Fitoterapia 82 (2011) 1057–1061

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Multihydroxylation of ursolic acid by *Pestalotiopsis microspora* isolated from the medicinal plant *Huperzia serrata*

Shao-bin Fu [a], Jun-shan Yang [a], Jin-long Cui [a], Qing-feng Meng [b], Xu Feng [c], Di-An Sun [a,*]

[a] Institute of Medical Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100193, China
[b] No 1 senior middle school of laiwu, Shandong, 271100, China
[c] Navy Medical Research Institute, Shanghai 200433, China

ARTICLE INFO

Article history:
Received 7 May 2011
Accepted in revised form 22 June 2011
Available online 2 July 2011

Keywords:
Biotransformation
Ursolic acid
Endophytic fungus
Huperzia serrata
Pestalotiopsis microspora

ABSTRACT

The structural modification of ursolic acid by an endophytic fungus *Pestalotiopsis microspora*, isolated from medicinal plant *Huperzia serrata* was reported for the first time. The structure diversity was very important for the SAR study of ursolic acid and its derivatives. Incubation of ursolic acid **1** with *P. microspora* afforded four metabolites: 3-oxo-15α, 30-dihydroxy-urs-12-en-28-oic acid (**2**), 3β, 15α-dihydroxy-urs-12-en-28-oic acid (**3**), 3β, 15α, 30- trihydroxy-urs-12-en-28-oic acid (**4**) and 3,4-seco-ursan-4,30-dihydroxy-12-en-3,28-dioic acid (**5**). All products were new compounds and their structures elucidation was mainly based on the spectroscopic data.

1. Introduction

Endophytes are bacterial or fungal microorganisms which colonize the healthy plant tissue inter- and/or intracellularly without causing any apparent symptoms of disease [1]. Endophytes commonly present in almost all plants are considered as a rich source of bioactive products many of which have been isolated so far. At least 100 thousand endophytes were speculated in nature [2]. Endophytes and host plants formed stable ecological relations through co-evolution. It is reported that endophytes can increase the production of metabolites generated by the hosts [3]. Additionally, endophytes can produce the same active compound [4,5]. In the hope of finding huperzine A, a very important drug for the treatment of Alzheimer disease [6], we isolated over 100 strains of the endophytes from *Huperzia serrata*. Unfortunately, although lots of natural products were obtained, none of them was huperzine A.

Considering the biotransformation ability of the microorganisms and the advantages of microbial transformation [7,8], we try to use these endophytes to transform ursolic acid in an attempt to obtain hydroxylated products.

Ursolic acid, a pentacyclic triterpene acid, is of great interest to scientists and pharmacists because of its cytotoxicity, induction of differentiation, anti-inflammatory anti-mutagenic, antiviral and anti-invasive activities [9]. The structural modifications of ursolic acid have been carried out by some researchers either by chemical methods [10,11] or by microbial transformation [12]. However, there is no report on microbial transformation of ursolic acid by *Pestalotiopsis microspora* from medicinal plant *H. serrata*. We reported here in this article the microbial transformation of ursolic acid by an endophytic fungus isolated from the medicinal plant *H. serrata*.

2. Experimental

2.1. General experimental

Mps were recorded on an X-4 microscopic melt point spectrometer (Beijing Tech instrument Co. Ltd, Beijing, China) that was not corrected. Optical rotations were measured using a Perkin-Elmer 341 apparatus at 509 nm and 20 °C. NMR spectra were acquired with Bruker DRX-600

* Corresponding author. Tel.: +86 10 57833298.
E-mail address: diansun@sina.com (D.-A. Sun).

0367-326X/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.fitote.2011.06.009

spectrometer operating at 600 MHz (for ^{1}H-NMR) and 150 MHz (for ^{13}C-NMR and APT using TMS as internal standard. High resolution mass (HR-ESI-MS) spectra were employed on a Thermo LTQ Orbitrap XL mass spectrometer. Column chromatography was carried out on silica gel (100–200 mesh, 300–400 mesh, Qingdao Oceanic Chemicals, Qingdao, China). TLC was performed on 0.25 mm thick silica prepared plates also from Qingdao Oceanic Chemicals, Qingdao, China. Spot visualizations were made by spraying with H_2SO_4/95% EtOH (9:1, v/v) followed by heating. Preparative HPLC was carried out using a SHIMADZU system with a LC-6 AD pump, a SPD-20A UV detector and a Grace ODS column (5 μm, 10×250 mm). Flow rate used was 3 mL/min and was monitored at 210 nm.

2.2. Substrate

The substrate ursolic acid (with purity >98%) was purchased from Changsha Staherb Natural Ingredients Co. Ltd, Changsha, China. Its structure was characterized by comparison its ^{1}H-NMR and ^{13}C-NMR spectra with those reported in the literature [13].

