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Microbial transformation of artemisinin to 5-hydroxyartemisinin by *Eurotium amstelodami* and *Aspergillus niger*

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Abstract Transformation of the anti-malarial drug artemisinin by the fungi *Eurotium amstelodami* and *Aspergillus niger* were investigated. Cultures were grown in sucrose/malt broth with artemisinin for 14 days and extracted with ethyl acetate. Extracts were characterized by liquid chromatography. Two metabolites from each fungal extract were isolated and identified using mass spectrometry and nuclear magnetic resonance. 5β-hydroxyartemisinin and 7β-hydroxyartemisinin were isolated in 63 and 32% yields, respectively, from the extract of *E. amstelodami*, and 80 and 19%, respectively, from the extract of *A. niger*.

Keywords Transformation · Artemisinin · Eurotium amstelodami · Aspergillus niger

Introduction

According to the World Health Organization, approximately 300 million people worldwide are affected by malaria and between 1 and 1.5 million people die from it every year. Previously extremely widespread, the malaria is now mainly confined to Africa, Asia, and Latin America. Chloroquine continues to be the drug of choice for the treatment of malaria, however the emergence of drug resistant strains of the parasite have complicated attempts to effectively control the disease [1, 2]. Although people living in endemic regions, gradually develop immunity to the disease, children remain highly vulnerable. Artemisinin (qinghaosu) is a naturally occurring sesquiterpene lactone peroxide cultivated from the plant *Artemisia annua* (Fig. 1).

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Although artemisinin leads to faster parasite and fever clearance than any other anti-malarial agent, there remain problems associated with water solubility of the compound and its potential for creating neurological lesions in the brainstem. These problems have created a search for new and effective artemisinin derivatives. Unfortunately, synthesis and derivatization of the natural product can be tedious and expensive, which ultimately result in derivatives too costly to be useful in third world countries. Microbial transformation offers the ability of replacing tedious synthetic manipulation of the parent chemical structure with an efficient cost-effective alternative.

Microbial biotransformations can provide an efficient and cost-effective alternative to synthetic derivatization. To date microbial transformation methodologies have been developed to efficiently and selectively derivatize a number of pharmacologically important positions on the artemisinin skeleton, positions that otherwise require inefficient synthetic derivatization. Biotransformations have been used to selectively hydroxylate the four-position [3–5], the six-position [3, 5], and the seven-position [4–6] of artemisinin. Here, we report the biotransformation of artemisinin by *Eurotium amstelodami* and *Aspergillus niger* to the novel 5-hydroxyartemisinin derivative.

Materials and methods

Aspergillus niger VKM F-1119 was obtained from the All-Russian collection of microorganisms (Moscow, Russia) and *E. amstelodami* A-51 was collected and isolated from the campus grounds of the University of Mississippi. The microorganisms were maintained on agar slants as previously described [7]. Cultures were grown in flasks (2,500 ml) containing 500 ml medium: 20 g malt extract (Difco, USA), 15 g sucrose, 10 g peptone (Fisher, Atlanta, GA, USA), and 1,000 ml deionized water, pH 6.0 with shaking at 180 rpm. Artemisinin (Fig. 1) (Mediplantex, Vietnam) was dissolved

Fig. 1 Structure of artemisinin and two metabolites formed by *E. amstelodami* and *A. niger*

(250 mg/10.0 ml) in methanol, filter-sterilized and 10.0 ml of this solution was added to each flask for a final concentration of 500 mg/l. Cultures and controls were incubated at 28 °C in environmental shakers. Well-grown cultures were harvested after 14 days and extracted exhaustively with ethyl acetate and evaporated to dryness [5].

Extracts were dissolved in methanol and analyzed using high-performance liquid chromatography (HPLC) [5], employing a Waters 2690 separation module (Waters, Milford, MA, USA) with a Waters XTerra RP_{18} 5 µm column (7.8×100 mm^2) and PL-ELS 1000 evaporative light-scattering (ELS) detector (Polymer Laboratories, Amherst, MA, USA) [5].

Metabolites were purified by flash-chromatography [4] and subjected to high-resolution mass spectra (HRMS), which was obtained using a Micromass Q-Tof Micro with lock spray source (Waters) [5]. ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra were recorded using a Bruker DPX 300 (Bruker AG, Fällanden, Switzerland) operating at 400 and 100 MHz, respectively [5]. Spectra were interpreted using distortionless enhancement by polarization transfer (DEPT) 135, correlation spectroscopy (COSY), nuclear Overhauser effect (NOESY), heteronuclear multiple bond correlation (HMBC), and heteronuclear multiple-quantum coherence (HMQC). Optical rotation was determined using an Autopol IV polarimeter (Rudolph, Flanders, NJ, USA) with a sample tube 0.05 ml of path length 10 mm (λ = 589 nm). Elemental analysis data were obtained with a Perkin Elmer series II 2400 CHNS/O analyzer (PerkinElmer, Boston, MA, USA). Melting points (mp) were determined using a FP62 Mettler-Toledo apparatus (Mettler-Toledo, Columbus, OH, USA) and were uncorrected. Infrared (IR) spectra were recorded using a Thermo Nicolet IR 300 FT-IR spectrometer (Thermo, San Jose, CA, USA) on a germanium crystal plate as neat solids or liquids.

