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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Biotransformation of artemisinin derivatives by *Glycyrrhiza glabra*, *Lavandula officinalis*, and *Panax quinquefolium*

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Abstract Biotransformation of α-artemether and dihydroartemisinin (DHA) by *Glycyrrhiza glabra* (Linn.), *Lavandula officinalis* (L.), and *Panax quinquefolium* was investigated. Two metabolites: tetrahydrofuran derivative (**3**) and a 13-carbon ring-rearranged product (**4**) were produced from α-artemether (**1**). DHA (**2**) provided metabolite **4**. The structure of the metabolites were characterized by proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance (NMR) imaging, fourier transform infrared spectroscopy, and mass spectroscopy. This is the first report that *G. glabra* and *L. officinalis* have the capability to biotransform α-artemether, and *P. quinquefolium* to biotransform DHA. Metabolite **3** is a new compound and metabolite **4** is reported here for the first time from artemisinin derivatives **1** and **2**. The presence of acetate function in the derivative **3** and hydroxyl and C-12 deoxo groups in **4** obtained in our study make them interesting synthones for further modification into new clinically potent molecules.

Keywords Plant cells · α-Artemether · Dihydroartemisinin · Metabolites · Biotransformation

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Introduction

Malaria still remains one of the most dangerous widespread parasitic diseases of the developing world although it is known to humankind since ancient times in different forms and exists over 100 countries including the United States (Brisibe *et al.*, 2008). What worsens the situation is the emergence of resistant malaria, caused by *Plasmodium falciparum* strains which are resistant to all well-established drugs like quinine, chloroquine, and sulfadoxine–pyrimethamine. Such a situation has called for an active search for novel antimalarial compounds from natural, semisynthetic, or synthetic routes. In 1972, a group of Chinese researchers isolated a new antimalarial drug, (+)-artemisinin, a sesquiterpene lactone of the amorphene sub-group of cadinene from traditional Chinese medicinal plant, *Artemisia annua.* The plant has been used for the treatment of fever and malaria since ancient times. Artemisinin and its derivatives are effective against multidrug-resistant *P. falciparum* strains. Dihydroartemisinin (DHA), the main active principle is the simplest and reduction product of artemisinin. α-Artemether (**1**) and β-artemether, the methyl ether derivatives of DHA were found to be more effective in vivo than artemisinin, probably due to its better lipophilicity and chemical stability of the trioxane system and well tolerated in moderately severe *P. falciparum* infections (Bhakuni *et al.,* 2002; Dondorp *et al.*, 2006; Klayman, 1985; Lee and Hufford, 1990; Martensson *et al.*, 2005). As a matter of fact, artemethers have lately become a renewed hope for combating the emerging generations of resistant malaria in underdeveloped countries. Therefore, intensive efforts are ongoing in many laboratories to modify artemisinin and its derivatives for value addition (Balint, 2001; Chaturvedi *et al.*, 2010; Jung *et al.*, 2004). In view of the highly promising clinical results of artemisinin

antimalarials, a biotransformation study was conducted by our group in order to characterize the major metabolites of artemisinin and β-artemether (Patel *et al.*, 2010; Patel *et al.*, 2011). This paper describes the biotransformation of α-artemether and DHA by *G. glabra, L. officinalis*, and *P. quinquefolium* to some novel analogs.

Materials and methods

General experimental procedures

Melting point was determined on a Toshniwal melting point apparatus and is uncorrected. IR spectra were recorded on a Perkin Elmer 1719 FT-IR spectrophotometer. Nuclear magnetic resonance (NMR) spectra were obtained in $CDCl_3$ on a Bruker Avance, 300 MHz instrument using TMS as internal standard. The chemical shift values are reported in ppm and coupling constants in Hz. ESI–MS spectra were recorded on a Perkin Elmer Turbo Mass/Shimadzu LC–MS. TLC analyses were carried out on precoated silica gel $60F_{254}$ plates (Merck) using solvent system, hexane:ethyl acetate (7:3). The compounds were visualized by either exposure of TLC plates to I_2 vapors or by spraying with vanillin–sulfuric acid reagent, followed by heating at 110 °C for 15 min. Si-gel, 60–120 mesh (spectrochem) was used in the column chromatography for the purification of metabolites. HPLC analyses were carried out on Waters Spherisorb ODS2 (250 × 4.6 mm^2 i.d.,10 μm) column using binary gradient elution with acetonitrile and water mobile phase 65:35 for 0–5 min, 70:30 for 5–20 min, and 75:25 for 20–30 min at a flow rate of 0.6 mL/min, column temperature of 25°, and UV detection at λ230 nm.

