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Biotransformation of 20(*S*)-protopanaxatriol by *Aspergillus niger* and the cytotoxicity of the resulting metabolites

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ABSTRACT

The microbial transformation of 20(*S*)-protopanaxatriol by cell suspension cultures of *Aspergillus niger* AS 3.1858 yielded metabolites **1**–**13**. The chemical structures of these transformed products were elucidated based on various spectroscopic analyses, including 1D and 2D NMR and HRESIMS. Metabolites **3**, **11**, and **13** are new compounds. Furthermore, metabolite **3** exhibited relative better activity profile toward the tested seven cancer cell lines (Du-145, Hela, K562, K562/ADR, SH-SY5Y, HepG2, and MCF-7) than substrate and preliminary structure-activity relationships were concluded.

1. Introduction

Ginseng, the root of *Panax ginseng* (Araliaceae), is a valuable traditional medicine and functional food. The beneficial effects of ginseng on diabetes, cardiovascular disease remedy as well as prevention cancer are well documented (Shibata, 2001; Surh et al., 2001). It is generally believed that ginsenosides and their metabolites are mainly responsible for the pharmacological activities of ginseng (Hsu et al., 2013; Jia et al., 2009). 20(*S*)-Protopanaxatriol (**PPT**) is one of the aglycone of ginsenosides such as ginsenoside Rg_1, Re and Rf, and exhibits strong cytotoxic activity against human leukemia THP-1 cells, Int-407 cells and Caco-2 cells (Hasegawa et al., 2002; Popovich and Kitts, 2002, 2004). Therefore, structural modification of 20(*S*)-protopanaxatriol may be of valuable to obtain new chemical derivatives for pharmacological screening. However, it seems very difficult to modify this compound with dammarane skeleton by conventional chemical method.

Biotransformation is regarded as an effective and useful technology in modification of natural products for finding the new chemical derivatives with the potent bioactivities and different physical–chemical characteristics (Bhatti and Khera, 2014; Muffler et al., 2011; Parra et al., 2009). It exhibited many advantages such as stereo- or regioselectivity, mild reaction conditions, avoiding complex protection, and deprotection steps over chemical synthesis (Deng et al., 2012; Li et al., 2011; Lv et al., 2013). In recent years, our research group has frequently reported structural modifications of natural functional products to obtain some new chemical entities for improving the solubilities and biological activities (Chen et al., 2007, 2008, 2013a, 2013b, 2013c). In present work, biotransformation of 20(*S*)-protopanaxatriol by *Aspergillus niger* AS 3.1858 was carried out. Thirteen metabolites, including three new transformed products, were isolated and identified (Fig. 1). In addition, the cytotoxicities of all transformed products were evaluated by using Du-145, Hela, K562, K562/ADR, SH-SY5Y, HepG2, MCF-7, and Vero cells and preliminary structure-activity relationships were concluded.

2. Results and discussion

The biotransformation of 20(*S*)-protopanaxatriol (**PPT**) with *A. niger* AS 3.1858 for five days yielded thirteen metabolites. Their chemical structures were identified as 12-oxo-20(*S*)-protopanaxatriol (**1**), 29-hydroxy-20(*S*)-protopanaxatriol (**2**), 24-methylene-20(*S*)-protopanaxatriol (**3**), (20*S*,24*R*)-epoxy-dammaran-3β,6α,12β,25-tetrol (**4**), (20*S*,24*S*)-epoxy-dammaran-3β,6α,12β,25-tetrol (**5**), 12-oxo-23β-hydroxy-20(*S*)-protopanaxatriol (**6**), 12-oxo-26-hydroxy-20(*S*)-protopanaxatriol (**7**), 12-oxo-27-hydroxy-20(*S*)-protopanaxatriol (**8**), 12-oxo-15α-hydroxy-20(*S*)-protopanaxatriol (**9**), 12-oxo-11β-hydroxy-20(*S*)-protopanaxatriol (**10**),

