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## Biotransformation of Artemisinin Mediated through Fungal Strains for Obtaining Derivatives with Novel Activities

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### Abstract

Artemisinin, a sesquiterpene lactone, is the active antimalarial constituent of *Artemisia annua*. Several fungal strains *Saccharomyces cerevisiae*, *Aspergillus flavus*, *Aspergillus niger* and *Picchia pastoris* were used to biotransform artemisinin. Among these strains, *A. flavus* was the only microorganism capable of transforming artemisinin to deoxyartemisinin in higher yields than the previous reports. The structure of deoxyartemisinin was elucidated by spectroscopy. Deoxyartemisinin showed antibacterial activity against *Staphylococcus aureus*, *S. epidermidis* and *S. mutans* at a minimum inhibitory concentration (MIC) of 1 mg/mL compared to artemisinin whose MIC was >2 mg/mL.

### Keywords

Biotransformation • Artemisinin • Deoxyartemisinin • *Aspergillus flavus* • Antibacterial

## Introduction

Artemisinin, a sesquiterpene lactone isolated from the aerial parts of *Artemisia annua* (Family: Asteraceae) is a potent therapeutic agent combating multidrug resistant *Plasmodium falciparum* strains and chloroquine-resistant parasites [1, 2]. The metabolism, chemical transformation, thermal rearrangement and decomposition studies of artemisinin were well established [3, 4]. Among known antimalarial compounds, artemisinin has a novel structure with a unique 1,2,4-trioxane ring system. But, the utility of artemisinin is limited in the biological systems due to its toxicity and water insolubility [5, 6]. Studies on modification of artemisinin through biological [4, 7–8] and chemical methodologies [9, 10] have been reported [11] to yield more effective and water soluble derivatives. Studies on quantitative structure activity relationship of artemisinin suggest that the structural modification of sesquiterpene lactone may yield desirable antimalarial analogues [12–14]. Biological conversions using microorganisms and their enzymes are most effective on sesquiterpene molecules due to their susceptibility to bioconversion at a cost effective rate.

Studies on the microbial transformation of artemisinin with *Nocardia corollina*, *Penicillium chrysogenum*, *Mucor polymorphus*, *Cunninghamella echinulata*, *Mucor ramannianus*, *Eurotium amstelodami*, *Streptomyces niger*, *Cunninghamella elegans* yielded 3 $\alpha$ -hydroxydeoxyartemisinin, deoxyartemisinin, artemisitone-9, 4 $\alpha$ -hydroxyartemisinin, 7 $\beta$ -hydroxyartemisinin, 6-hydroxyartemisinin, 5 $\beta$ -hydroxyartemisinin, 9 $\beta$ -hydroxyartemisinin, 3 $\beta$ -hydroxyartemisinin, 3 $\beta$ -hydroxydeoxyartemisinin, 1 $\alpha$ -hydroxydeoxyartemisinin, 7 $\beta$ -hydroxy-9 $\alpha$ -artemisinin, 4 $\alpha$ -hydroxy-1-deoxyartemisinin, 10 $\beta$ -hydroxyartemisinin and 3 $\alpha$ -hydroxydeoxyartemisinin respectively [4, 15–20]. The importance and usefulness of microbial transformation in carrying out a variety of chemical conversions is well documented in the previously published reports [21, 22].

Microorganisms are one of the most efficient biocatalytic agents with ability to metabolize a wide range of substrates. Several reports on microbial transformation of sesquiterpenes have been published earlier [23–26]. Biotransformation of artemisinin usually includes the processes such as hydroxylation of methyl, methyne and methylene groups deoxidation reactions, hydration reactions and breakdown of heterocyclic rings [8, 15, 16, 19, 27–32].

In the present study we have examined the bioconversion of artemisinin using different microorganisms. *A. flavus* was found to transform artemisinin to deoxyartemisinin in higher yield (30.5%) than the previous reports [4, 15, 31]. The biotransformed product was characterized by NMR, IR and Mass spectroscopy. Deoxyartemisinin was also showed antibacterial activity.

## Results and Discussion

Four different fungi, *Saccharomyces cerevisiae*, *Aspergillus flavus*, *Aspergillus niger* and *Picchia pastoris* were initially screened for their ability to biotransform artemisinin. Out of these, *A. flavus* efficiently converted artemisinin to deoxyartemisinin in 48h grown culture. The biotransformed product was isolated through column chromatography on silica gel. The product was identified on the basis of its melting point and spectroscopic data. In the ESI mass spectroscopy deoxyartemisinin showed molecular ion peaks at 289 [M+Na]<sup>+</sup>, 305 [M+K]<sup>+</sup> and 571.1 [2M+K]<sup>+</sup> in positive mode and at 265 [M–1]<sup>–</sup> in the negative ion

mode. In the EI mass spectra it showed molecular ion peak at 266 consistent with the molecular formulae  $C_{15}H_{22}O_4$  and further fragmentation pattern clearly indicating it as deoxyartemisinin. The compound's  $^1H$  and  $^{13}C$  NMR and different 2D spectral data were well in agreement with the earlier published reports [4].

