.7 (C)	120.9 (C)	118.5 (C)	114.4 (C)	114.7 (C)
7	131.8 (CH)	128.1 (CH)	156.4 (C)	155.5 (C)	154.7 (C)	155.4 (C)	153.4 (C)	152.5 (C)
8	116.9 (CH)	116.0 (CH)	99.9 (CH)	98.3 (CH)	98.9 (CH)	104.4 (CH)	104.1 (CH)	104.2 (CH)
8a	154.1 (C)	156.0 (C)	152.0 (C)	154.3 (C)	152.7 (C)	156.8 (C)	156.7 (C)	155.4 (C)
9			146.9 (CH)	144.5 (CH)	143.9 (CH)	77.7 (C)	76.5 (C)	76.1 (C)
10			106.4 (CH)	107.1 (CH)	106.0 (CH)	131.2 (CH)	128.0 (CH)	127.9 (CH)
11						120.8 (CH)	122.8 (CH)	121.8 (CH)
12						28.3 (CH_3)	28.1 (CH_3)	27.9 (CH_3)
13						28.3 (CH_3)	28.1 (CH_3)	27.9 (CH_3)

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

structure of metabolite **4** was established as 6,7-furano-hydrocoumaric acid. Compound **5** was shown to have a molecular formula, $C_{11}H_{12}O_3$ as determined by HR-EIMS. Comparison of this compound with metabolite **4** revealed significant similarity except for the disappearance of carbonyl group and the presence of oxygenated methylene in **5**. Compound **5** was therefore identified as completely reduced compound of **4**, 6,7-furano-*o*-coumaryl alcohol.

To clarify the time course of the microbial transformation of xanthyletin (**6**) by *G. cingulata*, a small amount of **6** 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, **6** was transformed to **7** and **8** at the conversion rate of 57 and 27% yields for 7 days (Fig. 4). To isolate these metabolites, larger scale incubation of **6** by *G. cingulata* was performed for 7 days. After the biotransformation, the culture was extracted, and metabolites **7** and **8** were isolated.

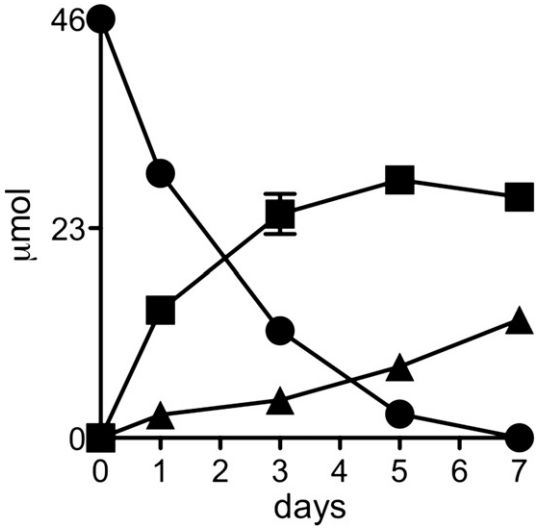

Fig. 4. Time course in the biotransformation of **6** by *G. cingulata*: (●) xanthyletin (**6**); (■) metabolite **7**; (▲) metabolite **8**.

HR-FABMS of compound **7** showed $[M+H]^+$ peaks at *m*/*z* 249.2828 (calcd for $C_{14}H_{17}O_4$, 249.2838), which established a molecular formula of $C_{14}H_{16}O_4$. The presence of a broad absorption band at 3359 cm^{-1} and a strong absorption band at 1707 cm^{-1} in the IR spectrum suggested the conversion of the original xanthyletin lactone into phenol and carboxylic acid functionalities. The ^{13}C NMR spectrum showed 14 resonances distributed as two primary, two secondary, four tertiary and six quaternary carbons. The conversion of original two olefinic carbons (C-3, δ_C 113.0 ppm and C-4, δ_C 143.3 ppm) into two aliphatic secondary carbons (C-3, δ_C 34.6 ppm, and C-4, δ_C 25.8 ppm) 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_H 6.22 ppm, d, J=9.3 Hz, H-3, and δ_H 7.58, d, J=9.2 Hz, H-4) into two triplets at δ_H 2.45 ppm (H-3) and δ_H 2.66 ppm (H-4). Thus, chemical structure of metabolite **7** was established as 9,9-dimethyl-6,7-pyrano-hydrocoumaric acid. Compound **8** was shown to have a molecular formula, $C_{14}H_{18}O_3$ as determined by HR-EIMS. Comparison of this compound with metabolite **7** revealed significant similarity except for the disappearance of carbonyl group, and the presence of oxygenated methylene in **8**. Compound **8** was therefore determined as completely reduced compound of **7**, 9,9-dimethyl-6,7-pyrano-*o*-hydrocoumaryl alcohol.

