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MICROBIAL TRANSFORMATION OF THE ANTIMALARIAL AND ANTICANCER DRUG ARTEMISININ BY WHITE-ROT BASIDIOMYCETES

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Abstract – We studied transformation of the potential anticancer drug artemisinin by the white-rot basidiomycetes *Pycnoporus sanguineus* and *Funalia trogii*. The fungi were grown on sucrose-malt broth in the presence of artemisinin for 14 days. Transformation products were extracted with ethyl acetate and analyzed by HPLC. Each of the fungi produced only a single metabolite from artemisinin. Products of transformation were identified by mass spectrometry and NMR methods. 5β-Hydroxyartemisinin was isolated from the incubation medium of *P. sanguineus* in 92.1% yield and 7β-hydroxyartemisinin was isolated from the incubation medium of F. trogii in 89.7% yield.

INTRODUCTION

Artemisinin (qinghaosu) is a natural product derived from the medicinal herb *Artemisia annua* (Fig. 1), which has long been used in traditional Chinese medicine for the treatment of fevers. A series of powerful antimalarial drugs have been obtained from artemisinin (Ho *et al*., 2014). However, recent reports show that artemisinin also has effective anticancer activity (Ho *et al*., 2014; Das, 2015). Artemisinin and its derivatives show high anticancer activity with both drug-sensitive and drug-resistant lines of cancer cells (Das, 1995). However, despite the effectiveness of artemisinin, there remain problems with its low water solubility, which makes the creation of an effective oral dosage form difficult (Balducci *et al.*, 2013), and with side effects associated with lesions in the brainstem at high doses in experimental animals (Genovese and Newman, 2008). These problems force us to search for new and effective artemisinin derivatives.

Unfortunately, the chemical synthesis of artemisinin derivatives is tedious and costly. The use of microbiological transformation makes it possible to replace tedious synthetic manipulations of the parent molecule by cheaper and simpler methods. To date, with the help of microbial transformation, effective processes have been developed for the preparation of artemisinin derivatives, which do not affect the basic skeleton of the parent molecule. Previously, with the help of microbiological transformation, we have obtained derivatives of artemisinin hydroxylated at the fourth carbon atom (Zhan *et al*., 2002a; Parshikov *et al*., 2004), the fifth carbon atom (Parshikov *et al*., 2006), the sixth carbon atom (Zhan *et al*., 2002a; Parshikov *et al*., 2004) and the seventh carbon atom (Parshikov *et al*., 2004; Zhan *et al*., 2002b; Parshikov *et al*., 2005). However, all processes lacked regioselectivity so that several transformation products were formed.

Here we report the regioselective hydroxylation of artemisinin by the basidiomycetous fungi *Pycnoporus sanguineus* CS 20 and *Funalia trogii* CS 63 at the fifth and seventh carbons, respectively, each with the formation of only one product in high yield. We were interested in the preparation of hydroxy derivatives of artemisinin to increase its solubility, on the one hand, with the preparation of a single product of transformation, on the other hand. The fulfillment of this task should help to significantly increase the efficiency of the process without the troublesome isolation of the desired product from a mixture of metabolites.

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MATERIALS AND METHODS

Strains of the fungi *P. sanguineus* CS 20 and *F. trogii* CS 63 (isolated from submontane forest, Cerro de la Silla, Nuevo Leon State, Mexico) were obtained from the collection of microorganisms of the biological faculty of the Autonomous University of Nuevo Leon, San Nicolás de los Garza, N.L., Mexico. They were maintained on agar slants as previously described (Parshikov *et al*., 1999).

Microorganisms were grown for 48 h on a circular shaker (200-220 rpm) at 28°C in flasks (2500 mL) each containing 500 mL of the following medium (g/L): sucrose - 15.0; malt extract (Difco, Detroit, MI, USA) - 20.0; peptone (Fisher, Atlanta, GA, USA) - 10.0; deionized water, pH 6.0. Artemisinin (Mediplantex, Vietnam) (Fig. 1) was dissolved in methanol at a concentration of 250 mg in 10.0 mL; 10.0 mL of this solution was added to each flask so that the final artemisinin concentration was 500 mg/L. The cultures were incubated for another 14 days on the shaker at 28°C. After 14 days, the biomass was separated by filtration with a paper filter on a Büchner funnel. The filtrate was extracted with ethyl acetate in a separatory funnel and evaporated to dryness (Parshikov *et al.*, 2004).