Plant cell suspension cultures and cultivation condition

Cell suspension cultures of *L. officinalis, G. glabra*, and *P. quinquefolium* were maintained and multiplied on a modified MS liquid medium (Patel *et al.*, 2011). The pH of all the media was adjusted to 5.8 before autoclaving. The inoculum to medium ratio of 1:10 was constantly used in all experiments. The suspension cultures were incubated at 26–28 °C on a rotary shaker (100 rpm) through a 15-, 12-, and 30-day culture passage for *L. officinalis, G. glabra*, and *P. quinquefolium*, respectively. α-artemether (**1**) was transformed by *L. officinalis* and *G. glabra* whereas DHA (**2**) was biocatalyzed only by *P. quinquefolium*. Substrate (300 mg) was dissolved in 9 ml of ethanol. The optimized dose of substrate, 7 mg/50 ml medium was added to 9-, 8-, and 15-day-old cultures of *L. officinalis, G. glabra*, and *P. quinquefolium* and kept for 5, 4, and 11 days, respectively. Cultures were taken out daily and analyzed by HPLC in order to determine the degree of transformation of substrate. In all experiments, medium without culture was also prepared to act as control. No metabolite was detected in the control.

Time course of substrate and metabolic product

The cultivation media were extracted with diethyl ether several times at various intervals. The crude extracts were analyzed by HPLC analysis. The ratios between the substrate and metabolic products determined on the basis of HPLC analysis are shown in Fig. 1.

Isolation of metabolic products

After incubation of substrate **1** with *G. glabra* and *L. officinalis* for 4 and 5 days, the individual incubated mixtures were pooled and extracted three times with diethyl ether, respectively. The combined respective extracts were dried over anhydrous Na_2SO_4 and the solvent was evaporated to dryness at 40 °C under reduced pressure to afford orange–brown crude extract. The extract was purified by column chromatography over a silica gel column, using a hexane–ethyl acetate gradient, followed by preparative TLC to obtain two colorless metabolites, a major **3**, Rf

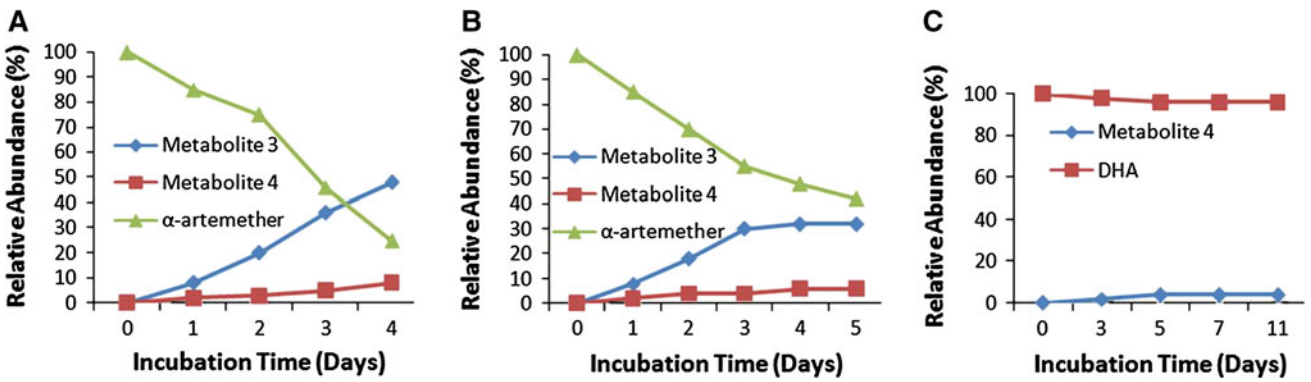

Fig. 1 Time course of biotransformation of α-artemether **(1)** by **a** *G.glabra*, **b** *L. officinalis*, and **c** DHA **(2)** by *P.quinquefolium*

0.55, 48 %, minor **4**, Rf 0.12, 8 % by *G. glabra* and **3** in 32 %, **4** in 6 % by *L. officinalis* w/w yields, respectively. The extract obtained after incubation of substrate **2** with *P. quinquefolium* for 11 days was purified as above to yield metabolite **4** in 4 % yield.

