

# Biotransformation of swertiamarin by *Aspergillus niger*

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**Abstract:** The biotransformation of swertiamarin has been carried out using *Aspergillus niger*. The results showed that 60% swertiamarin were metabolized into two metabolites during the 5 days of biotransformation. The metabolites were identified as erythrocentaurin and 5-ethylidene-8-hydroxy-3,4,5,6,7,8-hexahydro-1Hpyrano[3,4-c]-pyridine-1-one, a novel alkaloid, with NMR and MS. The hydrolysis of glucosidic bond catalyzed by  $\beta$ -D-glucosidase was found to be the rate-limiting reaction in pathway of biotransformation of swertiamarin.

**Keywords:** Swertiamarin; Biotransformation; Solid phase extraction; *Aspergillus niger*; Erythrocentaurin; 5-ethylidene-8-hydroxy-3,4,5,6,7,8-hexahydro-1Hpyrano[3,4-c]-pyridine-1-one; HPLC; NM.

## INTRODUCTION

Swertiamarin, a bitter secoiridoid glycoside from *Swertia mussotii* Franch, *Swertia franchetiana*, *Enicostemma axillare* subsp, has been used as an important herb drug in China for many years and is one of the main ingredients of some important Chinese traditional medicine (Takei *et al.*, 2001; Jaishree *et al.*, 2009). Its structure is shown in the fig. 1. Swertiamarin has been reported to have hepatoprotective effect (Jaishree and Badami, 2010; Mihailović, 2013), anti-hepatotoxic activity (Gupta *et al.*, 2012), antioxidant (Chen *et al.*, 2011), anticholinergic action (Yamahara *et al.*, 1991), gastroprotective effects (Niiho *et al.*, 2006), anti-hyperlipidaemic effect (Vaidya *et al.*, 2009), inhibition of the growth of *Serratia marcescens*, *Proteus mirabilis*, *Citrobacter freundii* and *Escherichia coli* (Kumarasamy *et al.*, 2003), depressing of human DNA lipase used as a effective CNS-depressant (Bhattacharya *et al.*, 1976) and have great effect on gastric emptying and gastrointestinal motility (Kimura and Sumiyoshi, 2011). Swertiamarin was reported to have the characteristics of high bioactivity, high bioavailability and low toxicity (Xu *et al.*, 2013). Swertiamarin, however, has no bioactivity before metabolized in intestinal tract by intestinal bacteria into active metabolites after oral administration (Németh *et al.*, 2003; El-Sedawy *et al.*, 1988) and can be used as pro-drug (Nishibe, 1994). To develop novel hepatoprotective drug, swertiamarin was biotransformed by *Aspergillus niger* and the metabolites were separated, purified and identified.

## MATERIALS AND METHODS

### Chemicals

Swertiamarin was separated and purified from *Swertia mussotii* Franch. The standard swertiamarin (Tianjin Institute for Drug Control, China) was over 98%.

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Acetonitrile and methanol used here were HPLC grade (Honeywell International Inc., USA).  $\rho$ -NPG ( $\rho$ -nitrophenyl- $\beta$ -D-glucoside) was purchased from Appli. Chem (German). Other chemicals used were all analytical grades.

### Microorganisms

*Aspergillus niger* were isolated from rotten apples and maintained in PDA medium. The cultures were transferred every 2 months.

### Medium and culture conditions

The medium used for biotransformation of swertiamarin was consisted of 5 g/L NaCl, 1 g/L  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 1 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 5 g/L  $\text{KH}_2\text{PO}_4$ , 5 g/L peptone, 8 g/L glucose, 5 g/L yeast extract and pH 6.5.

In each batch of experiment, three shake-flasks were used. One used for biotransformation containing swertiamarin, medium and culture of strain (biotransformation group, BG), two others were used for blank control containing medium and swertiamarin (control group, CG) and fermentation control containing medium and culture of strain (fermentation control group, FCK). One stage fermentation strategy was used to the biotransformation of swertiamarin. 10 ml of sterilized water was moved into a slant and equilibrium for 15 min. Two aliquots 0.2 ml of suspensions each containing  $10^7$  spores/ml were aseptically moved into 250ml shake-flasks of BG and FCK (each containing 50 ml medium), respectively. Then the cultures were incubated under condition of 28°C and 130 rpm. After 48 h of incubation, two aliquots 1 ml of stock solution of swertiamarin in methanol (50 mg/ml) was added into shake flasks of BG and CG. Then all of the three shake-flasks were continued to be cultivated under the same condition for another 5 days. At the end of incubation period, cultures were centrifuged at  $10000 \times g$  for 30 min and filtered. The supernatants were stored at 4°C for further SPE-HPLC analysis.