The dry residue was dissolved in methanol and analyzed by HPLC (Parshikov *et al*., 2004). The extracts were examined on a Waters 2690 chromatograph (Waters Corp., Milford, MA, USA) equipped with a PL-ELS 1000 evaporative light-scattering (ELS) detector (Polymer Laboratories, Amherst, MA, USA) and a Waters XTerra RP18 column of 5 μm (7.8 × 100 mm) (Parshikov *et al*., 2004). Metabolites were purified by flash chromatography (Parshikov *et al*., 2006).

HRMS were recorded on a Micromass Q-Tof Micro spectrometer with a LockSpray ionization source (Waters) (Parshikov *et al*., 2004). ^{1}H NMR and ^{13}C NMR spectra were recorded in a $CDCl_3$ solution on a Bruker DPX 300 instrument (Bruker AG, Fällanden, Switzerland) operating at 400 and 100 MHz, respectively (Parshikov *et al.*, 2004).

Optical rotation was measured on a Chemapol IV polarimeter (Rudolph, Flanders, NJ, USA) with a sample tube 0.05 mL of path length 10 mm (λ = 589 nm). Elementary analysis data were obtained using a CHNS/O Perkin Elmer 2400 Series II analyzer (PerkinElmer, Boston, MA, USA). Melting points (mp) were determined using a FP62 Mettler-Toledo instrument (Mettler-Toledo, Columbus, OH, USA). Infrared (IR) spectra were recorded using a Thermo Scientific Nicolet IR 300 FT-IR spectrometer (Thermo, San Jose, CA, USA) on germanium plates.

RESULTS

HPLC analysis of extracts obtained from *P. sanguineus* CS 20 and *F. trogii* CS 63 cultures showed only one metabolite in each culture; they had retention times of 14.8 and 13.2 min, respectively. Residual artemisinin was present in trace amounts and had a retention time of 19.1 min.

Analysis of both metabolites by HRMS showed that each of them had the value *m/z* 321.1314 $[M+Na]^+$. An additional 16 mass units in comparison with the initial artemisinin is explained by the presence of oxygen in the hydroxyl group. The additional oxygen atom was confirmed by elemental analysis data (not shown), which confirmed the molecular formula of $C_{15}H_{22}O_6$ for each metabolite.

Metabolite I, obtained from the *P. sanguineus* CS 20 culture, had a retention time of 14.8 min with a total peak area of 92.1% (as determined by the ELS detector). It had an optical rotation of [α]27D + 64° (*c* 1.0, methanol) and a melting point (mp) of 145-146°C. The IR spectrum showed a wide absorption band in the λ_{max} region of 3300 cm^{-1}, which is typical of the hydroxyl group.

^{1}H-NMR ($CDCl_3$, 400 MHz) δ 5.86 (1H, s, H-12), 4.38 (1H, dd, *J*=3.7, 9.5 Hz, H-5α), 3.4 (1H, m, H-9), 2.32 (1H, dd, *J*=4.7, 6.7 Hz, H-4α), 1.86 (1H, m, H-8α), 1.80 (1H, m, H-7β), 1.73 (1H, m, H-8a), 1.66 (1H, dd, *J*=5.4, 10.8 Hz, H-4β), 1.60 (1H, m, H-6), 1.47 (1H, m, H-5a), 1.46 (3H, s, CH_3-13), 1.2 (3H, d, *J*=7.0 Hz, CH_3-15), 1.12 (1H, m, H-8β), 1.08 (1H, m, H-7α), 1.02 (3H, d, *J*=5.5 Hz, CH_3-14).

^{13}C-NMR ($CDCl_3$, 100 MHz) δ 173.1 (C, C-10), 107.9 (C, C-3), 93.1 (C, C-12), 79.1 (C, C-12a), 74.2 (CH, C-5), 47.2 (CH, C-5a), 44.7 (CH, C-8a), 37.2 (CH, C-6), 35.2 (CH_2, C-4), 33.5 (CH_2, C-7), 33.0 (CH, C-9), 23.4 (CH_2, C-8), 21.1 (CH_3, C-13), 19.7 (CH_3, C-15), 12.5 (CH_3, C-14).

These data were in accordance with the structure of 5β-hydroxyartemisinin (Fig. 1).

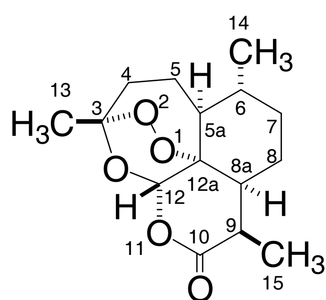
Metabolite II, obtained from the *F. trogii* CS 63 culture, had a retention time of 13.2 min with a total peak area (as determined by the ELS detector) of 89.7%. It had an optical rotation of [α]27D + 63° (*c* 1.0, methanol) and melting point (mp) of 193–195°C. The IR spectrum showed a wide absorption band in the λ_{max} region of 3481 cm^{-1}, which is typical of the hydroxyl group.

