NOTE

Microbial Transformation of Isopimpinellin by *Glomerella cingulata*

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Abstract: Microbial transformation studies conducted on isopimpinellin (**1**) by the fungus *Glomerella cingulata* have revealed that **1** was metabolized to give the corresponding reduced acid, 5,8-dimethoxy-6,7-furano-hydrocoumaric acid (**2**). The structure of metabolite **2** was elucidated by high-resolution mass spectrometry (HR-MS), extensive NMR techniques, including ^1H NMR, ^{13}C NMR, ^1H - ^1H correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond coherence (HMBC). The biotransformed product **2** showed weak *in vitro* β -secretase (BACE1) inhibitory effect.

Key words: biotransformation, isopimpinellin, *Glomerella cingulata*, β -secretase inhibitory activity.

1 INTRODUCTION

Isopimpinellin (5,8-dimethoxypsoralen, **1**) occurs in a number of plants of the Apiaceae family and in some members of the Rutaceae¹. It is usually present together with 5- and 8-methoxypsoralen (5-MOP and 8-MOP) in plants such as celery, parsley, and the seeds of bishop's weed, *Ammi majus*. Compound **1** possesses antifungal, insecticidal, and insect antifeedant activities²⁻⁴, as well as inhibition of cytochrome P450 enzymes activities^{5, 6}. However to our knowledge, biotransformation of isopimpinellin has not been previously reported.

Biotransformation is today considered by synthetic organic chemists to be an economically competitive technology for the development of new production routes of fine chemical, pharmaceutical, and agrochemical compounds⁷. Microorganisms are well known as efficient and selective catalysts. Previously, we studied the microbial transformation of furanocoumarins by *Glomerella cingulata* and evaluated the transformation products on the BACE1 inhibitory activity^{8, 9}. Therefore, it is envisioned that biotransformation of **1** may provide analogues which could be tested for new and improved activities. The goal of the present work was to transform isopimpinellin (**1**) using *G. cingulata* and to evaluate these compounds as BACE1 inhibitors (Fig. 1).

Fig. 1 Chemical structures of isopimpinellin derivatives.

2 EXPERIMENTAL PROCEDURE

2.1 General experimental procedures

Thin layer chromatography (TLC) was performed on pre-coated plates (Si gel 60 F₂₅₄, 0.25 mm, Merck). The mobile phase was hexane-EtOAc (1:1). Compounds were visualized by spraying plates with 0.5% vanillin in 96% H₂SO₄ followed by a brief heating. A SHIMAZU LC-10A HPLC system (SHIMAZU CO., LTD., Kyoto, Japan) comprised a quaternary solvent deliver system, an auto-sampler, a column temperature controller and a photo diode array (PDA) coupled with an 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) was 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

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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. The total runtime was 110 min. The injection volume was 10 μ L. Electron-ionization mass spectrometry (EIMS), high-resolution electron-ionization mass spectrometry (HREIMS) fast atom bombardment mass spectrometry (FABMS) and high-resolution fast atom bombardment mass spectrometry (HRFABMS) 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 125 or 100 MHz for ^{13}C on a JEOL ECA 500 or AL 400 spectrometer. Infrared (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 PanVera Co., USA.

2.2 Chemical

Isopimpinellin (**1**) was synthesized according to the methods reported previously¹⁰⁾.

2.3 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% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05% KCl, 0.1% K_2HPO_4 , and 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 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.

2.4 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. 120 rpm) for 3 days. After the growth of *G. cingulata*, **1** (10 mg, 46 μ mol) in 0.5 mL of dimethyl sulfoxide (DMSO) was added into the medium, 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 Fig. 2.

2.5 Preparative biotransformation of isopimpinellin (**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

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

medium or substrate dissolved in DMSO with medium. No metabolic product was observed in the two controls. After 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 (411 mg) was obtained. The extract was distributed between 5% NaHCO_3 aq. and EtOAc, and the EtOAc phase was evaporated to give a neutral fraction (256 mg). No metabolite was detected by TLC and HPLC. The alkali phase was acidified to pH 3 with 1N HCl and distributed between water and EtOAc. The EtOAc phase was evaporated, and the acidic fraction (155 mg) was obtained. Metabolites were detected in the acidic fraction by TLC and HPLC. 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 flash silica-gel column chromatography (silica gel 60, 230-400 mesh, Merck) with a hexane-Et₂O gradient (1:1) to yield compound **2a** (23 mg). Compound **2a** (20 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 1N HCl and distributed between EtOAc and water. The EtOAc phase was evaporated to yield **2** (15 mg).