*(3aS, 4R, 6aS, 7R, 8R, 10R, 10aR)-3, 3a, 4, 5, 6, 6a, 7, 8-octahydro-8-methoxy-4, 7-dimethyl-2H, 10H-furo [3, 2-i] benzopyran-10-yl acetate (**3**)*

It was obtained as white solid, mp 95–96 °C. FT-IR (KBr) λ_{max} cm^{-1} 1748(ester CO), 1455, 1370, 1223, 1038, 919 cm^{-1}. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 0.86 (3H, d, $J = 6.9$ Hz, H_3-13), 0.90 (3H,d, $J = 6.0$ Hz, H_3-14), 2.11 (3H, s, H_3-15), 3.43 (3H, s, OCH_3), 3.89 (1H, q, $J = 7.5$ Hz, Ha-3), 4.27 (1H, t, $J = 7.2$ Hz, Hb-3), 4.42 (1H, d, $J = 9.0$ Hz, βH-12), 6.06 (1H, s, H-5). ^{13}C NMR ($CDCl_3$, 75 MHz), δ 54.78 (C-1), 27.55 (C-2), 68.56 (C-3), 169.21 (C-4), 91.06 (C-5), 80.22 (C-6), 47.37 (C-7), 22.72 (C-8), 35.46 (C-9), 34.93 (C-10), 30.51 (C-11), 102.56 (C-12), 12.25 (C-13), 20.46 (C-14), 21.52 (C-15), 56.58 9($O\underline{C}H_3$). ESI–MS (Positive): 321$[M+Na]^{+,}$ $C_{16}H_{26}O_5$.

*(3aS, 4R, 6aS, 7R, 10R, 10aR)-3, 3a, 4, 5, 6, 6a, 7, 8-octahydro-4, 7-dimethyl-2H, 10H-furo [3, 2-i] [2]benzopyran-10-ol (**4**)*

It was obtained as white crystals, mp 102–103 °C. FT-IR (KBr) λ_{max} cm^{-1} 3442(OH). ^{1}H NMR ($CDCl_3$, 300 MHz,) δ 0.76 (3H, d, $J = 7.2$ Hz, H_3-12), 0.96 (3H, d, $J = 6.3$ Hz, H_3-12), 2.37 (1H, m, H-11), 3.46(1H, t, $J = 12.0$, Ha-10), 3.67 (1H, m, Hb-10), 3.87(1H, m, Ha-3), 4.17 (1H, t, $J = 8.1$, Hb-3), 5.01 (1H, s, H-5). ^{13}C NMR ($CDCl_3$, 75 MHz), δ 56.64 (C-1), 27.84 (C-2), 69.74 (C-3), 81.70 (C-4), 47.26 (C-5), 21.47 (C-6), 35.84 (C-7), 30.84 (C-8), 94.97 (C-9), 67.43 (C-10), 30.24 (C-11), 13.24 (C-12), 20.97 (C-13). ESI–MS (Positive): $[M+Na]^+$ 249, $C_{13}H_{22}O_3$.

Results and discussion

To investigate the bioconversion ability of *G. glabra, L. officinalis*, and *P. quinquefolium*, a small amount of α-artemether (**1**) and DHA (**2**) were tested. Incubation of substrate **1** with *G. glabra* and *L. officinalis* for 4 and 5 days, respectively, yielded two metabolites **3** and **4**. *P. quinquefolium* was not able to biotransform **1** whereas substrate **2** was converted to metabolites **4** only by *P. quinquefolium* upon incubation for 11 days. The time course of the reaction was measured by TLC and HPLC analysis. In order to isolate bioconversion products, the incubated culture media were combined and extracted with diethyl ether. The extracts were then chromatographed on silica gel repeatedly and metabolites **3** and **4** were isolated. Metabolite **3**, mp 95–96 °C was obtained as colorless crystals. The ion at m/z 321$[M+Na]^+$ in its ESI–MS(Positive) spectrum established the molecular formula $C_{16}H_{26}O_5$. The IR spectrum showed the presence of an ester carbonyl function (1,748 cm^{-1}) in the molecule. The ^{1}H-NMR and ^{13}C-NMR (DEPT) showed the presence of a methoxy group [δ_H 3.43 (3H, s), δ_c 56.58 (OCH_3)], an oxymethylene group [δ_H 3.89 (q, $J = 7.5$ Hz), 4.27 (1H, t, $J = 7.2$ Hz); δ_c 68.56 (CH_2-O)], two oxymethine groups [δ_H 4.42 (1H, d, $J = 9.0$ Hz, βH-12); δ_c 102.56 (CH-O)], 6.06 (1H, s); δ_c 91.06 (CH-O)], a tertiary oxycarbon [δ_c 80.22], an acetate function (δ_H 2.11 (3H, s), δ_c 21.52, 169.21 (CH_3CO)], two methyls, three methylenes, and four methines. Compound **3** showed correlation between (i) H_2-3/C-2 and C-6, (ii) H_3-13/C-7 and C-12, (iii) H-5/C-4 and C-12, (vi) H-11/C-8, (v) H-12/C-13 and C-16 in the HMBC spectrum and NOEs between (i) H-5/H-10 and H-12 (Fig. 2). Thus on the basis of these data the metabolite **3** was assigned as tetrahydrofuran acetate derivative of α-artemether. Metabolite **4** was a 12-carbon ring-rearranged derivative, characterized as 5-hydroxydeoxoartemether by the comparison of its physicospectral (mp, IR, ^{1}H, and mass spectral) data reported in the literature (Khalifa *et al.*, 1995; Medeiros *et al.*, 2002; Wei *et al.*, 2010). In the present study, the conversion efficiency of α-artemether into metabolites **3** and **4** was in 48 and 8 %, respectively.

