Microbial Metabolism of Artemisinin by *Mucor polymorphosporus* and *Aspergillus niger*

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Artemisinin (**1**) was transformed by *Mucor polymorphosporus* and *Aspergillus niger*. Five products were identified as 9β-hydroxyartemisinin (**2**), 3β-hydroxyartemisinin (**3**), deoxyartemisinin (**4**), 3β-hydroxydeoxyartemisinin (**5**), and 1α-hydroxydeoxyartemisinin (**6**). Products **2**, **3**, and **6** are new compounds.

Artemisinin (**1**), also called qinghaosu, is a sesquiterpene lactone endoperoxide isolated from the herbal plant *Artemisinin annua* L. by Chinese researchers[1,2] and an effective therapeutic agent combating multidrug-resistant *Plasmodium falciparum* strains.[3] It has been proposed that its highly reactive endoperoxide is converted to free radicals by the iron in the free heme molecules concentrated in the food vacuoles of malarial parasites, and the resulting free radicals lead to cellular destruction or alkylation damage of some target proteins in parasite cells.[4,5] As an antimalarial drug, artemisinin has the disadvantages of high recrudescence rate and poor solubility in water. Some studies on the modification of artemisinin (**1**) and its analogues by biological[6−12] and chemical methods[13−20] have been reported to yield more effective drugs such as artemether in recent years.[21] Because knowledge of mammalian metabolism is an essential feature of artemisinin's clinical pharmacology, microbial metabolism studies have been established as in vitro models to predict the mammalian metabolites of artemisinin and its derivatives (**1**).[22,23]

In this work, artemisinin was biotransformed by *Mucor polymorphosporus* AS 3.3443 and *Aspergillus niger* AS 3.1858 in potato medium. Five products were obtained, among which three were identified as new compounds.

Four products, **2**, **3**, **4**, and **5**, were isolated from the broth of *M. polymorphosporus* and three products, **4**, **5**, and **6**, from that of *A. niger*. Compound **4** was also found in the medium controls.

Compounds **2** and **3** are both more polar than the substrate (by TLC analyses). Both IR spectra showed strong hydroxyl group absorptions at 3400−3500 cm^{-1}, and the characteristic signals of the endoperoxide bridge at 835, 883, and 1113 cm^{-1} were observed. An $[M + H]^+$ ion in the positive high-resolution SIMS of **2** and **3** at 299.1492 and 299.1485, respectively, allowed a molecular formula of $C_{15}H_{22}O_6$ to be assigned to **2** and **3** (calcd 299.1489). All the above data suggested that **2** and **3** were possibly hydroxylated products of artemisinin. The ^{13}C NMR spectra of **2** and **3** showed new peaks at δ 73.5 and 74.2, respectively, further confirming that both compounds are hydroxylated products. DEPT analyses showed that the number of secondary carbons changed from 4 to 3, and the number of tertiary carbons increased from 5 to 6, which revealed that the hydroxyl group must be introduced at a secondary carbon. On the basis of their HMBC, HSQC, and ^{1}H−^{1}H COSY spectra, products **2** and **3** were identified as 9-hydroxyartemisinin and 3-hydroxyartemisinin. In the 2D ^{1}H−^{1}H NOESY spectrum, the correlation of 9α-H to 1α-H and 7α-H strongly suggested that the orientation of 9-OH is of the β-configuration. Similarly, the correlation of 3α-H to 1α-H and 2α-H suggested that the orientation of 3-OH is also β. Therefore, **2** and **3** were identified as 9β-hydroxyartemisinin and 3β-hydroxyartemisinin, respectively, which have not previously been reported.