5,8-Dimethoxy-6,7-furano-hydrocoumaric acid (**2**)

Pale yellow powder; IR (KBr) ν_{max} 3495 and 1705 cm^{-1} ; ^{13}C and ^1H NMR shown as Table 1; HR-FABMS (pos) m/z 267.0895 [$\text{M} + \text{H}$]⁺ (calcd. for $\text{C}_{13}\text{H}_{15}\text{O}_6$, 267.0869).

5,8-Dimethoxy-6,7-furano-hydrocoumaric acid methyl ester (**2a**)

Pale yellow powder; IR (KBr) ν_{max} 3419 and 1732 cm^{-1} ;

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

position	1 ^a		2 ^b	
	δ_{C}	δ_{H}	δ_{C}	δ_{H}
2	159.8 (C) ^c		174.7 (C)	
3	112.7 (CH)	6.30 1H, <i>d</i> , (9.6) ^d	34.3 (CH ₂)	2.42 2H, <i>m</i>
4	139.9 (CH)	8.13 1H, <i>d</i> , (9.6)	20.4 (CH ₂)	2.88 2H, <i>m</i>
4a	106.9 (C)		115.6 (C)	
5	144.5 (C)		147.2 (C)	
6	114.6 (C)		113.3 (C)	
7	149.6 (C)		147.4 (C)	
8	127.4 (C)		129.4 (C)	
8a	143.3 (C)		145.6 (C)	
9	146.5 (CH)	7.63 1H, <i>d</i> , (2.1)	143.8 (CH)	7.54 1H, <i>d</i> , (2.3)
10	105.9 (CH)	7.00 1H, <i>d</i> , (2.1)	105.6 (CH)	6.86 1H, <i>d</i> , (2.3)
5-OCH ₃	61.0 (CH ₃)	4.17 3H, <i>s</i>	60.9 (CH ₃)	3.87 3H, <i>s</i>
8-OCH ₃	61.5 (CH ₃)	4.17 3H, <i>s</i>	61.1 (CH ₃)	3.85 3H, <i>s</i>

^a Measured at 100 MHz for ^{13}C and 400 MHz for ^1H in CDCl_3 .^b Measured at 125 MHz for ^{13}C and 500 MHz for ^1H in Acetone- d_6 .^c ^{13}C multiplicities were determined by DEPT 135°^d The *J* values are Hz in parentheses.

^1H NMR (CDCl_3 , 500 MHz) δ 7.46 (1H, *d*, $J=2.3$ Hz, H-9), 6.82 (1H, *d*, $J=2.3$ Hz, H-10), 4.12 (3H, *s*, 8-OCH₃), 3.97 (3H, *s*, 5-OCH₃), 3.69 (3H, *s*, COOCH₃), 3.04 (2H, *m*, H-4), 2.62 (2H, *m*, H-3); ^{13}C NMR (CDCl_3 , 125 MHz) δ 174.5 (C, C-2), 146.2 (C, C-5), 145.7 (C, C-7), 143.7 (C, C-8a), 142.7 (CH, C-9), 128.2 (C, C-8), 114.2 (C, C-4a), 113.0 (C, C-6), 104.7 (CH, C-10), 60.9 (CH₃, 8-OCH₃), 60.7 (CH₃, 5-OCH₃), 51.7 (CH₃, COOCH₃), 33.9 (CH₂, C-3), 19.5 (CH₂, C-4); EIMS m/z 280 [M]⁺ (38), 248 (100), 206 (77), 191 (86), 163 (33); HR-EIMS m/z 280.0952 [M]⁺ (calcd. for $\text{C}_{14}\text{H}_{16}\text{O}_6$, 280.0947).

2.6 β -Secretase (BACE1) enzyme assay

The assay was carried out according to the supplied manual with modifications (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.

3 RESULTS AND DISCUSSION

Isopimpinellin (**1**) was administered to fungal cultures of *G. cingulata*, the cultures were incubated for 7 days. HPLC chromatograms of extracts from the cultures incubated with isopimpinellin (**1**) indicated that **1** was metabolized to **2** in 47% yield for 7 days (Fig. 2). Subsequently, to obtain sufficient quantities of each product for broth chemical characterization and bioassay, a larger scale mycelia incubated with **1** was performed, the culture was extracted as described in Materials and Methods, and methylated metabolite **2** (compound **2a**) was isolated. Metabolite **2** was obtained by the hydrolysis of **2a**. The structures of these compounds were determined by spectral data.