### Procedure of solid phase extraction

0.5 ml of cell-free supernatant was mixed with 1 ml phosphate buffer (0.2 mol/L, pH 7.0) and agitated for 5 min at room temperature. SPE cartridge (OASIS HLB ICC 30 mg, Waters, USA) was conditioned by methanol (1 ml) and equilibrated by water (1 ml). 0.5 ml sample was then loaded onto the SPE cartridge and consecutively washed with distilled water (1 ml, pH 7.0) and eluted with methanol (1 ml). The elution was dried under flow of air and the residue was redissolved with 100 $\mu$ L of 10% acetonitrile for HPLC analysis. In the whole clearance procedure, the flow rates of solvents were set at 1 ml/min.

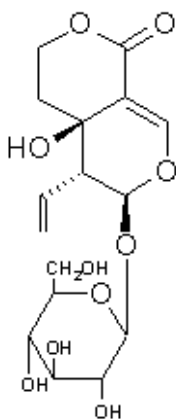


Fig. 1: Structure of swertiamarin.

### High pressure liquid chromatography condition

A Waters chromatography system constituted of a 2996 Photodiode Array Detector, a binary HPLC pump 90 and a manual injector was used to determine the concentration of swertiamarin and metabolites. The assay was performed on a C<sub>18</sub>, 5 $\mu$  column (200 mm $\times$ 4.6mm). The wavelength was set at 237 nm. The mobile phase was consisted of A (0.04% formic acid in acetonitrile) and B (0.04% formic acid in water). The gradient was as follows: 0 to 5 min, 10%A; 5 to 50 min, 10 to 30%A; 50 to 55 min, 30%A; 55 to 60 min, 30 to 10%A. The sample was filtered through 0.45  $\mu$ m membrane filter and injection column was 10 $\mu$ L and flow rate was set at 1 ml/min.

### Preparative scale biotransformation conditions

To purify the metabolites of swertiamarin in sufficient quality for structural identification, the preparative-scale biotransformation of swertiamarin was carried out with 40 shake flasks (250 ml) each containing 50 ml of medium on the same condition mentioned above. At the end of fermentation, pooled culture (approximately 2000 ml) was filtered and concentrated under reduced pressure at 60 $^{\circ}$ C into 200 ml. The concentrated culture was then extracted with the same volume of light petroleum and chloroform each three times. Anhydrous sodium sulfate was added into the combined organic layers of chloroform to remove water and then filtered. The filtrate was dried in

vacuum and the residue was dissolved in 15% acetonitrile (20 ml) for following semi-preparative HPLC procedure.

### Semi-preparative HPLC condition

a Hewlett-Packard 1100 system (Palo Alto Calif.) equipped with a binary pump, a UV2300 detector and a C<sub>18</sub>, 5 $\mu$  column (250 $\times$ 10 mm) was used to the semi-preparative HPLC purification of metabolites. The separation was divided into 2 stages. The first stage of purification was aimed to remove most of impurities and the second obtain the metabolites with enough purity. The mobile phase used for the first stage was gradient mixture of A (0.04% formic acid in acetonitrile) and B (0.04% formic acid in water). The linear gradient was as follows: 0~10 min, 15%A; 10~30 min, 15~24%A; 30~35 min, 30~100 %A; 35 to 50 min, 100%A; 50~55min, 100~15%A. The mobile phase of second stage was 10% acetonitrile in water. The injected volume was 1 ml and the flow rate 2 ml/min and the wavelength was set at 237 nm. The pooled elution of first stage was dried and dissolved in 10% acetonitrile for the second stage of purification. The pooled elution from second stage of purification was concentrated and lyophilized.

### NMR and high resolution MS conditions

To obtain the NMR spectra, purified metabolites were dissolved in CDCl<sub>3</sub> (metabolite 2, M<sub>2</sub>) or DMSO (metabolite 1, M<sub>1</sub>). The <sup>1</sup>H NMR, <sup>13</sup>C NMR and 2D NMR (HMBC, HSQC and COSY spectra) were performed at on a Bruker ARX300 NMR spectrometer. Chemical shifts were presented as  $\delta$  values relative to TMS as internal standard.