Time of flight mass spectroscopy (TOFMS) of **4** showed a molecular weight of 266, and the characteristic signals of a peroxide bridge were absent in the IR spectrum, which suggested that a single oxygen atom was lost. TLC analyses showed that **4** possesses the same polarity as deoxyartemisinin. Its ^{1}H and ^{13}C NMR data were in good agreement with those of deoxyartemisinin; therefore, **4** was confirmed to be deoxyartemisinin,[6,9] which is also one of the human metabolites of artemisinin.[24]

TOFMS of **5** suggested its molecular weight to be 282. The IR spectrum of **5** showed a strong absorption at 3495 cm^{-1}, and no characteristic signals of the peroxide bridge were observed. The comparison of the ^{1}H and ^{13}C NMR spectra with those of 3α-hydroxydeoxyartemisinin identified **5** as 3α-hydroxydeoxyartemisinin, which was previously reported to be a microbial transformation product of artemisinin by *Penicillium chrysogenum* (ATCC 9480).[9]The IR spectrum of **6** was similar to that of **5**, suggesting that **6** was also a hydroxylated product of deoxyartemisinin. High-resolution positive SIMS $[M + H]^+$ at 283.1540 was consistent with the formula $C_{15}H_{22}O_5$ (calcd 283.1540). A new peak at δ 71.2 was observed in its ^{13}C NMR spectrum, and DEPT analysis showed that the number of tertiary carbons decreased from 5 to 4 and the number of quaternary carbons increased from 3 to 4, indicating that one methine group must be hydroxylated. On the basis of its 1D and 2D spectral data, **6** was identified as 1α-hydroxydeoxyartemisinin. All proton and carbon signals were assigned accordingly.

Deoxyartemisinin **4** was also found in the substrate controls without any microorganisms, which indicated it to be possibly not a biotransformed product but a product of chemical reaction catalyzed by Fe^{2+} in potato medium. It could be inferred that *M. polymorphosporus* had the capability to biotransform artemisinin (**1**) and deoxyartemisinin (**4**), but *A. niger* can only transform deoxyartemisinin (**4**), which indicated the differences among the

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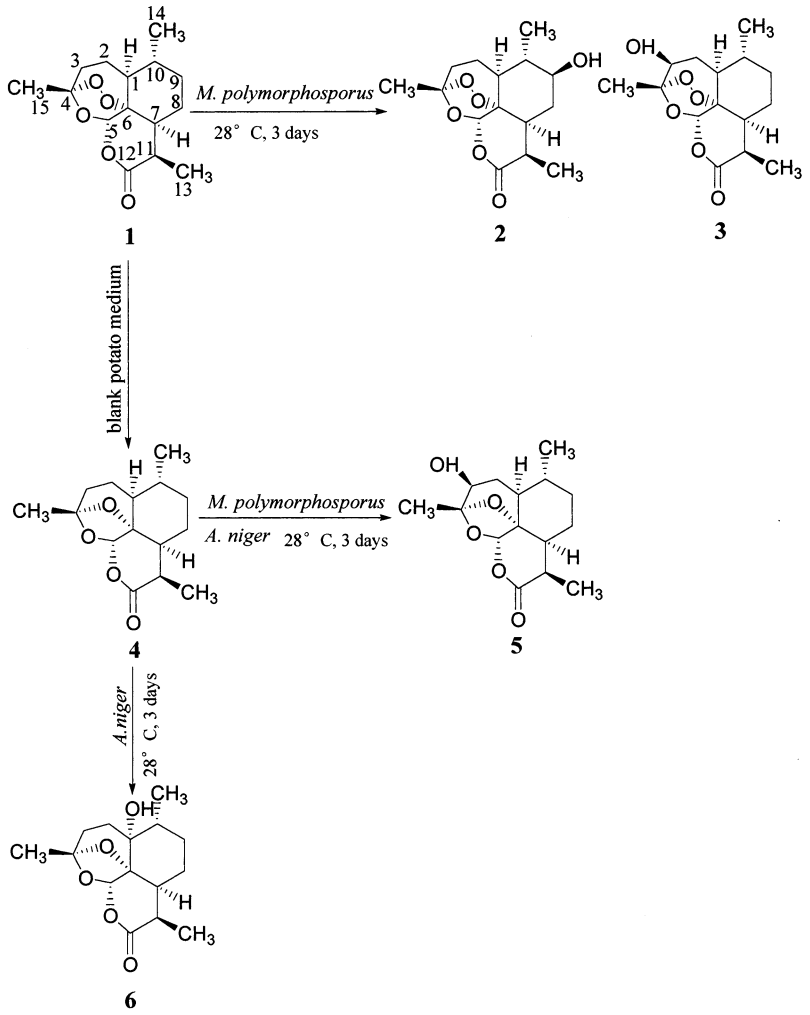

Figure 1. Possible biotransformation pathway of artemisinin by *M. polymorphosporus* and *A. niger*.

enzymes in the two strains of microorganisms. The bioactivities of the transformation products are under investigation.