Results of the metabolism of simple coumarin **1** and linear-type furano- or pyranocoumarins **3** and **6** by *G. cingulata* suggested that the main pathway of those metabolism involved reduction at α,β-unsaturated lactone ring of coumarin skeleton. Compared to the transformation of psoralen (**3**) and xanthyletin (**6**), the biotransformation of coumarin (**1**) in *G. cingulata* was a much slower process. The presence of linear-type furano- or pyrano-ring in these substrates boots the rate of bioconversion. Only linear-type furanocoumarin **3** and pyranocoumarin **6** were converted to completely reduction to give the corresponding reduced alcohols. However, previous studies with other linear-type furanocoumarins were not found to be a completely reduced metabolite. In the metabolism of coumarin (**1**), the *Psudomonas* sp., *Arthrobacter* sp., *Penicillium* sp., *Fusarium solani*, and *Bacillus cereus* were known to metabolize **1** by a reductive mechanism,[11–14] leading to hydrocoumaric acid (**2**), and *Colletotrichum capsici* completely reduced the lactone moiety of **1** to respective alcohol.[15] This is the first report that biotransformation completely reduced the lactone moiety of furanocoumarin and pyranocoumarin to respective alcohols. Although little is known about the enzymatic reduction of coumarins, it is interesting to speculate on the mechanism of production of the corresponding reduced acids. Because all coumarins are extremely stable in the lactone form, it would seem unlikely that the ring is opened before reduction unless the 3,4-double bond is immediately isomerized to the trans geometry yielding a stable hydroxy acid. In the lactone form, the 3,4-double bonds of coumarins are always more difficult to chemically hydrogenerate than are double bonds in a side chain or the non-nuclear double bond of a coumarins. If the fungus has overcome this difficulty, production of the corresponding reduced acids would follow because hydrolytic opening of the lactone ring of a 3,4-dihydrocoumarin gives a hydroxy acid with little tendency to lactonize. Therefore, fungal reduction of coumarins probably proceeds by opening of the lactone ring followed by isomerization and reduction or by hydrogenation of the 3,4-double bond followed by, or concomitant with, opening of the lactone ring. However, intermediate metabolites in each metabolic pathway were not detected in our biotransformation experiments. The data on the microbial transformed product, metabolite may be used for further pharmacological evaluation of coumarins.

Subsequently, the β-secretase (BACE1) inhibitory effects of all these compounds were evaluated to search for potential novel anti-Alzheimer agents. Results of evaluation of BACE1 inhibitory activity established that methyl ester derivatives **4a**, and **7a** inhibited 54.5±3.2, and 43.3±5.1% of the BACE1 activity at a concentration of 1 mM (Fig. 5), and the IC_{50} value of **4a** was 0.84±0.06 mM. The

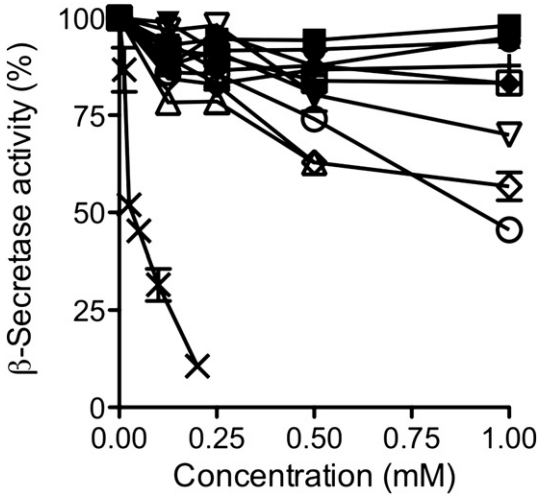

Fig. 5. β-Secretrase inhibitory activity of coumarin, psoralen, and xanthyletin derivatives. The bar is the mean of ± SD of three experiments. (●) **1**; (■) **2**; (▲) **2a**; (▼) **3**; (◆) **4**; (○) **4a**; (□) **5**; (△) **6**; (▽) **7**; (◇) **7a**; (+) **8**; (×) positive control.