Metabolite **3** is a new compound which is isomeric with the reported compound, obtained from the bioconversion of β-artemether with *G. glabra* and *L. officinalis* by us (Patel *et al.*, 2010). Metabolite **4** was also reported earlier by microbial transformations (Patel *et al.*, 2010, 2011) but it is the first report by cell suspension cultures from α-artemether **1** and DHA **2**.

The proposed biocatalytic pathway for metabolites **3** and **4** is mentioned in Fig. 3. It appears that the enzymes produced by *G. glabra* and *L. officinalis* strated the homolytic cleavage of the peroxide bond of **1**, producing a diradical which subsequently rearranges to the ring-contracted

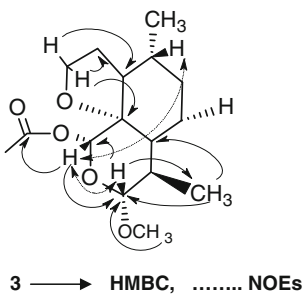

Fig. 2 HMBC (H → C) and NOEs correlations of compound **3**

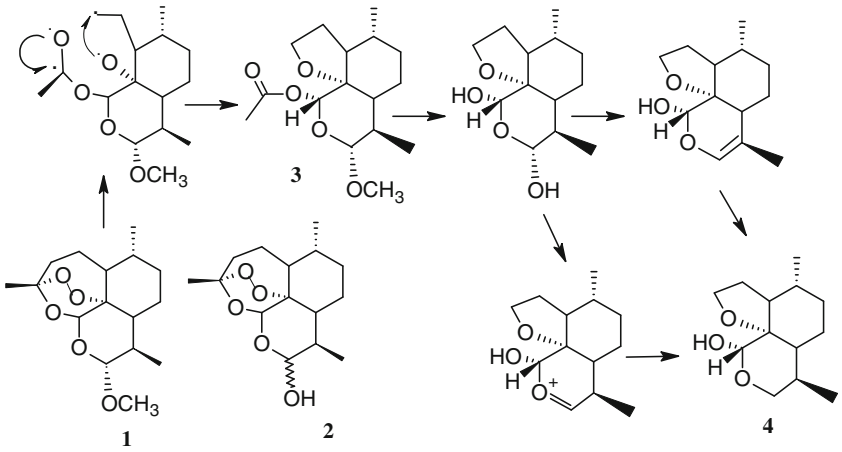

Fig. 3 Proposed bioconversion mechanism to obtain metabolites **3** and **4**

tetrahydyfuran acetate derivative **3** as also proposed by Kalita *et al.* (2003). The derivative **3** underwent hydrolysis of acetate and methoxy groups to C-5, C-12 hydroxyls which thereafter by the nucleophilic substitution of C-12 hydroxyl group by the hydride through reductase-utilizing NADPH/NADH co-factor or dehydration of hydroxyl to produce a double bond at C-12/C-13 followed by stereo-controlled reduction of the olefinic bond to deoxometabolite **4** (Musharraf *et al.*, 2012; Wu *et al.*, 1998). In the conversion of DHA (**2**) to **4** the matabolite **3** with hydroxyl at C-12 might be very unstable which quickly hydrolyzes the acetate group followed by substitution and reduction steps producing metabolite **4**. Since deoxoartemisin is more potent than artemisinin (Jung *et al.*, 1990), the presence of C-12 deoxo and C-5 hydroxyl in **3** and acetate function in the derivative **3** make them interesting synthones for further modification into new clinically potent molecules. Plant cell suspension cultures and cell-free enzyme extracts have been frequently employed as efficient biocatalysts to carry out complex regiospecific or stereospecific chemical reactions that are otherwise difficult or very costly to perform through synthetic mechanisms (Giri *et al.*, 2001; Shams *et al.*, 2005, 2007; Yang *et al.*, 2008). The present study constitutes the first report on the successful utilization of cell cultures of *G. glabra* and *L. officinalis* in the bioconversion of antimalarial compound α-artemether into its THF–acetate **3** and 12-carbon rearranged derivative **4**, and *P.quinquefolium* in the bioconversion of antimalarial compound DHA into derivative **4**. This work may contribute in understanding the metabolism of artemisinin derivatives (**1,2**) in the biological system (Park *et al.*, 1998) and designing new artemisinin derivatives with better biological profile.

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