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# Biotransformation of 20(*S*)-protopanaxadiol by *Aspergillus niger* AS 3.1858

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ABSTRACT

The biotransformation of 20(*S*)-protopanaxadiol (**1**) by *Aspergillus niger* AS 3.1858 was conducted. Seven metabolites 26-hydroxyl-20(*S*)-protopanaxadiol (**2**); 23, 24-en-25-hydroxyl-20(*S*)-protopanaxadiol (**3**); 25, 26-en-20(*S*)-protopanaxadiol (**4**); (*E*)-20, 22-en-25-hydroxyl-20(*S*)-protopanaxadiol (**5**); 25, 26-en-24(*R*)-hydroxyl-20(*S*)-protopanaxadiol (**6**); 25, 26-en-24(*S*)-hydroxyl-20(*S*)-protopanaxadiol (**7**); and 23, 24-en-25-ethoxyl-20(*S*)-protopanaxadiol (**8**) were afforded. Among them, **6**, **7**, and **8** are new compounds. The chemical structures of these metabolites were elucidated based on extensive spectral data including 2D NMR and HRMS. In addition, the cytotoxicity of substrate and all transformed products was evaluated by MTT assay using a panel of seven human tumor cell lines (Du-145, Hela, K562, K562/ADR, SH-SY5Y, HepG2, and MCF-7 cells) and one normal cell line Vero.



## 1. Introduction

20(*S*)-Protopanaxadiol (**1**) is the glycone of ginsenosides such as ginsenoside $Rb_1$, $Rb_2$, Rc, Rd, $Rg_3$, and $Rh_2$, and exhibits powerful pleiotropic anti-cancer effects in several cancer cell lines including the inhibition of metastasis [1,2]. Previous investigations have found that 20(*S*)-protopanaxadiol had the most potent anticancer activity among known ginsenosides [3]. It has been paid more attention for its distinguished anti-cancer activity and significant inhibition of P-glycoprotein in tumor cells, and extremely low toxicity [4]. Furthermore, 20(*S*)-protopanaxadiol could induce apoptosis through mitochondria-mediated apoptotic pathway in Caco-2, U937, THP-1, and SMMC7721 cancer cells [5]. It also could induce programmed cell death in two human glioma cell lines through caspase-dependent and independent pathways [6]. In 2008, it has been reported to be successfully entered phase I clinical trials as an anticancer drug candidate [7]. Therefore, structural modification of 20(*S*)-protopanaxadiol may be of value to obtain new chemical derivatives for improving the biological activities and physical–chemical properties, or preparing the metabolic standard substances for the further metabolism of human.

Nowadays, microbial transformation is regarded as an effective and useful technology in modification of nature products for finding the new chemical derivatives with the potent bioactivities and physical–chemical characteristics. It exhibited many advantages such as stereo- or regioselectivity, mild reaction conditions and avoiding complex protection and deprotection steps over chemical synthesis [8,9]. In our previous research work, microbial biotransformation of some bioactive natural products as the unique and inexpensive resources has been widely reported to modify their structures and obtain some new chemical entities for improving the solubilities and biological activities [10–13].

In the present work, biotransformation of 20(*S*)-protopanaxadiol by *Aspergillus niger* AS 3.1858 was carried out. Seven metabolites including three new transformed products were isolated and identified. In addition, the cytotoxicities of compounds **1**–**8** against Du-145, Hela, Vero, K562, K562/ADR, SH-SY5Y, HepG2, and MCF-7 cells were evaluated by MTT assay.

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## 2. Experimental

### 2.1. General experimental procedures

1D and 2D NMR spectra were carried out on a Bruker DRX-500 spectrometer operating at 500 MHz (for $^1$H) and 125 MHz (for $^{13}$C) using pyridine-$d_5$ as solvent and internal reference. Chemical shifts are expressed in $\delta$ (ppm) and coupling constants ($J$) were given in hertz (Hz). HR-ESI-MS was recorded on a Finnigan LCQ$^{DECA}$ instrument (Thermo Finnigan, San Jose, CA, USA). Optical rotations in $CH_3OH$ were measured on a Perkin-Elmer 341 polarimeter. IR spectra were measured on a Nicolet 5700 FT-IR microscope spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Reversed-phase preparative HPLC was performed on an Shimadzu LC-20A instrument with a Thermo $C_{18}$ column (250 mm × 10 mm, i.d. 5 μm) and a Shimadzu SPD-20A detector at the flow rate 3.0 mL/min. Column chromatography was carried out on silica gel (300–400 mesh; Qingdao Oceanic Chemicals, China). Analytical TLC was carried out on pre-coated silica gel GF-254 plates (0.25 mm thick, Qingdao Oceanic Chemicals, China), and the developing solvent was chloroform–ethanol (10:1, v/v). The visualization of TLC plates was performed by spraying with 10% $H_2SO_4$ in EtOH followed by heating at 120 °C. All solvents were of analytical reagent grade (Sinopharm Chemical Reagent Co., China).

### 2.2. Substrate and organisms

The substrate 20(*S*)-protopanaxadiol (with purity >98%) was purchased from Shanghai Tauto Biotech Co., Ltd. in China. The fungal strain, *A. niger* AS 3.1858, was purchased from China General Microbiological Culture Collection Center.