HR-FABMS of compound **2** showed [M + H]⁺ peaks at m/z 267.0895 (calcd. for $\text{C}_{13}\text{H}_{15}\text{O}_6$ 267.0869), which established a molecular formula of $\text{C}_{13}\text{H}_{14}\text{O}_6$. The presence of a broad absorption band at 3495 cm^{-1} and a strong absorption band at 1705 cm^{-1} in the IR spectrum. Comparing ^1H NMR data of **2** with those of **1**, the signals ascribed to the

β -enone system were found to be absent in **2**. Instead two sets of methylene protons were observed at δ_{H} 2.87 and 2.59 ppm, respectively. Methylene peaks at δ_{C} 34.4 and 26.4 ppm were detected by ^{13}C NMR spectrum. This showed that some chemical changes have occurred on the lactone moiety of the coumarin ring, as no changes in NMR were detected for the benzofuran moiety of furanocoumarin. The chemical structure of **2** was shown to be identical to 5,8-dimethoxy-6,7-furano-hydrocoumaric acid (**2**). The protons and carbons assignments were unambiguously made from the H-H COSY, HMQC and HMBC spectra. This is the first report of the spectral data of **2**.

Previously, we had incubated furanocoumarins bergapten, with a methoxy group at C_5 position, and xanthotoxin, with a methoxy group at the C_8 position, by *G. cingulata*⁹⁾. In the biotransformation of bergapten and xanthotoxin, fungal reduction progressed not only bergapten but also xanthotoxin. In addition a demethylated product was produced as a minor metabolite in the biotransformation of xanthotoxin. The reduction product of **1** by *G. cingulata* was identified in this study. However a demethylated metabolite was not detected in this biotransformation. These results indicate that the presence of an alkoxy group at the C_5 position was involved in the dealkylation of the alkoxy group at the C_8 position in the furanocoumarin skeleton by *G. cingulata*.

Subsequently, the β -secretase (BACE1) inhibitory effects of compounds **1**, **2**, and **2a** were evaluated to search for potential novel anti-Alzheimer agents. Compounds **1**, **2**, and **2a** showed weak inhibitory effects against BACE1. BACE1 inhibitory effects of compounds **1**, **2**, and **2a** were 11.2, 19.1, and 20.9% at concentration of 0.5 mM, respectively.

In summary, the present study demonstrated that the fungus *G. cingulata* was able to transform isopimpinellin (**1**) to a corresponding reduced acid enzymatically. Since there are no reports on mammalian metabolites of isopimpinellin (**1**), the data on the microbial transformed product **2** may be useful for further pharmacological evaluation of **1**. They may also be used as analytical standards for detection in biological fluids.

References

- 1) Murray R. D.; Mendez J.; Brown S. A. *The Natural Coumarins: Occurrence, Chemistry and Biochemistry*. John Wiley & Sons, Ltd., New York (1982).
- 2) Montagner C.; de Souza S. M.; Groposo C.; Delle M. F. S.; Elza F. A.; Smania A. Jr. Antifungal activity of coumarins. *Z. Naturforsch., C: J. Biosci.* **63**, 21-28 (2008).
- 3) Miyazawa M.; Tsukamoto T.; Anzai J.; Ishikawa Y. Insecticidal effect of phthalides and furanocoumarins from *Angelica acutiloba* against *Drosophila melanogaster*. *J. Agric. Food Chem.* **52**, 4401-4405 (2004).
- 4) Yajima T.; Munakata K. Phloroglucinol-type furocoumarins, a group of potent naturally-occurring insect antifeedants. *Agric. Biol. Chem.* **43**, 1701-1706 (1979).
- 5) Cai Y.; Bennett D.; Nair R. V.; Ceska O.; Ashwood-Smith M. J.; DiGiovanni J. Inhibition and inactivation of murine hepatic ethoxy- and pentoxysresorufin O-dealkylase by naturally occurring coumarins. *Chem. Res. Toxicol.* **6**, 872-879 (1993).
- 6) Uesawa Y.; Mohri K. Quantitative structure-activity relationship (QSAR) analysis of the inhibitory effects of furanocoumarin derivatives on cytochrome P450 3A activities. *Pharmazie* **65**, 41-46 (2010).
- 7) Davis B. G.; Boyer V. Biocatalysis and enzymes in organic synthesis. *Nat. Prod. Rep.* **18**, 618-640 (2001).
- 8) Marumoto S.; Miyazawa M. Biotransformation of isoperatorin and imperatorin by *Glomerella cingulata* and β -secretase inhibitory activity. *Bioorg. Med. Chem.* **18**, 455-459 (2010).
- 9) Marumoto S.; Miyazawa M. Biotransformation of bergapten and xanthotoxin by *Glomerella cingulata*. *J. Agric. Food Chem.* **58**, 7777-7781 (2010).
- 10) Wulff H.; Rauer H.; Duering T.; Hanselmann C.; Ruff K.; Wrisch A.; Grissmer S.; Haensel W. Alkoxy-psoralens, novel nonpeptide blockers of shaker-type K^+ channels: synthesis and photoreactivity. *J. Med. Chem.* **41**, 4542-4549 (1998).