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Short communication

Selective oxidation-reduction and esterification of asiatic acid by *Pestalotiopsis microspora* and anti-HCV activity

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ABSTRACT

Microbial transformation of asiatic acid (AA) by an endophytic fungus, Pestalotiopsis microspora, yielded six metabolites: 2-oxo-3β,15α,23-trihydroxyurs-12-ene-28-oic acid (**1**); 2-oxo-3β,15α,22α,23-tetrahydroxyurs-12-ene-28-oic acid (**2**); 2-oxo-3β,15α,23,30-tetrahydroxyurs-12-ene-28-oic acid (**3**); 2α,3β,15α,23,30-pentahydroxyurs-12-ene-28-oic acid methyl ester (**4**); 2α,3α,15α,23-tetrahydroxyurs-12-ene-28-oic acid (**5**); 2α,3α,15α,23,30-pentahydroxyurs-12-ene-28-oic acid (**6**). The structure elucidation of these products was confirmed based on the spectroscopic data. Compounds 2–6 were new. A possible biotransformation pathway is proposed. The anti-HCV activity of compounds 1–6 was also evaluated.

1. Introduction

Pestalotiopsis microspora is an endophytic fungus that was isolated from the medicinal plant *Huperzia serrata*. Endophytic fungi can produce secondary metabolites that have antibacterial and antitumor pharmacological activities (Ma et al., 2014; Pretsch et al., 2014; Qian et al., 2014; Wang et al., 2011; Xiao et al., 2014). Endophytic fungi also have the ability to transform endogenous and exogenous substances (Fu et al., 2011a; Zeng et al., 2014; Zikmundova et al., 2002). Using endophytic fungi to transform active natural products can enhance the diversity of natural products while providing technology to discover new derivatives with better pharmacological activities. In this way, microbial transformation is used as an important tool for finding new and unusual compounds. Thus, enzymes are considered to be more satisfactory tools than conventional chemical methods from an economical and environmental point of view (Ishige et al., 2005). Some reactions that are difficult to achieve via chemical modification, such as hydroxylation at saturated carbons, were relatively easy via microbial biotransformation. Asiatic acid (**AA**), a pentacyclic triterpene acid, mainly exists in *Centella asiatica.* **AA** has been reported to have anti-tumor (Park et al., 2007; Yun et al., 2008), neuroprotective (Jew et al., 2000), anti-inflammatory and anti-depressant properties. The structural modification of **AA** has been carried out either by chemical methods (Jeong et al., 2007) or by biotransformation (Guo et al., 2013; He et al., 2010; Huang et al., 2012; Wu et al., 2012). The endophytic fungus *Pestalotiopsis microspora* was used to transform asiatic acid in an attempt to obtain some new and useful compounds for further pharmacological study. Herein, we report in this article the selective oxidation, oxidation-reduction and methyl esterification of asiatic acid by the endophytic fungus *Pestalotiopsis microspore.* A possible transformation pathway is described (Fig. 1). The anti-HCV activities of the identified products were also tested.

2. Experimental

2.1. General experimental procedures

NMR spectra were recorded on a Bruker DRX-600 spectrometer operating at 600 MHz (for ^{1}H) and 150 MHz (for ^{13}C) in pyridine-d_5 with tetramethylsilane (TMS) as an internal standard. HR-ESI–MS spectra were measured on a Thermo LTQ Orbitrap XL mass spectrometer. Preparative HPLC was conducted on a SHIMADZU system with an LC-6 AD pump, an SPD-20A UV detector and a Grace Smart RP_{18} column (5 μm, 10 × 250 mm).