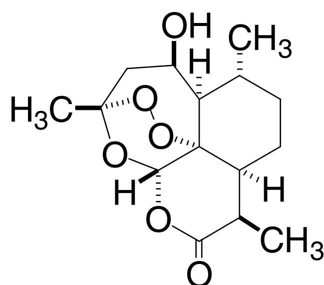
^{1}H-NMR ($CDCl_3$, 400 MHz) δ 5.90 (1H, s, H-12),

3.33 (1H, dq, *J*=7.5, 6.0 Hz, H-9), 3.24 (1H, dt, *J*=11.0, 5.0 Hz, H-7α), 2.40 (1H, ddd, *J*=14.5, 13.0, 4.0 Hz, H-4α), 2.11 (1H, m, H-8α), 2.05 (1H, ddd, *J*=14.5, 5.0, 4.0 Hz, H-4β), 2.00 (1H, ddd, *J*=13.0, 4.0, 4.0 Hz, H-5α), 1.87 (1H, dt J=14.0, 5.0, 5.0 Hz, H-8a), 1.52 (1H, m, H-5β), 1.48 (1H, m, *J*=5.0, 10.0 Hz, H-5a), 1.42 (3H, s, CH_3-13), 1.35 (1H, m, *J*=4.5, 10.5 Hz, H-6), 1.19 (1H, m, H-8β), 1.19 (3H, d, *J*=7.5 Hz, CH_3-15), 1.09 (3H, d, *J*=6.5 Hz CH_3-14)

^{13}C-NMR ($CDCl_3$, 100 MHz) δ 172.5 (C, C-10); 105.9 (C, C-3); 93.9 (C, C-12); 79.2 (C, C-12a); 73.9 (CH, C-7); 48.4 (CH, C-5a); 44.9 (CH, C-6); 42.6 (CH, C-8a); 36.2 (CH_2, C-4); 33.5 (CH_2, C-8); 33.0 (CH, C-9); 25.6 (CH_3, C-13); 25.2 (CH_2, C-5); 15.9 (CH_3, C-14); 13.0 (CH_3, C-15).

These data were in accordance with the structure of 7β-hydroxyartemisinin (Fig. 1).





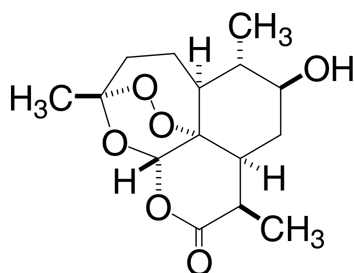


Fig. 1. The structures of artemisinin and metabolites **I** and **II**, formed by *P. sanguineus* CS 20 and *F. trogii* CS 63, respectively.

DISCUSSION

At present, the need for an economical, non-toxic, oral derivative of artemisinin for the treatment of malaria and oncological diseases is evident. Since the chemical synthesis of artemisinin derivatives does not fully provide a viable approach to the development of new drugs, we propose a new, logical approach to the synthesis of water-soluble artemisinin derivatives. The low costs associated with microbial transformation suggest that it may be an excellent approach to produce hydroxylated artemisinin derivatives.

The pharmacological activity of artemisinin against malaria and oncological diseases is associated with an endoperoxide fragment (Das, 2015). The introduction of a polar hydroxyl group at different positions should increase the hydrophilicity of the artemisinin molecule and increase the solubility of the drug in water while maintaining its pharmacological activity.

In addition, there is evidence that the introduction, at the location of the hydroxyl group, of radicals such as ethyl or *n*-propyl increases the pharmacological activity of the artemisinin molecule (Avery *et al.*, 2003). Also, some substituted derivatives obtained by chemists from 7â-hydroxyartemisinin have shown increased solubility in water and excellent antimalarial activity *in vitro* (Pabbisetty *et al*., 2012). It is not known yet how such substituents will behave at the C-5 position of the artemisinin molecule.

Previously, artemisinin transformation processes using various microorganisms have been reported (Parshikov, 2016), but the processes were not regioselective. Mixtures of several metabolites were formed, for which further separation into individual substances was required.

For the transformation of artemisinin, we used the white-rot basidiomycetes *P. sanguineus* CS 20 and *F. trogii* CS 63. The results were excellent and each fungus produced only one metabolite with a high yield of the transformation product: 5β-hydroxyartemisinin (92.1%) and 7β-hydroxyartemisinin (89.7%), respectively.

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