BACE1 inhibitory effects of methyl ester derivatives **4a** and **7a** were stronger than those of corresponding acids, and methyl ester derivative **2a**, indicating the presence of methyl ester and furan- and pyran-systems were important for the inhibitory activity. Psoralen (**2**) and its reduced biotransformation product by *G. cingulata* were found to significantly lower BACE1 inhibitory activity than did isoimperatorin, having an additional prenyloxy group at C_5-position, and its biotransformation products.[8] Bergapten, with a methoxy group at C_5-position, and its reduced biotransformation product were similar inhibitory activity to psoralen and its biotransformation product,[9] indicating an additional prenyloxy group at C_5-position on furanocoumarin skeleton and its corresponding reduced acid derivative seems to have significant influence.

In conclusion, one metabolite **2** was obtained from the incubation of coumarin (**1**) with *G. cingulata*, two new compounds **4** and **5** were obtained from the incubation of psoralen (**3**), and two new compounds **7** and **8** were obtained from the incubation of xanthyletin (**6**). The results suggested that *G. cingulata* possess the characteristics of reaction of the coumarin skeleton as we previously reported.[8,9] The reactions involved the reduction of α,β-unsaturated lactone ring of coumarin skeleton to give the corresponding reduced acid. In addition, bioconversions of simple linear-types furanocoumarin and pyranocoumarin, those substrates were completely metabolized in ca. 5 days, and to give both the corresponding reduced alcohols and acids. These new metabolites will be useful reference standards for our continuing studies on structure modification and pharmacological evaluation of coumarins. This investigation also demonstrates that biotransformation is a powerful tool for the structural modification of natural products.

3. Experimental

3.1. General experimental details

A thin layer chromatography (TLC) was performed on precoated plates (Si gel 60 F_{254}, 0.25 mm, Merck). 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 photo diode array (PDA) coupled with analytical works station. A YMC-Pack ODS-AQ (4.6 ×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 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, 70% A for 10 min at 1.0 mL/min. 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 400 or 500 MHz for ^1H and 100 or 125 MHz for ^{13}C on a JEOL AL 400 or ECA-500 spectrometer. 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 Pan Vera Co. (United States).

3.2. Chemicals

Coumarin (**1**) and psoralen (**3**) were purchased from Sigma–Aldrich (Tokyo, Japan). Xanthyletin (**6**) was synthesized according to the methods reported previously.[16]

3.3. 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, 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*, coumarins **1**, **3**, and **6** (46 μmol) in 0.5 mL of dimethyl sulfoxide (DMSO) were added into the medium, respectively, and cultivated in 7 days. Every other day, 2 mL of the culture mediums were extracted with EtOAc, respectively. These extracts were 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 of **1**, **3**, and **6** are shown in Figs. 2–4.

3.5. Preparative biotransformation of coumarin (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 dimethyl sulfoxide (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. 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 (423 mg) was obtained. The extract was distributed between 5% $NaHCO_3$ aq and EtOAc, and EtOAc phase was evaporated to give a neutral fraction (159 mg). No metabolite was detected 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 (264 mg) was obtained. Metabolites were detected from both fractions by TLC and HPLC, respectively. The acidic fraction was dissolved in acetone (5 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 (CC) (silica gel 60, 230–400 mesh, Merck) with a *n*-hexane–Et_2O gradient (9:1 to 1:4) to yield compound **2a** (25 mg). Compound **2a** (13 mg) was dissolved in MeOH (1 mL), 1% NaOH (2 mL) 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** (9 mg, R_t=6.6 min).

*3.5.1. Hydrocoumaric acid (**2**).* White powder; IR (KBr) ν_{max} 3264, 1698 cm^{-1}; ^1H NMR (acetone-d_6, 500 MHz) δ 7.11 (1H, dd, J=7.5, 1.7 Hz, H-5), 7.01 (1H, ddd, J=8.0, 7.5, 1.7 Hz, H-7), 6.81 (1H, dd, J=8.0, 1.2 Hz H-8), 6.73 (1H, dt, J=7.5, 1.2 Hz, H-6), 2.87 (2H, m, H-4), 2.59 (2H, m, H-3); ^{13}C NMR shown as Table 1; HR-FABMS (pos) m/z 167.1817 $[M+H]^+$ (calcd for $C_9H_{10}O_3$, 167.1827).