Experimental Section

General Experimental Procedures. Melting points were determined on a micromelting point apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer 243 polarimeter using MeOH as solvent. IR spectra were determined with a Perkin-Elmer 983 FT-IR spectrometer and recorded in KBr pellets. 1D and 2D NMR spectra were recorded in $CDCl_3$ with an INOVA-500 instrument at 500 MHz using TMS as internal standard. The chemical shift values (δ) were given in parts per million (ppm), and the coupling constants were in Hz. Abbreviations for NMR signals are as follows: s = singlet, d = doublet, m = multiplet. High-resolution positive SIMS were obtained with a Bruker Apex II FI-ICR mass spectrometer. Time of flight mass spectra (TOFMS) were obtained with a Perkin-Elmer QSTAR mass spectrometer. TLC analyses were performed on silica gel G. Separation and purification were carried out by column chromatography on silica gel (200−300 mesh). Silica gels were obtained from Qingdao Haiyang Chemical Group Co., People's Republic of China.

Microorganisms. Both *Mucor polymorphosporus* AS 3.3443 and *Aspergillus niger* AS 3.1858 were purchased from the China General Microbiological Culture Collection Center.

Medium. All culture and biotransformation experiments were performed in potato medium. Potato medium was prepared by the following procedure: 200 mg of minced, husked potatoes was boiled in water for 1 h, then the solution was filtered, and the filtrate was added to water to 1 L.

Culture and Biotransformation Procedures. Screening scale biotransformation of artemisinin by *M. polymorphosporus* and *A. niger* were carried out in 250 mL Erlenmeyer flasks containing 50 mL of potato medium. Microorganisms were transferred into the flasks from the slants. The flasks were placed on rotary shakers, operating at 180 rpm at 28 °C. The substrate was dissolved in acetone with a concentration of 20 mg/mL. After 48 h of culture, 0.3 mL of the solution was added into the fermentation flasks and these flasks were maintained under the same conditions for an additional 3 days. Culture controls consisted of fermentation blanks in which microorganisms were grown without substrate but with the same amount of acetone alone. Substrate controls consisted of sterile medium containing the same amount of substrate and incubated under the same conditions. When the fermentation finished, the broths were filtered and the filtrates were extracted with the same volume of ethyl acetate three times. The cells were extracted with ethyl acetate by supersonic. The extracts were evaporated to dryness under reduced pressure, and the residues were dissolved in acetone. The solutions were spotted on silica gel plates, which were developed by petroleum ether (60−90 °C)−acetone (5:2) and visualized by spraying with 10% H_2SO_4 solution, followed by heating. TLC analyses revealed that both strains could biotransform the substrate.

Preparative scale biotransformation of artemisinin by *M. polymorphosporus* and *A. niger* was carried out in 20 1-L Erlenmeyer flasks containing 250 mL of potato medium, respectively. A total of 300 mg of **1** was transformed with each strain. Other procedures were the same as screening scale biotransformations.

Isolation and Characterization of Biotransformed Products. A total of 850 mg of yellow residue was obtained from the fermented broth of *M. polymorphosporus* and 650 mg from that of *A. niger*. The residues were chromatographed on silica gel columns. Both columns were eluted with petroleum ether (60−90 °C)−acetone (10:1). Biotransformation of **1** by *M. polymorphosporus* resulted in 67 mg of **2**, 48 mg of **3**, 10 mg of **4**, and 51 mg of **5**, while biotransformation by *A. niger* resulted in 12 mg of **4**, 79 mg of **5**, and 39 mg of **6**; 110 mg and 140 mg of substrate were left after both biotransformations, respectively.

In addition, 80 mg of substrate was added into four 1-L flasks containing 250 mL of blank medium, which were also maintained on the rotary shakers under identical conditions. A total of 1 mg of **4** was isolated from the medium, and 70 mg of the untransformed substrate was also obtained.