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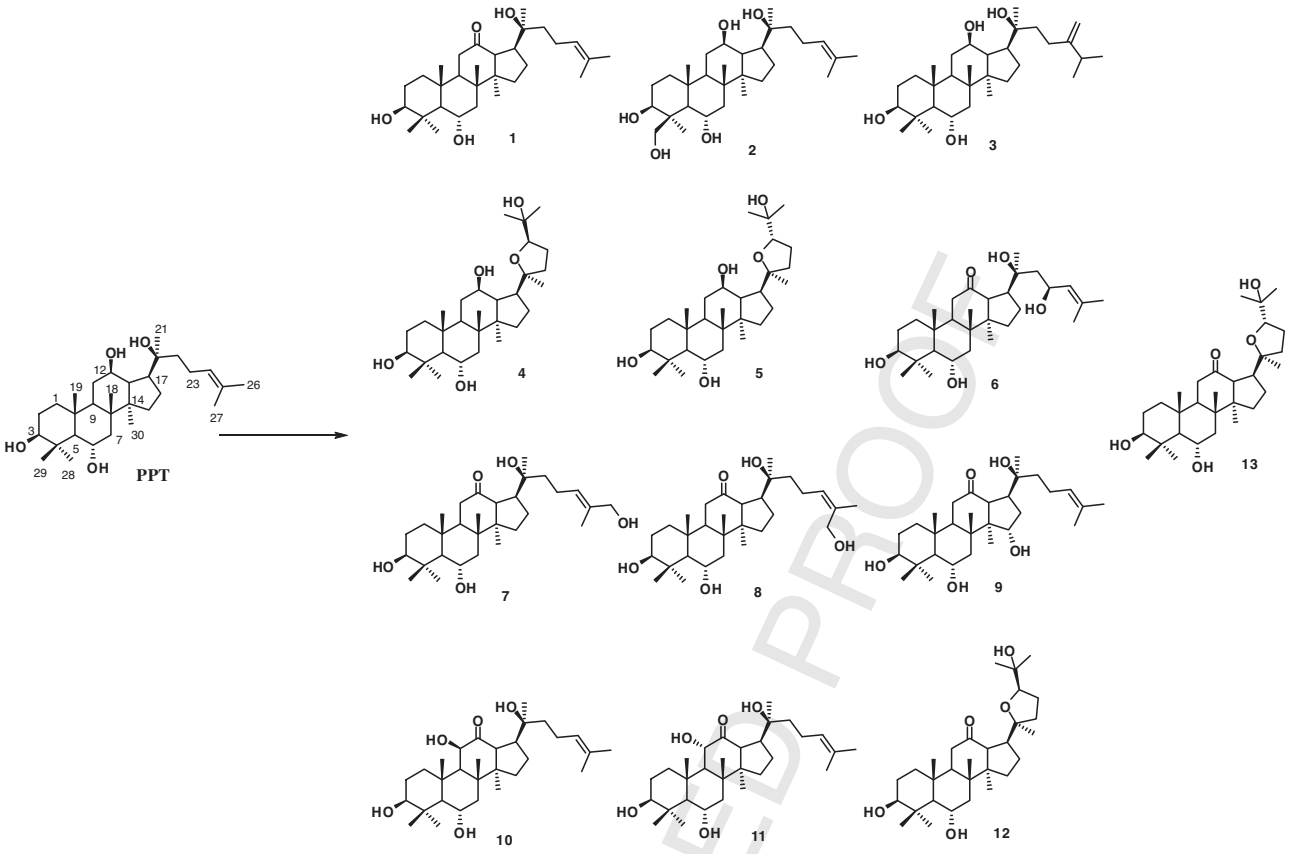

Fig. 1. Biotransformation of 20(*S*)-protopanaxatriol by *Aspergillus niger* AS 3.1858.

12-oxo-11α-hydroxy-20(*S*)- protopanaxatriol (**11**), (20*S*,24*R*)-epoxy-3β,6α,25-trihydroxy-dammarane-12-one (**12**), and (20*S*, 24*S*)-epoxy-3β,6α,25-trihydroxy-dammarane-12-one (**13**). Among them, **3**, **11**, and **13** were elucidated as new compounds by mass spectroscopic analyses. The ^{1}H and ^{13}C NMR signals of all new compounds were fully assigned based on their HSQC, HMBC, and NOESY spectra, as listed in Tables 1 and 2.