Artemisinin was biotransformed to an extent of 26–32% into deoxyartemisinin which was separated through column chromatography to obtain 95% purity with a 30.50% yield. The purity was evaluated using RP-HPLC area normalization method.

Biotransformation performed by *Aspergillus flavus* with our protocol was much better in yield (30.50%) than the previously reported methods [4, 15]. The compound deoxyartemisinin showed *invitro* antibacterial activity against *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus mutans* at a minimum inhibitory concentration (MIC) of 1mg/mL, whereas MIC of artemisinin was found to be more than 2 mg/mL.

For the last few years, research work on artemisinin and its analogues was focused on to decipher the enzymes involved in the proposed biosynthetic pathway [35–37]. In this direction, the present study was aimed to identify the possible conversions of artemisinin using microorganisms and to explore the transformation similarities between the present microbial model and previously reported systems. Such a microbial system could then further be used as a model to predict and generate metabolites of artemisinin and its analogues for novel biological activities.

In conclusion, the formation of the deoxyartemisinin suggests that *A. flavus* has the potential to serve as a microbial model for generating metabolites of artemisinin and its related analogues of varied structural diversity. Thus, it appears that *A. flavus* may be a useful tool to prepare various metabolites of artemisinin and its related analogues. The biological activity obtained with deoxyartemisinin may provide preliminary information for the design of novel antibacterial agents.

## Experimental

### Substrate

Artemisinin (Fig. 1) was isolated from the leaves of *Artemisia annua* and characterized by melting point, NMR and Mass spectroscopy (1). Artemisinin was dissolved in sterile DMSO (100mg/ml) to prepare a stock solution and 625  $\mu$ l of it was added to 125 ml of Sabouraud Dextrose Broth (SDB) to make the final concentration of 0.5mg/ml.

### Microorganisms used

The Microorganisms tested for the biotransformations were *Saccharomyces cerevisiae*, *Aspergillus flavus*, *Aspergillus niger* and *Picchia pastoris*. Among these strains, *A. flavus* showed promising results with the formation of a distinct product.

### Media preparation

All the fermentation experiments using *A. flavus* were carried out in the defined medium of SDB comprising of Peptone (10 grams) and dextrose (40 grams) in 1litre of distilled water.

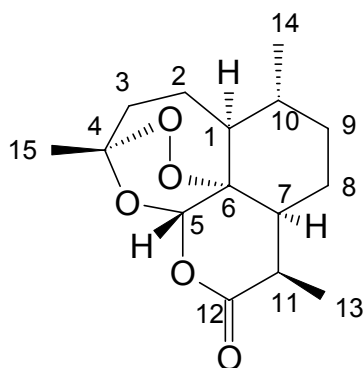
The pH was adjusted to 5.8 before autoclaving at 121°C for 15 minutes. Stock cultures of the fungal strains were stored on the slants of Sabouraud Dextrose Agar at 4°C.

### **Fermentation procedure**

The cultures of *A. flavus* were grown on Sabouraud Dextrose Agar plates at 28°C for a week. Well developed mycelia were removed from the plates, suspended in 10 ml of normal saline and used to inoculate 125ml of SDB in a 500ml shaker flask. The substrate artemisinin was added to the broth at the concentration of 0.5mg/ml at the time of inoculation. Cultures were grown for 48 hours on rotary shaker incubator (Kuhner shaker) at 28°C with shaking speed at 200 rpm. Culture control consisted of fermentation blank in which microorganism was grown under identical conditions except for no substrate. Substrate control consisted of sterile medium containing the same amount of substrate incubated under the same conditions.

### **Isolation of metabolites**

The cultures were harvested by centrifugation at 10,000 rpm for 25 minutes in centrifuge (REMI). The broth and mycelia were separated using coarse filter paper (Whatmann no.1) in a funnel. The culture broth was extracted thrice with equal volumes of ethyl acetate and evaporated under vacuum. The residue was washed with chloroform and evaporated under vacuum. The chloroform soluble portion (980mg) was column chromatographed (3x 50cm) over silica gel (60–120 mesh size). A mixture of hexane–ethyl acetate was used as eluent with increasing polarity of ethyl acetate (2%, 4%) in hexane. Fractions collected in 8% ethyl acetate–hexane were checked on TLC using artemisinin spray reagent to confirm deoxyartemisinin developed in green color. These fractions were evaporated in vacuum and the residue was recrystallised with chloroform–hexane (1:3) to get deoxyartemisinin (**Fig. 2**) as a colorless solid. Compound **2** (Yield= 282mg (30.5%). m.p. = 111–112°C) showed IR (KBr): 2938, 1748, 1389, 1139, 1016  $\text{cm}^{-1}$ ; EI MS (MeOH): 266 [ $\text{M}^+$ ], 237, 224, 195, 165, 164. ESI MS (MeOH): Positive mode: 289 [ $\text{M}+\text{Na}^+$ ], 305 [ $\text{M}+\text{K}^+$ ], 571.1 [ $2\text{M}+\text{K}^+$ ]; Negative mode: 265 [ $\text{M}-1^-$ ]. NMR data given in Table 1. The purity of (**2**) was obtained using peak area normalization of high performance liquid chromatographic analysis method [column– Phenomenex  $\text{C}_{18}$  4.6 x 250mm, 5 $\mu\text{m}$ ; mobile phase– acetonitrile:  $\text{CH}_3\text{COONH}_4$  (10mM) +0.1%  $\text{HCOOH}$  (85:15); Flow– 1mL/min; detection–210nm PDA].