To obtain the mass spectra of metabolites, electrospray ionization (ESI) source (Agilent Corp., USA) was used in positive mode with a spray voltage of 9 kv and source temperature of 400 $^{\circ}$ C. The scan rate was set at 2s/scan, full ion scan was applied in the range of 50~400 amu. Nitrogen was used as the nebulizer and curtain gas (0.8 torr, 20 ml/min).

## RESULTS

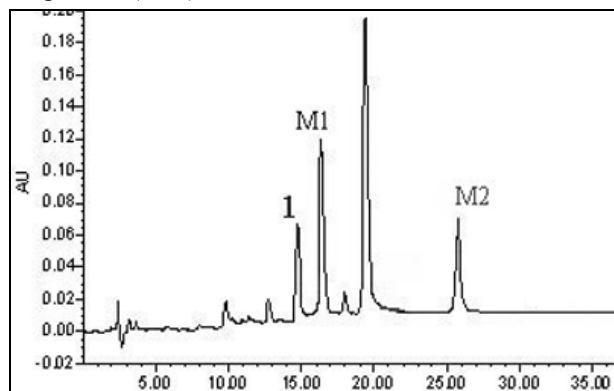
### Biotransformation

The HPLC chromatography of BG (fig. 2) showed that after 5 days of biotransformation, two metabolites were determined in the culture. The retention times for swertiamarin, M<sub>1</sub> and M<sub>2</sub> were 14.8 min, 16.8 min and 25.4 min, respectively. 60% swertiamarin was found to be transformed by *Aspergillus niger* by calculation of ratio of peak area of swertiamarin to that of total peaks area.

### The purification of metabolites with semi-preparative HPLC

At the end of preparative-scale biotransformation, cultures were pooled, filtered and extracted with light petroleum and chloroform each three times with equal

volume. After pooled chloroform layer was dried with rotary evaporation, 3 g of residue was obtained. The residue was then dissolved in 5 ml of 15% acetonitrile and filtered through 0.45  $\mu\text{m}$  membrane filter. The resulted solution was used to isolate the two metabolites with semi-preparative HPLC. Finally, 11 mg of  $M_1$  (96%) and 2 mg of  $M_2$  (94%) were obtained.



**Fig. 2:** HPLC of swertiamarin and its major metabolites. 1. Swertiamarin;  $M_1$  and  $M_2$  are the two metabolites of swertiamarin.

#### Identification of structures of metabolites

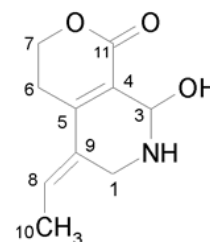
Structure data of  $M_2$ : Colorless needles. MS  $m/z$ : 175.88 (100), 145.89 (42), 116.91 (59), 90.87 (49).  $^1\text{H}$  NMR (400MHz,  $\text{CDCl}_3$ )  $\delta$ : 3.41 (2H, t,  $J=6.0\text{Hz}$ , 6- $\text{H}_2$ ), 4.36 (2H, t,  $J=6.0\text{Hz}$ , 7- $\text{H}_2$ ), 7.42 (1H, t,  $J=7.7\text{Hz}$ , 10-H), 7.87 (1H, dd,  $J=7.7\text{Hz}$ ,  $J=1.3\text{Hz}$ , 8-H), 8.23 (1H, dd,  $J=7.7\text{Hz}$ ,  $J=1.3\text{Hz}$ , 3-H), 10.3 (1H, s, 1-H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100MHz)  $\delta$ : 24.2 (C-6), 66.6 (C-7), 127.1 (C-5), 127.8 (C-10), 132.6 (C-4), 135.8(C-3), 138.2 (C-8), 141.0 (C-9), 164.7 (C-11), 191.5(C-1). After compared the data of  $M_2$  with that of authenticated compound, it was found that the data of  $M_2$  was same as that of erythrocentaurin (Yamahara *et al.*, 1991). Thus  $M_2$  was identified as erythrocentaurin.

Structure data of  $M_1$ : yellow needles, MS: 194.2165(100), 178.1823 (81), 150.2017 (62), 132.1857 (12), 104.1323 (51), 90.105(52), 76.0785(79).  $^1\text{H}$  NMR (400MHz, DMSO)  $\delta$ : 1.77(3H, d,  $J=7.2\text{Hz}$ , H-12), 2.6(2H, q, H-3), 4.3(2H, H-4), 4.46(2H, s, H-9), 5.52(1H, s, H-7), 6.11(1H, q,  $J=7.2\text{Hz}$ , H-11);  $^{13}\text{C}$  NMR(100MHz, DMSO)  $\delta$ : 55.86 (C-9), 85.75 (C-7), 121.87 (C-6), 145.41 (C-5), 21.98 (C-4), 65.16 (C-3), 128.56 (C-11), 130.27 (C-10), 13.62 (C-12), 163.29 (C-1). The data of HMBC, HSQC and COSY of  $M_1$  were listed in Table.1.  $M_1$  was identified as 5-ethylidene-8-hydroxy-3,4,5,6,7,8-hexahydro-1H-pyrano[3,4-c]pyridine-1-one (fig. 3), a novel alkaloid.

