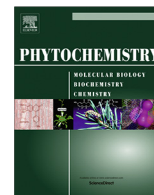
ARTICLE IN PRESS

Phytochemistry xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Phytochemistry

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



In vitro antimalarial studies of novel artemisinin biotransformed products and its derivatives

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ARTICLE INFO

Article history:
Received 2 May 2014
Received in revised form 28 July 2014
Accepted 4 August 2014
Available online xxxx

Keywords:
Antimalarial
Artemisinin
Metabolism
In-vitro

ABSTRACT

Biotransformation of antimalarial drug artemisinin by fungi *Rhizopus stolonifer* afforded three sesquiterpenoid derivatives. The transformed products were 1α-hydroxyartemisinin (**3**), 3.0%, a new compound, 10β-hydroxyartemisinin, 54.5% (**4**) and deoxyartemisinin (**2**) in 9% yield. The fungus expressed high-metabolism activity (66.5%). The chemical structures of the compounds were elucidated by 1D, 2D NMR spectrometry and mass spectral data. The major compound 10β-hydroxyartemisinin (**4**) was chemically converted to five new derivatives **5**–**9**. All the compounds **3**–**9** were subjected for *in vitro* anti-malarial activity. 10β-Hydroxy-12β-arteether (**8**), IC_{50} at 18.29 nM was found to be 10 times better active than its precursor **4** (184.56 nM) and equipotent antimalarial with natural drug artemisinin whereas the α-derivative **9** is 3 times better than **4** under *in vitro* conditions. Therefore, the major biotransformation product **4** can be exploited for further modification into new clinically potent molecules. The results show the versatility of microbial-catalyzed biotransformations leading to the introduction of a hydroxyl group at tertiary position in artemisinin in derivative (**3**).

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1. Introduction

Artemisinin (**1**) derivatives have achieved great importance as potential antimalarials for their action against multi-drug resistant *Plasmodium falciparum* malaria which cannot be treated with quinoline based and antifolate drugs (Sriram et al., 2004). In order to discover more potent anti-malarial leads than the existing lead molecules, artemisinin was transformed into value-added products by chemical and microbial methods in the last decade and resulted in the development of more effective anti-malarial drugs (Bhakuni et al., 2002; Goswami et al., 2010; Liu et al., 2006).

Microbial transformations can be considered as "green chemistry" for their ecological technology using mild conditions such as an aqueous medium, moderate pH and low temperature (Vézina, 1987). They can be used to modify chemical structures of biologically active products (Zhang et al., 2011), to prepare products which are difficult to obtain by conventional chemical methods (Adelin et al., 2012), to study the metabolism of xenobiotics (Abourashed et al., 1999) as well as to develop structure–activity relationship (SAR) models (Zhang et al., 2007). Till now, there are several reports on microbial transformations of sesquiterpene lactones (SLs) by fungi (Galal et al., 1999; Kumari et al., 2003; Lamare and Furstoss, 1990; Parshikov et al., 2006; Rocha et al., 2012). Some microorganisms are well known for their hydroxylation of artemisinin producing active metabolites (Khalifa et al., 1995). The main enzymatic reactions of SLs, catalyzed by *Rhizopus stolonifer*, are simple hydrogenations, hydroxylations, acetylations and reductions. Based on the potential of this fungus, metabolism of artemisinin (**1**) was investigated in order to obtain new potentially bioactive derivatives and, if possible, to study their structure–activity relationships.

In the continuation of our earlier biotransformation studies on bioactive compounds (Patel et al., 2010, 2011; Gaur et al.,

Abbreviations: DEPT, distortionless enhancement by polarization transfer; COSY, correlation spectroscopy; HSQC, heteronuclear single quantum correlation; HMBC, heteronuclear multiple-bond correlation spectroscopy.