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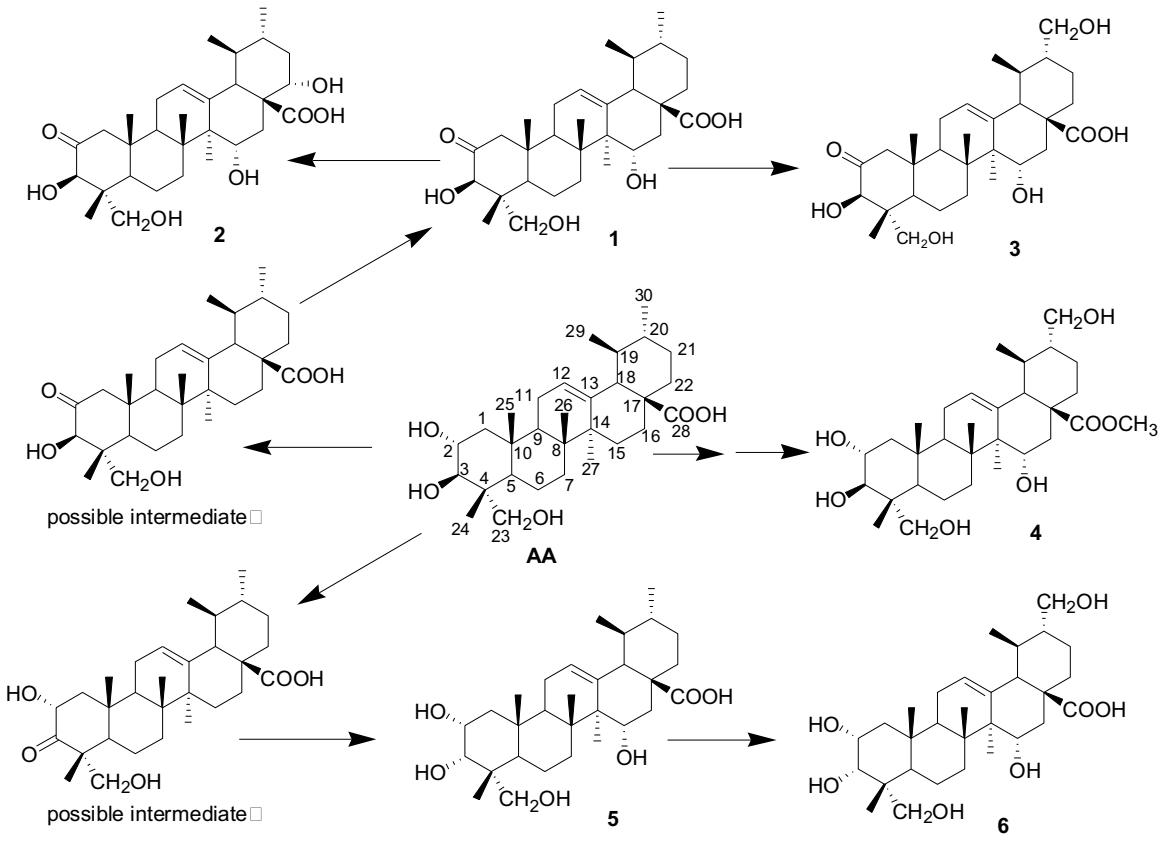

Fig. 1. Microbial transformation of asiatic acid (AA) by *Pestalotiopsis microspora* and its possible transformation pathway.

2.2. Chemicals and materials

The substrate asiatic acid was purchased from Zelang Company (Nanjing, China). Silica gel (200–300 mesh, Qingdao Oceanic Chemicals, Qingdao, China) was used for open column chromatography. GF254 plates (0.25-mm-thick pre-coated silica gel, Qingdao Oceanic Chemicals, China) were used for TLC analyses. HPLC-grade MeOH was purchased from Beijing Chemical Industry Company (Beijing, China). All the general solvents were analytical grade and were purchased from Beijing Chemical Industry Company (Beijing, China).

2.3. Microorganisms and culture medium

Endophytic fungus *Pestalotiopsis microspora* was previously isolated from the medical plant *Huperzia serrata* in our laboratory. From its rDNA- ITS area and morphology characteristics, it was identified as *Pestalotiopsis microspora* (Fu et al., 2011b). All culture media used in the experiments were prepared from potato dextrose broth as follows: approximately 1000 ml of distilled water was added to 200 g of mincing husked potato, and the mixture was boiled for half an hour before it was filtered. Then, 20 g of glucose and distilled water was added to the filtrate to bring the volume to 1000 ml. The medium was sterilized for half an hour at 121 °C before use.

2.4. Biotransformation procedures

The preliminary screening experiments were conducted in 100-ml Erlenmeyer flasks containing 40 ml of potato medium. The seed cultures were added to the Erlenmeyer flasks and incubated on a rotary shaker for 2 days at 28 °C and 160 rpm. The substrate asiatic acid (1.0 mg dissolved in 0.2 ml methanol) was then added to each flask for another 8-day incubation under the same conditions. The cultures were homogenized and then extracted with an equal volume of ethyl acetate (3 times). The extracts were evaporated under reduced pressure on a rotary evaporator (45 °C) and then evaluated by TLC. Two controls were utilized: a substrate control without fungus and a culture control with the fungus but without the substrate. The conditions on preparative scale were the same as described in the preliminary screening experiment except that 1000-ml Erlenmeyer flasks containing 500 ml of medium and 20 mg of substrate were used. Additionally, two batches on a preparative scale were prepared using 400 mg of substrate. The final extract was 700 mg for the first batch and 650 mg for the second batch.

2.5. Isolation of metabolites of endophytic fungus Pestalotiopsis microspora

The crude extract (first batch, 700 mg) was purified using a silica gel column chromatography (200–300 mesh, 20 g) and eluted with chloroform-methanol in a gradient from 40:1 to 0:1. Twenty fractions were collected. Fractions 1–3 (125 mg) were mainly pigments. Fraction 15 (25 mg) was purified by semi-preparative HPLC and eluted with MeOH—H_2O (70:30, v/v) to yield 2 mg of compound (**1**). Fractions 8–10 were combined and purified by semi-preparative HPLC and eluted with MeOH—H_2O (70:30, v/v) to yield 4 mg of compound (**2**). The crude extract (second batch, 650 mg) was purified with silica gel column chromatography (200–300 mesh, 21 g) and eluted with a chloroform/methanol gradient from (40:1) to methanol, with sixteen fractions collected.

Fraction 5 was purified by using HPLC and eluted with MeOH—H_2O (69:31, v/v) to yield 2.5 mg of compound (**5**). Fraction 15 was purified by HPLC eluted with MeOH—H_2O (65:35, v/v) to yield 1.8 mg of compound (**4**) and 2.2 mg of compound (**6**).