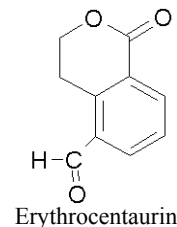
## DISCUSSIONS

*Aspergillus niger* is one of major fungal for production  $\beta$ -D-glucosidase and has been the focus of finding high activity high glucose-tolerant  $\beta$ -D-glucosidase (Gunata and Vallier, 1999; Busto *et al.*, 1997; Seidle and Huber,

2005).  $\beta$ -D-glucosidase from *Aspergillus niger* is prior to other microorganisms in production of  $\beta$ -D-glucosidase activity (Woodward and Wiseman, 1982). *Aspergillus niger* can metabolize some glycosides (Bhattacharya *et al.*, 1976; He, *et al.*, 2006). In this paper, *Aspergillus niger* showed high catalytic activity metabolizing swertiamarin into two metabolites. Swertiamarin was previous reported to transformed into three products e.g. gentianine, erythrocentaurin and 5-hydroxymethyl isochroman-1-one by intestinal bacteria (Yamahara *et al.*, 1991). In our study of biotransformation of swertiamarin, Gentianine and 5-hydroxymethyl isochroman-1-one were not determined in this study but a novel product, 5-ethylidene-8-hydroxy-3,4,5,6,7,8-hexahydro-1Hpyrano[3,4-c]-pyridine-1-one was separated and purified from the biotransformation culture.



5-ethylidene-8-hydroxy-3,4,5,6,7, 8-hexahydro-1H-pyrano-[3,4-c]pyridine-1-one



**Fig. 3:** Structures of 5-ethylidene-8-hydroxy-3,4,5,6,7,8-hexahydro-1H-pyrano-[3,4-c] pyridine-one and Erythrocentaurin

The reason of the different in metabolites is not clear and should be further investigated. According to the structure of swertiamarin, it is readily be catalytic hydrolyzed by  $\beta$ -D-glucosidase into aglycone (El-Sedawy *et al.*, 1988) which will be further metabolized by enzymes, e. g. hydroxylase, from cells into other metabolites. However, the corresponding aglycone was not determined in the culture. This may be explained that the reaction of hydrolysis catalyzed by  $\beta$ -D-glucosidase is the rate-limiting reaction, and once the aglycone is produced, it was catalytic transformed by e. g. hydroxylase or CYP<sub>450S</sub> existing in *Aspergillus niger*. Basing on this hypothesis, it could be further concluded that in order to raise the transformation efficiency, the activity of  $\beta$ -D-glucosidase in the culture should be kept at a higher level during biotransformation and the optimization of biotransformation condition should be centered around how to make the activity of  $\beta$ -D-glucosidase as possible as high.

**Table:** The data of 2D NMR of HMBC, HSQC and COSY of M1

	$\delta_H$	HMBC(H→C)	HSQC (H→C)	COSY(H→H)
H-7	4.23(H <sup>a</sup> )	C-5 C-11	C-7	H-6
H-1	4.46	C-3 C-8 C-9 C-5	C-1	-
H-3	5.52	C-1 C-4 C-5	C-3	-
H-6	2.60	C-7 C-4 C-5	C-6	H-7
H-7	4.33(H <sup>b</sup> )	C-5 C-11	C-7	H-6
H-8	6.11	C-1 C-5 C-10	C-8	H-10
H-10	1.77	C-8 C-9 C-5	C-10	H-8

## CONCLUSION

*Aspergillus niger* was found being a high effective strain metabolizing swertiamarin and valid methods for transformation of swertiamarin, for the determination of the two metabolites, for the preparative scale biotransformation and isolation of metabolites have been successfully developed. The structures of the two metabolites were identified as erythrocentaurin and 5-ethylidene-8-hydroxy-3,4,5,6,7, 8-hexahydro-1H-pyrano-[3,4-c]pyridine-1-one. The latter was a novel alkaloid.

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