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http://dx.doi.org/10.1016/j.phytochem.2014.08.004
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Please cite this article in press as: Gaur, R., et al. *In vitro* antimalarial studies of novel artemisinin biotransformed products and its derivatives. Phytochemistry (2014), http://dx.doi.org/10.1016/j.phytochem.2014.08.004

2014a,b), five fungi *Alternaria alternata*, *Aspergillus flavus*, *Aspergillus niger*, *Penicillium citrinum* and *R. stolonifer* were used for the metabolism of **1** in the present study. *R. stolonifer* was selected for detailed study because of its high metabolic activity. Three microbial metabolites deoxyartemisinin **2**, 1α-hydroxyartemisinin **3** and 10 β-hydroxyartemisinin **4** of **1** were isolated (Fig. 1). 1D and 2D-NMR data showed that the yield of the new metabolite **3** was 4.6%. The major compound (54.5%), metabolite **4**, was chemically converted to five new derivatives (**5–9**). The isolates and derivatives were studied for *in vitro* activities against *P. falciparum*. The *in vitro* results indicated that 10β-hydroxy-12β-arteether (**8**) is ten times better active than its precursor **4**, and equipotent antimalarial with natural drug artemisinin whereas the α-derivative **9** is three times better than **4**.

2. Material and methods

2.1. General experimental procedures

Melting points obtained with a Toshniwal melting point apparatus are uncorrected. Infrared spectra of the samples were performed with a Perkin Elmer Bx infrared spectrophotometer and recorded in KBr pellets. Specific rotations, $[\alpha]_D$ were measured with a polarimeter (SEPA-300, HORIBA). ^1H and ^{13}C NMR spectra were determined on a Bruker Avence-300 instrument at 300 MHz and 75 MHz, respectively in $CDCl_3$ using TMS as an internal standard. The chemical shift values are in ppm units, and Hz denotes the coupling constants. The NMR signals abbreviations are: s (singlet), d (doublet), dd (double doublet), br (broad), t (triplet), dt (double triplet), q (quartet), dq (double quartet), m (multiplet). COSY, DEPT experiments were performed using standard pulse sequence. Electrospray ionization (ESI) mass spectra, obtained from Shimadzu LC–MS system were used to determine the molecular weight or molecular formula of the metabolites. HRMS spectra were recorded on JEOL-AccuTOF JMS-T100LC mass instrument using dry Helium for ionization. Artemisinin, used for the metabolism study was isolated from *Artemisia annua* (Tandon et al., 2003).

2.2. Chromatographic conditions

For the detection and quantification of the isolates, CAMAG HPTLC system with WINCATS software was used. Samples of 10 μl of bio-transformed products, except metabolite **3** 20 μl were spotted using ATS-4 CAMAG, Switzerland on HPTLC plates from Merck and scanned the plates at 620 nm using CAMAG TLC scanner 3, Switzerland. HPLC data were obtained, to check the purity of compounds, from a Shimadzu LC-10 instrument using an SPD-10A UV–Vis detector and a CBM- 10A interface module. The data were analyzed using class LC-10 Work Station. The sample analysis was carried out through reverse phase chromatography (RP 18 column, 250 mm, 5 μm, SymmetryShield®TM) using acetonitrile and water mobile phase (75:25), flow rate 1 ml/min at λ 220 nm.

2.3. Microorganisms and culture media

Alternaria alternate, *A. flavus*, *A. niger*, *P. citrinum* (all lab isolates, confirmed from Commonwealth Mycological Institute, Kew, Surrey, England.) and *Rhizopus stolonifer* (confirmed by the Indian Agricultural Research Institute, New Delhi) (Shukla et al., 2006) were used in this experiment. All the cultures and biotransformation experiments using filamentous fungi were performed in potato medium consisting of peeled potato extract (200 g), dextrose (10 g) and distilled H_2O (1000 ml).