*3.5.2. Hydrocoumaric acid methyl ester (**2a**).* White powder; IR (KBr) ν_{max} 3398, 1714 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 7.10 (2H,

m, H-5, 8), 6.86 (2H, m, H-6, 7), 3.69 (3H, s, $COOCH_3$), 2.91 (2H, m, H-4), 2.72 (2H, m, H-3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 176.0 (C, C-2), 154.2 (C, C-8a), 130.5 (CH, C-5), 128.0 (CH, C-7), 127.2 (C, C-4a), 120.8 (CH, C-8), 117.1 (CH, C-6), 52.2 (CH_3, $COOCH_3$), 34.9 (CH_2, C-3), 24.6 (CH_2, C-4); HR-FABMS (pos) m/z 181.2081 $[M+H]^+$ (calcd for $C_{10}H_{13}O_3$, 181.2093).

3.6. Preparative biotransformation of psoralen (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 dimethyl sulfoxide (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. 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 (423 mg) was obtained. The extract was distributed between 5% $NaHCO_3$ aq and EtOAc, and EtOAc phase was evaporated to give a neutral fraction (159 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 (264 mg) was obtained. Metabolites were detected from both fractions by TLC and HPLC, respectively. The acidic fraction was dissolved in acetone (5 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 (CC) (silica gel 60, 230–400 mesh, Merck) with a *n*-hexane–Et_2O gradient (9:1 to 1:4) to yield compound **4a** (25 mg). Compound **4a** (13 mg) was dissolved in MeOH (1 mL), 1% NaOH (2 mL) 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 (9 mg, R_t=28.2 min). The neutral fraction was subjected to CC using a gradient of *n*-hexane–EtOAc (1:0 to 1:9) of increasing polarity gave to metabolite **5** (11 mg, R_t=27.9 min).

*3.6.1. 6,7-Furano-hydrocoumaric acid (**4**).* White powder; IR (KBr) ν_{max} 3264, 1698 cm^{-1}; ^1H NMR (acetone-d_6, 500 MHz) δ 7.61 (1H, d, J=2.3 Hz, H-9), 7.38 (1H, s, H-5), 6.99 (1H, br s, H-8), 6.73 (1H, dd, J=2.3, 1.2 Hz, H-10), 2.98 (2H, t, J=7.7 Hz, H-4), 2.65 (2H, t, J=7.7 Hz, H-3); ^{13}C NMR shown as Table 1; HR-FABMS (pos) m/z 207.0685 $[M+H]^+$ (calcd for $C_{11}H_{11}O_4$, 207.0658).

*3.6.2. 6,7-Furano-o-hydrocoumaryl alcohol (**5**).* White powder; IR (KBr) ν_{max} 3317 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 7.49 (1H, d, J=2.3 Hz, H-9), 7.29 (1H, s, H-5), 7.02 (1H, br s, H-8), 6.64 (1H, dd, J=2.3, 0.9 Hz, H-10), 3.66 (2H, t, J=5.7 Hz, H-2), 2.87 (2H, t, J=6.6 Hz, H-4), 1.92 (2H, m, H-3); ^{13}C NMR shown as Table 1; EIMS m/z 192 $[M]^+$(57), 174 (53), 147 (100), 146 (24), 131 (18), 118 (14), 91 (21), 77 (14); HR-EIMS m/z 192.0782 $[M]^+$ (calcd for $C_{11}H_{12}O_3$, 192.0786).

*3.6.3. 6,7-Furano-hydrocoumaric acid methyl ester (**4a**).* White powder; IR (KBr) ν_{max} 3398, 1714 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 7.48 (1H, d, J=2.3 Hz, H-9), 7.28 (1H, s, H-5), 7.04 (1H, br s, H-8), 6.63 (1H, dd, J=2.3, 0.9 Hz, H-10), 3.68 (3H, s, $COOCH_3$), 2.99 (2H, t, J=6.5 Hz, H-4), 2.76 (2H, t, J=6.5 Hz, H-3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 176.0 (C, C-2), 154.8 (C, C-7), 152.2 (C, C-8a), 144.1 (CH, C-9), 123.9 (C, C-4a), 121.6 (C, C-5), 121.0 (C, C-6), 106.0 (CH, C-10), 99.8 (CH, C-8), 52.2 (CH_3, $COOCH_3$), 35.5 (CH_2, C-3), 24.8 (CH_2, C-4); EIMS m/z 220 $[M]^+$(31), 188 (100), 160 (82), 147 (48), 146 (75), 131 (28), 118 (34); HR-EIMS m/z 220.0724 $[M]^+$ (calcd for $C_{12}H_{12}O_4$, 220.0736).