2.1. Identification of metabolites *3*, *11*, and *13*

Compound **3** was obtained as white amorphous powder (MeOH). Its molecular formula was determined to be $C_{31}H_{54}O_4$ on the basis of HRESI-MS. Compared to the ^{1}H NMR spectrum of **PPT**, the specific signal of H-24 at about 5.17 ppm was disappearing, along with two new methylene protons at δ_H 4.77 (1H, s) and 4.71 (1H, s) that were observed. According to ^{13}C NMR, DEPT and HSQC spectra of **3**, the presence of two methylene carbon signals at δ_C 106.9 and 156.8, and a new methane carbon signal at δ_c 34.2, was confirmed. In the HMBC spectrum, two methylene protons at δ_H 4.77 and 4.71 (H-1′) correlated with the methane carbon at δ_C 34.2 (C-25), the methyl protons at δ_H 1.04 (H-26) and 1.05 (H-27) correlated with the carbons at δ_C 156.8 (C-24) and 34.2 (C-25) (Fig. 2). These evidences implied that the methylene group was at C-24. Consequently, compound **3** was identified as 24-methylene-20(*S*)-protopanaxatriol.

Compound **11** was isolated as a colorless powder (MeOH). The molecular formula was determined to be $C_{30}H_{50}O_5$ on the basis of HRESI-MS. By comparing with the ^{13}C NMR spectrum of **PPT**, a new carbonyl signal at δ_C 211.7 and a new oxygenated methane signal at δ_C 77.5 were observed which indicated that **11** was a hydroxylated and oxidized product of **PPT**. In the HMBC spectrum, carbonyl signal showed long-range correlations with H-11 (δ_H 3.91, t, J = 10.5 Hz), H-13 (δ_H 2.87, d, J = 9.5 Hz) and H-17 (δ_H 2.31) which suggested that the position of the carbonyl group was at C-12. Analysis of the HMBC data showed that hydroxylation had taken place at C-11, because its corresponding proton signal (δ_H

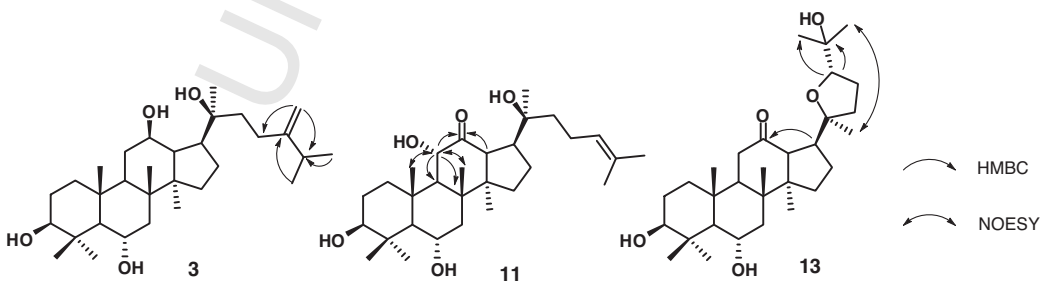

Fig. 2. Key 2D NMR correlations of metabolites **3**, **11**, and **13**.

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3.91) had correlations with C-8 (δ_C 44.9), C-9 (δ_C 61.4), and C-13 (δ_C 50.1) (Fig. 2). The NOE enhancement of H-11 with H-18 (δ_H 1.34) and H-19 (δ_H 1.03) indicated the α-configuration for 11-OH. From the above results, **11** was determined as 12-oxo-11α-hydroxy-20(*S*)-protopanaxatriol.

Compound **13** was isolated as a colorless powder (MeOH). HRESIMS suggested a molecular formula of $C_{30}H_{50}O_5$. Compared with **PPT**, ^{13}C NMR spectrum of **13** exhibited an additional carbonyl signal at δ_C 210.9. In the HMBC experiment, this carbonyl signal showed long-range correlation with H-17 (δ_H 2.53) (Fig. 2). In addition, the chemical shift of C-11 (δ_C 40.0) and C-13 (δ_C 57.2) enabled it to be assigned to C-12. Further compared ^{13}C NMR data with **PPT** showed that significant differences were in the carbon signals from C-20 to C-27. The side-chain double bond (C24–C25) in **PPT** was replaced with a new oxygenated quaternary resonance (δ_C 70.3) and a new oxygenated tertiary signal (δ_C 88.0). The C-20 resonated at a much lower field (δ_C 85.5) and NOE enhancements were observed between 21-CH_3 (δ_H 1.03) and H-26 (δ_H 1.09), and H-27 (δ_H 1.19), indicating cyclization of the side-chain. The structure and stereochemistry of the side-chain was established by comparison of the ^1H NMR and ^{13}C NMR spectra of **13** with data reported in the literature (Zhou et al., 2013). Comparison of the ^{13}C NMR spectrum of **13** with that of (20S,24S)-epoxy-3β, 25-dihydroxy-dammaran-12-one showed a close similarity between the C-20 and C-27 data. Thus, the structure of **13** was determined as (20*S*,24*S*)-epoxy-3β,6α,25-trihydroxy-dammarane-12-one.