**Fig. 1.** Artemisinin

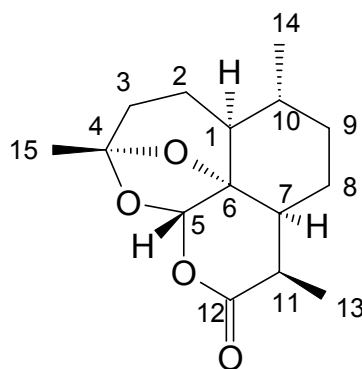


Fig. 2. Deoxyartemisinin

### Characterization of transformed products

Tab. 1.  $^1\text{H}$  and  $^{13}\text{C}$ NMR data of 1 and 2 (both in  $\text{CDCl}_3$ ,  $\delta$  values)<sup>a,b</sup>

Assignment	Artemisinin (1)		Deoxyartemisinin (2)	
	$^{13}\text{C}$	$^1\text{H}$	$^{13}\text{C}$	$^1\text{H}$
1	50.17	1.40 (m)	45.10	1.27 (m)
2	24.87	1.47 (m), 2.03 (m)	22.43	1.23 (m), 1.88 (m)
3	35.96	2.43 (ddd), 2.07 (ddd)	34.41	1.59 (m), 1.77 (m)
4	105.32	—	109.51	—
5	93.68	5.87 (s)	100.01	5.88 (s)
6	79.48	—	82.80	—
7	45.05	1.77 (m)	42.88	2.44 (dt)
8	23.39	1.88 (m), 1.12 (m)	23.90	1.07 (m), 1.88 (m)
9	33.66	1.81 (m), 1.09 (m)	33.91	1.11 (m), 1.77 (m)
10	37.63	1.43 (m)	35.75	1.24 (m)
11	32.89	3.38–3.42 (qd)	33.13	3.39 (distorted quartet)
12	171.82	—	171.97	—
13	12.49	1.23 (d, 7.2 Hz)	12.93	1.21 (d, 7.2 Hz)
14	19.74	0.99 (d, 6.0 Hz)	18.87	1.01 (d, J=6.0 Hz)
15	25.15	1.46 (s)	24.28	1.46 (s)

<sup>a</sup> Assignments are based on DEPT,  $^1\text{H}$ – $^1\text{H}$  COSY, HSQC and HMBC experiments.

<sup>b</sup> Signal multiplicity and coupling constants (Hz) are in parentheses.

Melting points were determined using Toshniwal melting point apparatus and were uncorrected. Biotransformation reactions were monitored on Merck aluminium thin layer chromatography (TLC) plates. Visualisation was accomplished by dipping TLC plates with artemisinin reagent (Glacial acetic acid: Sulphuric acid: Anisaldehyde, 500:10:5) and charring them at higher temperatures. Column chromatography was carried out on silica gel (60–120 mesh, Thomas Baker Chemicals).  $^1\text{H}$  NMR and  $^{13}\text{C}$ NMR analysis was

performed on Bruker Avance–300 MHz spectrometer using tetramethylsilane as internal standard. Chemical shifts are given in  $\delta$  ppm values (Table 1). Electron impact ionization (EI) mass spectra were recorded on Perkin Elmer GC–MS system after dissolving the compounds in methanol. Electrospray ionization (ESI) mass was recorded on Shimadzu LC–MS system in both positive and negative modes after dissolving compound in methanol.

### **Biological activity**

Antibacterial activity (Table 2) of artemisinin and deoxyartemisinin were performed as per standardized protocols [33, 34].

**Tab. 2.** Minimum inhibitory concentration (MIC) of artemisinin and deoxyartemisinin against *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus mutans*.

Pathogenic Bacteria	Minimum Inhibitory Concentration (mg/ml)	
	Artemisinin	Deoxyartemisinin
<i>Staphylococcus aureus</i>	>2	1
<i>Staphylococcus epidermidis</i>	>2	1
<i>Streptococcus mutans</i>	>2	1

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### **Authors' Statement**

#### **Competing Interests**

The authors declare no conflict of interest.

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