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A novel dihydroxylated derivative of artemisinin from microbial transformation

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ABSTRACT

Microbial transformation of artemisinin (**1**) by *Cunninghamella elegans* was investigated. Four isolated products were identified as 6β-hydroxyartemisinin (**2**), 7α-hydroxyartemisinin (**3**), 7β-hydroxyartemisinin (**4**), and 6β,7α-dihydroxyartemisinin (**5**). The structures were elucidated by spectroscopic and X-ray crystallographic analysis. Product **5** is a novel compound and being reported here for the first time. It features two hydroxyl groups in its structure, and this is the first report on dihydroxylation of the artemisinin skeleton. Quantitative structure-activity relationship and molecular modeling studies indicate the modification of artemisinin skeleton will increase antimalarial activity and water solubility. The chemical syntheses of artemisinin derivatives at C6 or C7 position are impossible due to the lack of functional groups. 6β,7α-Dihydroxyartemisinin is hydroxylated at both 6β- and 7α-positions of artemisinin skeleton at the same time. Therefore, this new compound would be a good scaffold for further structural modification in the search for more potent antimalarial drugs.

1. Introduction

Artemisinin (Fig. 1) **1**, a sesquiterpene lactone endoperoxide and an antimalarial drug, is effective against chloroquine-resistant parasites; but its toxicities and low solubility in water hamper its therapeutical use. Quantitative structure-activity relationship (QSAR) studies suggested that structural modification of artemisinin skeleton may provide more effective antimalarial agents [1,2]. The complexity of the natural molecule makes many synthetic modifications impossible, but there are reports showing that artemisinin is transformed to a variety of derivatives by microorganisms [3–9].

In this work, we report the bioconversion of **1** by *Cunninghamella elegans* ATCC 9245. Four hydroxylated products were isolated, among which one was identified as a novel dihydroxylated compound. It is the first report on dihydroxylation of artemisinin by microbial transformation. This new compound could be used as a new lead molecule for further structural modification by biological and chemical methods to discover more effective antimalarial agents.

2. Materials and methods

2.1. General experimental procedures

^{1}H NMR and ^{13}C NMR spectra were recorded with a Bruker DRX-400 spectrometer at 400 and 100 MHz, respectively. HRMS (high-resolution mass spectra) were obtained with a Thermo LTQ Orbitrap XL mass spectrometer. The X-ray diffraction experiments were performed using a Bruker SMART CCD diffractometer with Cu Kα radiation. The crystals of metabolites were obtained from ethanol by slow evaporation of solvent at room temperature.

Methanol (MeOH) was used to dissolve the residue for analysis by HPLC using a Waters 2690 instrument with a Phenomenex Gemini

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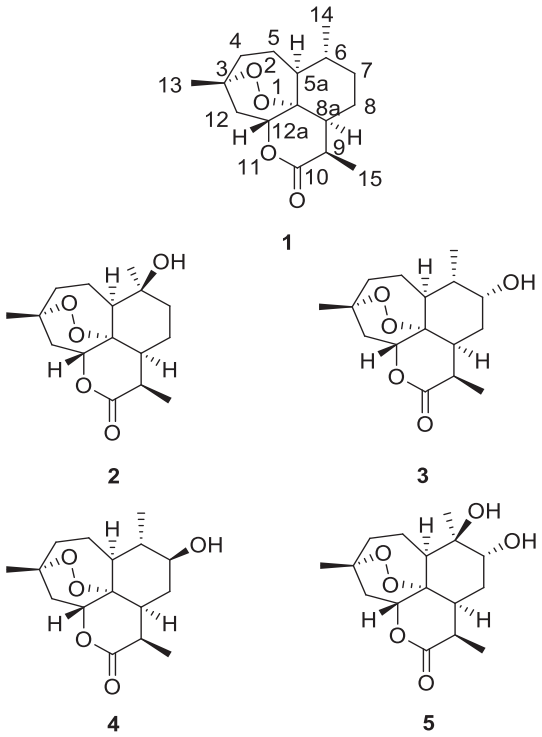

Fig. 1. Structures of artemisinin and four metabolites from biotransformation of artemisinin by *Cunninghamella elegans*.

column (5 μm NX-C18 110 Å, 250 × 4.6 mm) and a PL-ELS 2100 evaporative light scattering (ELS) detector. The detector gas flow rate was 1.0 SLM, and the evaporator and nebulizer temperatures were 70 °C and 50 °C, respectively. The mobile phase were MeOH and H_2O (flow rate 1.00 mL/min) with a gradient increasing from 40% to 90% MeOH in 20 min followed by decreasing to 40% MeOH in 5 min and additional 5 min at 40% MeOH.

2.2. Microorganism

Cunninghamella elegans ATCC 9245 was obtained from American Type Culture Collection.

2.3. Medium

The following medium was used in the culture and bioconversion experiments [10]: 20 g Sabouraud Dextrose Broth (Difco), 10 g peptone, 15 g sucrose, and 1000 mL deionized water.