2.2. Biotransformation characteristics

In the biotransformation of 20(*S*)-protopanaxatriol by *A. niger* AS 3.1858, it was observed that the dehydrogenation, hydroxylation, and side-chain oxidation–reduction were the main reaction type. The 12β-hydroxyl groups were selectively dehydrogenated into carbonyl groups, while the 3β-OH and 6α-OH remained intact. These results indicated that enzymes in the *Aspergillus* culture could specifically and efficiently catalyze dehydrogenation of 12β-OH. Moreover, these enzymes could catalyze hydroxylation of methylene (C-11, C-15 or C-23) and methyl (C-26, C-27 or C-29) groups. These highly specific reactions may be difficult in chemical synthesis. In addition, we found a new methylene reaction which has never been reported in the biotransformation of dammarane-type compounds.

2.3. Cytotoxicity testing

The cytotoxic activities of thirteen products were evaluated with seven cancer cell lines Du-145, Hela, K562, K562/ADR, SH-SY5Y, HepG2, and MCF-7 and one normal cell line Vero. The results expressed as IC_{50} values are summarized in Table 3.

As illustrated in Table 3, metabolite **3** exhibited relative better activity profile toward the tested seven cancer cell lines than 20(*S*)-protopanaxatriol. Among the tested cell lines, metabolite **3** displayed the most potent cytotoxicity against Hela, K562, K562/ADR, and SH-SY5Y cell, with IC_{50} values of 7.3 μmol/L, 12.6 μmol/L, 9.9 μmol/L, and 11.4 μmol/L. This result indicated that the 24-methylene side-chain would increase the cytotoxicities. The metabolite **10** showed the lower potent inhibitory effects against tested cells than metabolite **1**, which suggested that the hydroxylation at C-11β could significantly reduce the cytotoxicities. The results in Table 3 also shown that metabolite **11** had more potent cytotoxicity toward the all tested cell lines than metabolite **10**. This result suggested that the hydroxylation at C-11α would increase the activities. The pharmacological significance of new biotransformed products obtained in this study will be further evaluated.

3. Experimental

3.1. General experimental produces

1D and 2D NMR spectra were carried out on a Bruker DRX-500 spectrometer operating at 500 MHz (for ^1H) and 125 MHz (for ^{13}C) using $CDCl_3$ as solvent and internal reference. HRESI-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. Reversed-phase preparative HPLC was performed on a Shimadzu LC-20A instrument with a Thermo C_{18} column (250 mm × 10 mm, i.d. 5 μm) and a Shimadzu SPD-20A detector. Melting points were measured on an XT4A apparatus (Dianguang Corp., Shanghai, China) and were uncorrected. All organic solvents were of analytic grade and were obtained from Sinopharm Chemical Reagent Co., (China). For HPLC experiments, chromatographic grade acetonitrile and methanol (Merck, Darmstadt, Germany) were used. Thin-layer chromatography (TLC) analyses were carried out on pre-coated silica gel GF-254 plates (0.25 mm thick), which was purchased from Qingdao Marine Chemical Corporation, China. Octadecylsilane (ODS) was purchased from YMC Co., Ltd. (Kyoto, Japan).

3.2. Substrate

20(*S*)-protopanaxatriol (500 mg) was purchased from Shanghai Tauto Biotech Co., Ltd in China. Its purity was determined to be 98% by HPLC analysis.

3.3. Microorganisms

Aspergillus niger AS 3.1858 was purchased from the Chinese General Microbiological Culture Collection Center, Beijing, China. All of the culture and biotransformation experiments were performed in liquid potato medium. Minced and husked potato (200 g) was boiled in water for 0.5 h, and the solution was filtered. The filtrate was added to 1 L of water after adding 20 g of glucose.