2.4. Biotransformation procedures

The media for preliminary screening and scale-up experiments contained potato infusion 200 g, dextrose 10 g and appropriate amount of distilled water to make the final volume of 1 l, pH 7.0. The screening experiments using five fungi were conducted in conical flasks (125 ml each) containing 25 ml of sterilized potato dextrose broth (PDB) medium. The metabolism experiments were performed by inoculation and incubation of the cultures on orbital shaker (model 3020, GFL mbH, Germany) at 160 rpm and 23 ± 1 °C using literature procedures (Elmarakby et al., 1986; Shukla et al., 1997). Usually, 6% solution of the substrate in acetone was added to the 48 h old stage II culture media at 0. 2 mg/ml concentration. The substrate controls contained only sterile media, and the culture controls were experimented under similar conditions in the absence of substrate.

2.5. Bioconversion of artemisinin

R. stolonifer was cultured in 50 ml of PDB medium inside 250 ml conical flasks. For ascertain the toxicity tolerance of the fungus against artemisinin (**1**), the substrate was added at different concentrations, i.e. from 0.2 mg/ml to 2 mg/ml (Table 1), to the 48 h old stage II culture in triplicates. After five days, the bioconversion mixtures were monitored by TLC analysis. The higher amounts of bio-products with the highest biomass were obtained up to 0.8 mg/ml concentration of artemisinin, but its 1.6 mg/ml and 2 mg/ml concentrations produced less fungal biomass. Percent increase/decrease over the initial inoculum on dry weight basis is denoted as growth index (GI). The bioconversion mixtures of three flasks from 3rd to 10th day of inoculation were mixed to observe the time course of biotransformation. All the experiments were performed two times each in three repetitions/treatments. In the scale up experiment, artemisinin (1 g) acetone (6.2 ml) solution was distributed equally in twenty-five 250 ml culture flasks, each containing 50 ml PDB of 48 h old *R. stolonifer* cultures. After seven days of incubation, the total mixtures were filtered, washed the mycelia mates with water and combined. The combined aqueous filtrate (1.2 l) was extracted three times with an equal volume of ethyl acetate. The ethyl acetate layer was dried (using Na_2SO_4), filtered and evaporated under reduced pressure to yield a dark brown residue (1.1 g).

2.5.1. Isolation and characterization of deoxyartemisinin 2

The residue showed a major and two minor spots on TLC in hexane–ethyl acetate (70:30). The residue (1.1 g) was subjected to chromatography on a silica-gel column (12.5 g, 17 .5 × 0.8 cm), eluting with different proportions of hexane:ethyl acetate. Fractions of 15 ml each were collected and monitored by TLC. Hexane–ethyl acetate (85:15) fractions 28–36, which showed two spots on TLC were pooled and evaporated to dryness (305 mg); 100 mg from that was purified by preparative TLC in hexane–ethyl acetate (70:30) to yield 30 mg of metabolite **2** (9.1%, Rf = 0.43) and 65 mg of unreacted artemisinin (19.8%, Rf = 0.38). Metabolite **2**, mp 110–111 °C, $[\alpha]_D^{23} = -131.6$ (c = 0.6 DCE); IR (KBr) λ_{max}, cm^{-1} 2938, 2886, 1748(CO), 1595, 1458, 1386, 1138, 1021; ESI-MS (positive): m/z 267 $[M+H]^+$, 289 $[M+Na]^+$, (Calc. for $C_{15}H_{23}O_4$); ^1H NMR: δ 0.94 (3H, d, J = 3.3 Hz, H_3-14), 1.02 (1H, m, H-8), 1.13 (2H, m, H_2-9), 1.19 (3H, d, J = 7.2 Hz, H_3-13), 1.21 (2H, m, H_2-2), 1.23 (1H, m, H-10), 1.26 (1H, m, H-1), 1.52 (3H, brs, H_3-15), 1.64 (1H, m, H-3), 1.75–1.83 (2H, m, H-3, H-9), 1.94 (1H, m, H-2, H-8), 2.01 (1H, m, H-7), 3.18 (1H, m, H-11), 5.70 (1H, s, H-5); ^{13}C NMR data (see Table 2). It was identified as deoxyartemisinin by comparison of its spectral data with literature (Lee et al., 1989) and Co-TLC with an authentic sample.

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