2.3. Isolation and identification of endophytic fungi

Endophytic fungi were isolated from healthy stems and leaves of medicinal plant *H. serrata* collected from Chongqing, China with a slightly modified method described by Strobel et al. [5]. Endophytes were isolated by a surface sterilization method. Sample leaves and stems of the plant were washed under running water for 2 h, and rinsed 5 times with sterile distilled water. The leaves were dipped with 75% ethanol (v/v) for 1 min and 0.1% mercuric chloride (v/v) for 5 min to be surface-sterilized. The stems were sterilized with 0.1% mercuric chloride (v/v) for 8 min. And then the stems and leaves were rinsed 5 times again in sterilized distilled water. Segments about 1 cm long were aseptically cut with a sterile knife. The cut segments were placed on Petri dishes containing PDA media (PDA media: potato, 200 g; glucose, 20 g; agar, 16 g; distilled water, 1 L) and were incubated at 28 °C in darkness. The Petri dishes with the last sterilized distilled water as the control incubated in the same conditions. After several times purification, cultures were then transferred to PDA slants.

The fungi were identified by the sequence of the nuclear ribosomal internal transcribed region (rDNA-ITS). The universal primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) were used to amplify the ITS regions from the DNA extract. The PCR reaction was performed with the following cycles: (1) 94 °C for 3 min; (2) 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and (3) 72 °C for 10 min.

2.4. Preliminary screening experiments

One hundred endophytic fungi strains from the PDA slants were cultured in PDB liquid medium in 100 mL flasks containing 40 mL of medium at 28 °C with rotary shaking at 160 rpm. The substrate ursolic acid dissolving in DMSO: ethanol = 1:1 (v/v) was added into the media 3 days later. The metabolites were extracted with EtOAc after another 10-days incubation. Culture controls consisted of microorganisms without substrate and substrate controls composed of sterile medium without the microorganisms were incubated in the same conditions as described above.

2.5. Microbial transformation of UA on preparative-scale fermentation

The preparative scale fermentation procedure was carried out in 1000 mL Erlenmeyer flasks with 400 mL of PDB medium on rotary shakers at 160 rpm and 28 °C. The microorganisms were first cultured for 3 days under the conditions described above. Then evenly to the 16 flasks was added 490 mg substrate ursolic acid in total (dissolved in DMSO: ethanol = 1:1 v/v). The cultures were incubated for another 10 days. At last, the mycelia were filtered off and were ultrasonic extracted with EtOAc for 40 min. The broth was extracted with the same solvent three times. The extracts were combined and evaporated to give a crude extract (500 mg).

The crude extracts were subjected to silica gel column chromatography (300–400 mesh, 18 g, Qingdao Oceanic Chemical, Qingdao, China) and eluted with mixtures of petroleum ether/acetone/acetic acid (100/2.5/0.1) to acetone. Fraction A (28 mg), fraction C (49 mg) and fraction D (37 mg) were obtained. 41 mg (8.4%) of compound **2** were obtained after recrystallization with acetone from fraction C. Fraction A was further purified by HPLC on semi-preparative column (MeOH–H_2O = 88:12) to yield compound **3** (6 mg, 1.2%). Fraction D was purified by using HPLC (MeOH/H_2O = 85/15) to afford compound **4** (8 mg, 1.6%) and **5** (6 mg, 1.2%).

2.6. Compound data

2.6.1. Compound 2

White solid, mp 219–220 °C, [α]20 D +16.7° (c = 2.27×10^{-1}, EtOH) HR-ESI-MS: 485.3268 [M-H]$^-$, (calcd 485.3262), 971.6608 [2 M-H]$^-$. ^{1}H-NMR (500 MHz, pyridine-d_5) and ^{13}C-NMR (150 MHz, pyridine-d_5) see Tables 1 and 2.

2.6.2. Compound 3

White solid, mp 280–282 °C, [α]20 D +29.7° (c = 1.345×10^{-1}, EtOH), HR-ESI-MS: 471.3478 [M-H]$^-$, (calcd 471.3469), 943.7047 [2 M-H]$^-$. ^{1}H-NMR (500 MHz, pyridine-d_5) and ^{13}C-NMR (150 MHz, pyridine-d_5) see Tables 1 and 2.

2.6.3. Compound 4

White solid, mp 284–285 °C, [α]20 D +16.8° (c = 1.19×10^{-1}, EtOH), HR-ESI-MS: 487.3427 [M-H]$^-$ (calcd 487.3418). ^{1}H-NMR (500 MHz, pyridine-d_5) and APT (150 MHz, pyridine-d_5) see Tables 1 and 2.

2.6.4. Compound 5

White solid, mp 277–278 °C, [α]20 D +21.3° (c = 1.64×10^{-1}, EtOH), HR-ESI-MS: 503.3372 [M-H]$^-$, (calcd 503.3367), 1007.6817 [2 M-H]$^-$. ^{1}H-NMR (500 MHz, pyridine-d_5) and APT (150 MHz, pyridine-d_5) see Tables 1 and 2.