2.4. Culture and biotransformation procedures

Fungal mycelia of the slants were transferred into 1000-mL shake flasks containing 200 mL of medium. After 48 h of incubation at 28 °C and 180 rpm on a rotary shaker, the cultures were used to inoculate 4-L Erlenmeyer flasks containing 1 L of medium. The inoculated flasks were again incubated for 48 h on rotary shakers at 28 °C and 180 rpm before the addition of artemisinin (Mediplantex, Vietnam). The substrate artemisinin was dissolved in MeOH with a concentration of 25 mg/mL, and 20 mL of the solution was added into each flask. A total of 10 g of substrate was used in the biotransformation. The cultures were incubated under the same condition for an additional 14 days. When the fermentation finished, the mycelia and broth were separated by filtration and the mycelia were discarded. The filtrate was extracted with an equal volume of ethyl acetate (EtOAc) three times, and the extract was evaporated under vacuum to afford a brown residue.

2.5. Chromatographic conditions

The residue was purified by chromatography carried out on silica gel 60, pore size 60 Å, particle size 32–63 μm, using gradient EtOAc-hexanes as mobile phase, eluting from 10 to 50% EtOAc.

3. Results

The bioconversion of artemisinin by *Cunninghamella elegans* gave 955 mg of product **2**, 534 mg of product **3**, 470 mg of product **4**, and 225 mg of product **5**. Their structures were identified by HRMS, ^{1}H NMR, ^{13}C NMR, DEPT 135, ^{1}H−^{1}H COSY, HSQC, HMBC, and X-ray crystallographic analysis.

HPLC analysis of the extract (Fig. 2) showed four metabolites (**2**, **3**, **4** and **5**), eluted at 14.0, 11.3, 12.6 and 9.8 min, respectively, but no substrate artemisinin. The elution time of artemisinin should be 18.5 min (Fig. 2).

The HRMS data of product **2** had an ion at *m/z* 321.1315 [M + Na]$^+$, with 16 mass units more than that of **1**, suggesting the introduction of a hydroxyl group. The ^{13}C NMR spectrum of product **2** showed a new peak at δ 72.2, further confirming that the compound is a hydroxylated product. DEPT 135 analysis of metabolite **2** showed that in comparison to **1**, the number of quaternary carbons changed from three to four, while the number of tertiary carbons changed from five to four, which revealed it is hydroxylation on a tertiary carbon. The methyl group of C-14 appeared as a singlet, so hydroxylation had occurred at C-6. X-ray diffraction experiment revealed metabolite **2** is 6β-hydroxyartemisinin (Fig. 1). The X-ray structure of metabolite **2** is shown in Fig. 3. The ^{1}H and ^{13}C NMR data of metabolite **2** were in good agreement with the literature data [6].

The HRMS data of product **3** had an ion at *m/z* 321.1313 [M + Na]$^+$, with 16 mass units more than that of **1**, which suggested the introduction of a hydroxyl group. The ^{13}C NMR spectrum of product **3** had a new peak at δ 68.7, further confirming that the compound is a hydroxylated product. DEPT 135 analysis of metabolite **3** showed that in comparison to **1** the total number of tertiary carbons changed from five to six, while the number of secondary carbons decreased from four to three, which indicated hydroxylation of a secondary carbon. On the basis of its ^{1}H−^{1}H COSY, HMBC and HSQC spectra, metabolite **3** was identified as 7-hydroxyartemisinin. X-ray diffraction experiment revealed metabolite **3** is 7α-hydroxyartemisinin (Fig. 1). The X-ray structure of metabolite **3** is shown in Fig. 3. The ^{1}H and ^{13}C NMR data of metabolite **3** were in good agreement with the literature data [11].

The HRMS data of product **4** had a peak at *m/z* 321.1312 [M + Na]$^+$, with 16 mass units more than that of **1**, suggesting the introduction of a hydroxyl group. The ^{13}C NMR spectrum of product **4** had a new peak at δ 73.5, further confirming that the compound is a hydroxylated product. DEPT 135 analysis of metabolite **4** showed that the number of tertiary carbons changed from five to six, and the number of secondary carbons changed from four to three, which indicated hydroxylation on a secondary carbon. X-ray diffraction experiment revealed metabolite **4** is 7β-hydroxyartemisinin (Fig. 1). The X-ray structure of metabolite **4** is shown in Fig. 3. The ^{1}H and ^{13}C NMR data of metabolite **4** were in good agreement with the literature data [6].

The HRMS data of metabolite **5** had a peak at *m/z* 337.1284 [M + Na]$^+$, with 32 mass units more than that of **1**, which suggested the introduction of two hydroxyl groups and a molecular formula of $C_{15}H_{22}O_7$. The ^{13}C NMR spectrum of **5** had new peaks at δ 75.1 and 74.4, further confirming the compound is a dihydroxylated product. DEPT 135 analysis of metabolite **5** showed that in comparison to **1**, the number of secondary carbons had changed from four to three, and the number of quaternary carbons changed from three to four, which indicated the hydroxyl groups were introduced at a tertiary carbon and a secondary carbon. The methyl group of C-14 appeared as a singlet, so hydroxylation had occurred at C-6. On the basis of its ^{1}H−^{1}H COSY,