Results

High-performance liquid chromatography analysis (Fig. 2) of the ethyl acetate extract of the cultures of both *E. amstelodami* and *A. niger* revealed the presence of two metabolites eluting at 14.8 and 13.2 min with residual artemisinin eluting at 19.1 min.

The HRMS analysis of both metabolites indicated that each metabolite had an identical *m/z* of 321.1314 $[M + Na]^+$. The additional 16 mass units were attributed to hydroxylation. Monohydroxylation was confirmed using elemental analysis, which provided a molecular formula of $C_{15}H_{22}O_6$ for each metabolite.

Metabolite I obtained from cultures of *E. amstelodami* and *A. niger* eluted from the HPLC column after 14.8 min with a total peak area (as determined by ELSD) of 63 and 80%, respectively (Fig. 2). The metabolite gave an optical rotation of $[\alpha]_D^{27}$ + 64° (c 0.5, MeOH), and a melting point of 145–146 °C.

^{1}H and ^{13}C-NMR as well as two-dimensional COSY, NOESY, HMBC, and HMQC spectra of the metabolite I were used to determine the specific site of hydroxylation and its spatial arrangement. Spectral assignments of the metabolite from *E. amstelodami* and *A. niger* were identical. ^{1}H and ^{13}C-NMR spectra of the metabolite clearly showed that the hydroxyl functionality was attached at C-5 and resulted in the downfield chemical shift of the adjacent proton H-5 and C-5, respectively (4.39 and 74.24 ppm). The presence of the intact protons on the neighboring C-4 (NOESY) and C-5a and their proximity (COSY) confirmed the site of hydroxylation to be C-5. The NOESY correlations with H-4 and H-5a indicated that the hydroxyl group was attached to C-5 in the *β*-orientation (Fig. 1).

^{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-7*β*), 1.73 (1H, m, H-8a), 1.66 (1H, dd, J = 5.4, 10.8 Hz, H-4*β*), 1.60 (1H, m, H-6), 1.47 (1H, m, H-5a), 1.46 (3H, s, C*H3*-13), 1.2 (3H, d, J = 7.0 Hz, C*H3*-15), 1.12 (1H, m, H-8*β* 1.08 (1H, m, H-7α), 1.02 (3H, d, J = 5.5 Hz, C*H3*-14).

^{13}C-NMR ($CDCl_3$, 100 MHz) δ173.1 (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 (CH2, C-4), 33.5 (CH2, C-7), 33.0 (CH, C-9), 23.4 (CH2, C-8), 21.1 (CH3, C-13), 19.7 (CH3, C-15), 12.5 (CH3, C-14).