3.7. Preparative biotransformation of xanthyletin (6)

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 **6** in 1.0 mL of dimethyl sulfoxide (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. 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 (391 mg) was obtained. The extract was distributed between 5% $NaHCO_3$ aq and EtOAc, and EtOAc phase was evaporated to give a neutral fraction (188 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 (203 mg) was obtained. The acidic fraction was dissolved in acetone (5 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 CC (silica gel 60, 230–400 mesh, Merck) with a *n*-hexane–Et_2O gradient (9:1 to 1:4) to yield compound **7a** (42 mg). Compound **7a** (18 mg) was dissolved in MeOH (1 mL), 1% NaOH (2 mL) 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 **7** (15 mg, R_t=45.2 min). The neutral fraction was subjected to CC using a gradient of *n*-hexane–EtOAc (1:0 to 1:9) of increasing polarity gave to metabolite **8** (8 mg, R_t=44.8 min).

*3.7.1. 9,9-Dimethyl-6,7-pyrano-hydrocoumaric acid (**7**).* White powder; IR (KBr) ν_{max} 3359, 1707 cm^{-1}; ^1H NMR (acetone-d_6, 500 MHz) δ 6.67 (1H, s, H-5), 6.16 (1H, s, H-8), 6.16 (1H, d, J=9.8 Hz, H-11), 5.37 (1H, d, J=9.8 Hz, H-10), 2.66 (2H, t, J=7.5 Hz, H-4), 2.45 (2H, t, J=7.5 Hz, H-3), 1.23 (6H, s, H-12, 13); ^{13}C NMR shown as Table 1; HR-FABMS (pos) m/z 249.2828 $[M+H]^+$ (calcd for $C_{14}H_{17}O_4$, 249.2838).

*3.7.2. 9,9-Dimethyl-6,7-pyrano-o-hydrocoumaryl alcohol (**8**).* White powder; IR (KBr) ν_{max} 3319 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 6.70 (1H, s, H-5), 6.32 (1H, s, H-8), 6.24 (1H, d, J=9.8 Hz, H-11), 5.45 (1H, d, J=9.8 Hz, H-10), 3.64 (2H, t, J=6.0 Hz, H-2), 2.67 (2H, t, J=6.6 Hz, H-4), 1.83 (2H, m, H-3) 1.40 (6H, s, H-12, 13); ^{13}C NMR shown as Table 1; EIMS m/z 234 $[M]^+$ (19), 219 (100), 201 (30), 181 (13), 163 (13), 91 (7); HR-EIMS m/z 234.1254 $[M]^+$ (calcd for $C_{14}H_{18}O_3$, 234.1256).

*3.7.3. 9,9-Dimethyl-6,7-pyrano-hydrocoumaric acid methyl ester (**7a**).* White powder; IR (KBr) ν_{max} 3397, 1740 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 6.67 (1H, s, H-5), 6.33 (1H, s, H-8), 6.22 (1H, d, J=9.8 Hz, H-11), 5.45 (1H, d, J=9.8, 0.9 Hz, H-10), 3.69 (3H, s, $COOCH_3$), 2.80 (2H, m, H-4), 2.67 (2H, m, H-3) 1.40 (6H, s, H-12, 13); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 176.2 (C, C-2) 155.0 (C, C-8a), 152.9 (C, C-7), 128.1 (CH, C-10), 127.9 (CH, C-5), 121.7 (CH, C-11), 119.3 (C, C-4a), 114.8 (C, C-6), 105.1 (CH, C-8), 76.1 (C, C-9), 52.2 (CH_3, $COOCH_3$), 35.2 (CH_2, C-3), 28.0 (CH_3, C-12, 13), 23.9 (CH_2, C-4); EIMS m/z 262 $[M]^+$(12), 248 (6), 247 (34), 230 (13), 216 (15), 215 (100), 187 (39), 173 (18); HR-EIMS m/z 262.1203 $[M]^+$ (calcd for $C_{15}H_{18}O_4$, 262.1205).

3.8. β-Secretase (BACE1) enzyme assay

The assay was carried out according to the supplied manual with modifications.[6,8,9] 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. 6,7-Furano-8a-methoxy-5-prenyloxy hydrocoumaric acid methyl ester was used as positive control.[8,9]

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