9β-Hydroxyartemisinin (2): colorless needles (CH_3COCH_3); mp 195−196 °C; $[\alpha]^{20}_D$ +52.0° (*c* 0.20, MeOH); IR (KBr) ν_{max} 3491 (OH), 1741 (C=O), 835, 883, and 1113 (peroxide bridge) cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 5.93 (1H, s, H-5), 3.38 (1H, m, H-11), 3.29 (1H, m, H-9), 2.43 (1H, m, H-3), 2.12 (1H, m, H-8), 2.07 (1H, m, H-3), 2.00 (1H, m, H-2), 1.91 (1H, dq, J = 14.0, 5.0 Hz, H-7), 1.52 (2H, m, H-1, H-2), 1.46 (3H, s, H-15), 1.39 (1H, m, H-10), 1.23 (3H, d, J = 7.0 Hz, H-13), 1.18 (1H, m, H-8), 1.12 (3H, d, J = 6.0 Hz, H-14); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 171.6 (s, C-12), 105.5 (s, C-4), 93.4 (d, C-5), 78.8 (s, C-6), 73.5 (d, C-9), 47.9 (d, C-1), 44.4 (d, C-10), 42.2 (d, C-7), 35.7 (t, C-3), 32.5 (d, C-11), 32.1 (t, C-8), 25.2 (q, C-15), 24.7 (t, C-2), 15.5 (q, C-14), 12.6 (q, C-13); High-resolution positive SIMS *m*/*z* 299.1492 $[M + H]^+$.

3β-Hydroxyartemisinin (3): colorless needles (CH_3COCH_3); mp 167−168 °C; $[\alpha]^{20}_D$ +62.1° (*c* 0.29, MeOH); IR (KBr) ν_{max} 3491 (OH), 1748 (C=O), 835, 883, and 1113 (peroxide bridge) cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 5.87 (1H, s, H-5), 4.39 (1H, m, H-3), 3.37 (1H, m, H-11), 2.33 (1H, m, H-2α), 1.90 (1H, m, H-8), 1.81 (1H, m, H-9), 1.75 (1H, m, H-7), 1.47 (3H, s, H-15), 1.46 (1H, m, H-2β), 1.44 (2H, m, H-1, H-10), 1.21 (3H, d, J = 7.5 Hz, H-13), 1.10 (1H, m, H-8), 1.08 (1H, m, H-9), 1.03 (3H, d, J = 5.5 Hz, H-14); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 171.8 (s, C-12), 107.8 (s, C-4), 93.1 (d, C-5), 79.1 (s, C-6), 74.2 (d, C-3), 47.1 (d, C-1), 44.6 (d, C-7), 37.2 (d, C-10), 35.1 (t, C-2), 33.4 (t, C-9), 33.0 (d, C-11), 23.4 (t, C-8), 21.2 (q, C-15), 19.7 (q, C-14), 12.5 (q, C-13); high-resolution positive SIMS *m*/*z* 299.1485$[M + H]^+$.

1α-Hydroxydeoxyartemisinin (6): colorless needles (CH_3COCH_3); mp 169−171 °C; $[\alpha]^{20}_D$ −158.3° (*c* 0.18, MeOH); IR (KBr) ν_{max} 3549 (OH), 1735 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 6.41 (1H, s, H-5), 3.16 (1H, m, H-11), 2.01 (1H, m, H-7), 1.81 (1H, m, H-2), 1.79 (1H, m, H-9), 1.78 (1H, m, H-3), 1.74 (1H, m, H-8), 1.65 (1H, m, H-2), 1.57 (2H, m, H-3, H-10), 1.55 (1H, m, H-9), 1.52 (3H, s, H-15), 1.45 (3H, d, J = 5.5 Hz, H-14), 1.44 (1H, m, H-8), 1.21 (3H, d, J = 7.0 Hz, H-13); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 171.8 (s, C-12), 108.8 (s, C-4), 100.3 (d, C-5), 82.1 (s, C-6), 71.2 (s, C-1), 46.1 (d, C-10), 42.8 (d, C-7), 40.0 (t, C-9), 34.1 (t, C-2), 32.6 (d, C-11), 28.8 (q, C-14), 24.0 (q, C-15), 19.3 (t, C-8), 16.9 (t, C-3), 12.7 (q, C-13); high-resolution positive SIMS *m*/*z* 283.1540 $[M + H]^+$.

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