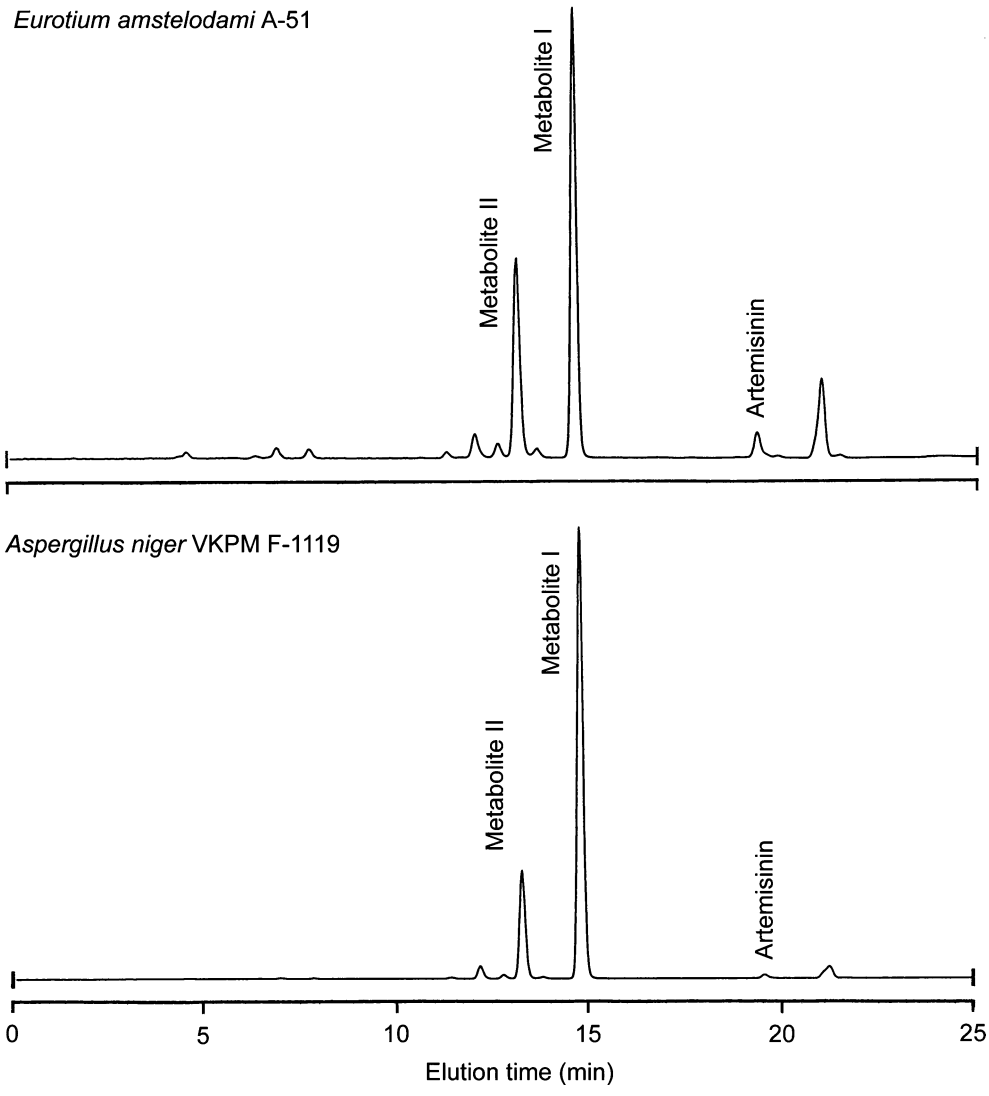

Fig. 2 High-performance liquid chromatography chromatograms, obtained with ELS detector, for the metabolites produced from artemisinin by *E. amstelodami* and *A. niger*

A DEPT-135 experiment revealed the hydroxylation to have occurred on a secondary carbon atom as evident from the decreased primary and secondary carbon atom from 4 to 3 and increased tertiary carbon atoms from 4 to 5. IR spectroscopy of the metabolite showed a broad absorption at 3,300 per centimeter that is characteristic of hydroxylation.

Metabolite II obtained from cultures of *E. amstelodami* and *A. niger* eluted from the HPLC column after 13.2 min with a total peak area (as determined by ELSD) of 32 and 19%, respectively (Fig. 2). ^{1}H and ^{13}C-NMR data of this metabolite were described previously [5] as 7β-hydroxyartemisinin (Fig. 1).

Discussion

Artemisinin has proved to be a clinically effective agent for the treatment of chloroquine-resistant malaria, however its low-water solubility has prevented the formulation of an effective oral dosage form, which in turn has greatly limited its widespread use. In addition, artemisinin exhibits several detrimental neurological side effects at high doses in animal models. The need for an economical, non-toxic, oral anti-malarial agent that is effective against chloroquine-resistant malaria that can be employed in clinics throughout Africa and Asia is obviously great. Since total synthesis of artemisinin does not provide a viable approach for drug design, experiments using artemisinin as a natural product scaffold for synthetic manipulation offers a logical design approach. Difficulties associated with the synthetic approaches to attaching 'synthetic handles' to artemisinin are a limitation to an economically viable production of any potentially effective artemisinin derivatives. Therefore, the low costs associated with microbial transformation offers an excellent approach for producing hydroxylated scaffolds. It is clear that anti-malarial activity associated

with artemisinin requires the presence of the peroxide ring system [8]. In addition, it has been reported that substitution with polar moieties in the nine-position of artemisinin can be used to increase the water solubility of the compounds while retaining the anti-malarial activities. Obviously, the addition of polar hydroxyl groups in the five-position helps to increase the hydrophilicity of the artemisinin skeleton. Also, substitution with such groups as ethyl or *n*-propyl at the three-position appears to increase anti-malarial activity [9]. We have also examined several C-7 substituted derivatives that present increased water solubility profiles and excellent in vitro anti-malarial activity (unpublished). To date, little is known about the activity and substitution of the C-5 analogs and these derivatives will be essential in establishing a complete structure-activity profile for artemisinin.

We have examined artemisinin transformations using a variety of microorganisms [5] in an effort to identify specific transformations that occur in relatively high yield. Molecular modeling and structure–activity relationship experiments indicate that derivatization of artemisinin in positions 4–7 offer the greatest potential of increasing water solubility yet retaining anti-malarial activity. Here we report the production of 5β-hydroxyartemisinin, which completes our library of biotransformation experiments that provide hydroxylated products in the 4–7 positions. Although there exists a report for the production of 5β-hydroxy-10-deoxoartemisinin [10], the 5β-hydroxyartemisinin derivative represents a novel compound.

We have now produced several semi-synthetic derivatives that exhibit excellent water solubility profiles as well as increased anti-malarial activities (non-human). Pharmacokinetic profiles of these compounds are currently being attained, which will be revealed at a later time.

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