3.4. Biotransformation procedures

Preliminary screening scale biotransformation was carried out in 250 mL Erlenmeyer flasks containing 100 mL of potato dextrose medium. 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 5 days. Culture controls consisted of fermentation blanks in which fungi were grown without substrate. Substrate controls were composed of sterile medium with substrate, and they were incubated without microorganisms. The preparative scale biotransformation of **PPT** was carried out in twenty 1000 mL flasks each containing 400 mL of potato dextrose medium. The fungus was incubated for 2 days before 20 mg of substrate **PPT** (1 mL, ethanol) was fed to each flask. Incubation conditions and the extraction process were the same as described above.

3.5. Extraction, purification and identification of biotransformed products

The culture was filtered and the filtrate was extracted three times with equal volumes of ethyl acetate (EtOAc), and the extractions were evaporated in vacuo and afforded a crude extract (1.3 g). The extract was subjected to column chromatography on a silica gel column (60.0 g), with CH_2Cl_2-EtOH (30:1–1:1) as solvent, which yielded fractions A-G. Fraction B was purified by semi-preparative

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HPLC (mobile phase: 55% acetonitrile; flow rate: 1.5 mL/min) to yield metabolite **1** (8.6 mg). Fraction C was purified using semi-preparative HPLC (mobile phase: 60% methanol; flow rate: 3.0 mL/min) to afford metabolite **2** (11.4 mg). Purification of fraction D by semi-preparative HPLC with 64% acetonitrile as mobile phase and flow rate of 2.0 mL/min yielded compounds **3** (10.2 mg), **4** (9.8 mg), and **5** (7.5 mg). Fraction E was purified by semi-preparative HPLC with 80% methanol as mobile phase and flow rate of 1.5 mL/min led to **6** (9.3 mg), **7** (8.9 mg), **8** (12.3 mg), and **9** (10.5 mg). Fraction F was further subjected to semi-preparative HPLC with 68% acetonitrile as mobile phase and flow rate of 1.5 mL/min yielded to **10** (14.4 mg), **11** (5.7 mg), **12** (9.3 mg), and **13** (6.8 mg).

*3.5.1. 24-Methylene-20(S)-protopanaxatriol (**3**)*

White amorphous powder, m.p. 152–154 °C, $[\alpha]_D^{22}$ +48.3° (c = 0.1, MeOH). ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) see Tables 1 and 2. HRESIMS $[M+Na]^+$ m/z 513.3927 (calcd. for $C_{31}H_{54}O_4Na$, 513.3920).

*3.5.2. 12-oxo-11α-hydroxy-20(S)-protopanaxatriol (**11**)*

Colorless powder, m.p. 137–139 °C, $[\alpha]_D^{22}$ +46.5° (c = 0.1, MeOH). ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) see Tables 1 and 2. HRESIMS $[M+Na]^+$ m/z 513.3558 (calcd. for $C_{30}H_{50}O_5Na$, 513.3556).

*3.5.3. (20S,24S)-epoxy-3β,6α,25-trihydroxy-dammarane-12-one (**13**)*

Colorless powder, m.p. 127–129 °C, $[\alpha]_D^{22}$ +38.6° (c = 0.1, MeOH). ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) see Tables 1 and 2. HRESIMS $[M+Na]^+$ m/z 513.3553 (calcd. for $C_{30}H_{50}O_5Na$, 513.3556).

3.6. Bioassay

Seven cancer cell lines, Du-145, Hela, K562, K562/ADR, SH-SY5Y, HepG2, MCF-7 and one normal cell line Vero, 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 survival rates of the cancer cells were evaluated by the MTT method. Cytotoxicities were determined as IC_{50} values, namely, the concentration of test compounds required to provide 50% inhibition of cell growth. The results were expressed as the mean value of triplicate determinations.

4. Conclusions

The incubation of **PPT** with *A. niger* AS 3.1858 yielded thirteen products in total, including three new compounds. The enzymatic reactions included dehydrogenation, hydroxylation, and side-chain. In addition, we found a new methylene reaction which has never been reported in the biotransformation of dammarane-type compounds. Thus, biotransformation is a potent approach to diversify the structures of natural products and preparing a variety of derivatives for the search of new lead compounds.

Q4 Uncited references

He et al. (2014), Tian et al. (2005) and Zhang et al. (2007).

Acknowledgements

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