### 2.3. Preliminary screening test

Preliminary screening scale biotransformation was carried out in 250 mL Erlenmeyer flasks containing 100 mL of potato dextrose medium (potato 200 g/L, glucose 20 g/L, water 1 L). The flasks were placed on a rotary shaker operating at 160 rpm at 26 °C. A standard two-stage fermentation protocol was employed in all experiments. After 2 days of incubation, the substrates 2 mg (dissolved in 0.5 mL ethanol) were added into each flask. These flasks were maintained under fermentation conditions for 10 days, and then the cultures were pooled and filtered. The filtrates were extracted three times with equal volumes of EtOAc, and the extractions were evaporated in vacuo and analyzed by TLC and HPLC. Culture controls consisted of fermentation blanks in which fungi were grown without substrate but fed with the same amount of ethanol.

### 2.4. Preparative biotransformation

The preparative scale biotransformation of **1** was carried out in forty 1000 mL flasks each containing 400 mL of potato dextrose medium. The fungus was incubated for 2 days before 20 mg of substrate **1** (1 mL, ethanol) was fed to each flask. Incubation conditions and the extraction process were the same as described above and afforded a crude extract (2.2 g). The extract was subjected to column chromatography on a silica gel column (80.0 g), with $CH_2Cl_2$–EtOH (50:1–1:1) as solvent, which yielded fractions A–D. Fraction A was purified by preparative HPLC with MeOH:$H_2O$ = 90:10 to yield metabolite **2** (16.2 mg) and metabolite **3** (14.4 mg). Fraction B was purified using HPLC with MeOH:$H_2O$ = 83:17 to afford metabolite **4** (17.2 mg), metabolite **5** (11.3 mg), metabolite **6** (8.1 mg) and metabolite **7** (13.4 mg). Fraction C was subjected to further chromatography on HPLC with MeOH:$H_2O$ = 87:13 to yielded metabolite **8** (19.2 mg).

#### 2.4.1. 25, 26-en-24(R)-hydroxyl-20(S)-protopanaxadiol

White solid; $[\alpha]_D^{20}$ +16° (*c* 0.14, MeOH); IR (KBr) $\nu_{max}$ 3319, 2958, 1423, 1054 cm$^{-1}$; The $^1$H NMR (pyridine-$d_5$, 500 MHz) and $^{13}$C NMR (pyridine-$d_5$, 125 MHz) spectroscopic data see Table 1; HR-ESI-MS *m/z* 499.3765 $[M + Na]^+$ (calcd for $C_{30}H_{52}O_4Na$, 499.3763).

#### 2.4.2. 25, 26-en-24(S)-hydroxyl-20(S)-protopanaxadiol

White amorphous powder; $[\alpha]_D^{20}$ +42° (*c* 0.17, MeOH); IR (KBr) $\nu_{max}$ 3309, 2925, 1408, 1102 cm$^{-1}$; The $^1$H NMR (pyridine-$d_5$, 500 MHz) and $^{13}$C NMR (pyridine-$d_5$, 125 MHz) spectroscopic data see Table 1; HR-ESI-MS *m/z* 499.3772 $[M + Na]^+$ (calcd for $C_{30}H_{52}O_4Na$, 499.3763).

#### 2.4.3. 23, 24-en-25-ethoxyl-20(S)-protopanaxadiol

White amorphous powder; $[\alpha]_D^{20}$ +32° (*c* 0.11, MeOH); IR (KBr) $\nu_{max}$ 3243, 2967, 1427, 1089 cm$^{-1}$; The $^1$H NMR (pyridine-$d_5$, 500 MHz) and $^{13}$C NMR (pyridine-$d_5$, 125 MHz) spectroscopic data see Table 1; HR-ESI-MS *m/z* 527.4081 $[M + Na]^+$ (calcd for $C_{32}H_{56}O_4Na$, 527.4076).

### 2.5. Cytotoxicity assay

Seven cancer cell lines, Du-145, Hela, K562, K562/ADR, SH-SY5Y, HepG2, MCF-7 and one normal cell line Vero, were kindly provided by Shanghai Jiao Tong University. All cells were maintained in RPMI 1640 medium or DMEM, supplemented with 10% (v/v) neonatal bovine serum or 10% fetal bovine serum. The culture was maintained at 37 °C, 5% $CO_2$ and grown in 96-well microtiter plates for the assay. All media were supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin.

The cytotoxic activity in vitro was measured using the MTT assay. The MTT solution (10.0 mL/well) was added in culture media after cells were treated with various concentrations of compounds for 72 h, and cells were incubated for further 4 h at 37 °C. The purple formazan crystals were dissolved in 100 mL DMSO. After 10 min, the plates were read on an automated microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT) at 570 nm and 630 nm. Assays were performed in triplicate on three independent experiments. The concentration required for 50% inhibition of cell viability ($IC_{50}$) was calculated using the software "Dose–Effect Analysis with Microcomputers". The tumor cell lines panel consisted of Du-145, Hela, K562, K562/ADR, SH-SY5Y, HepG2, and MCF-7. In all of these experiments, three replicate wells were used to determine each point.

## 3. Results and discussion

The biotransformation of 20(*S*)-protopanaxadiol (**1**) by *A. niger* AS 3.1858 yielded four known metabolites and three