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Bio-transformation of artemisinin using soil microbe: Direct C-acetoxylation of artemisinin at C-9 by *Penicillium simplissimum*

Abhishek Goswami [a], Partha P. Saikia [a], Nabin C. Barua [a,*], Manobjyoti Bordoloi [a], Archana Yadav [b], Tarun C. Bora [b], Binod K. Gogoi [b], Ajit Kumar Saxena [c], Nithasa Suri [c], Madhunika Sharma [c]

[a] *Natural Products Chemistry Division, North-East Institute of Science & Technology (CSIR), Jorhat 785 006, Assam, India*
[b] *Biotechnology Division, North-East Institute of Science & Technology (CSIR), Jorhat 785 006, Assam, India*
[c] *Pharmacology Division, Indian Institute of Integrative Medicine (CSIR), Jammu 180 001, India*

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ABSTRACT

Potent antimalarial compound artemisinin, **1** was bio-transformed to C-9 acetoxy artemisinin, **2** using soil microbe *Penicillium simplissimum* along with C-9 hydroxy derivative **3**. The products were characterized using high field NMR and MS–MS data. The absolute stereochemistry of the newly generated chiral centers has been ascertained by COSY and 1D NOESY experiments. This is the first Letter of direct C-acetoxylation of artemisinin using microbial strains.

Artemisinin, **1** is a sesquiterpene lactone endoperoxide isolated[1] in 1971 from the Chinese medicinal plant *Artemisia annua* L. Due to their potent antimalarial activity, low toxicity, and fast action, artemisinin and a few of its analogue viz. artemether, artether, artesunate have gained importance over the years as a new generation antimalarial drugs, especially in the treatment of multi-drug-resistant malaria strains.[2] However, sensitive nature of the artemisinin molecule has restricted extensive chemical transformation on this naturally occurring endoperoxide for developing more potent antimalarial derivatives. In this context, several authors have reported findings on bio-transformation of artemisinin which include introduction of a keto function in the C-9 position of artemisinin by *Streptomyces griseus* ATCC 13273,[3] conversion to 3α-hydroxy-deoxyartemisinin and deoxyartemisinin by *Mucor polymorphosporus* and *Aspergillus niger*,[4,5] conversion to 9β-hydroxy artemisinin and 3α-hydroxy artemisinin,[5–7] conversion to 10-hydroxy artemisinin and 9β-hydroxy-11α-artemisinin,[7] and conversion to 5β-hydroxy artemisinin.[8] However, direct C-acetoxylation with microorganism has not been previously known.

In connection with our ongoing program on bio-transformation of phytochemicals for value addition using microbial strains[9] we screened several strains isolated from untapped mega biodiversity hot zone of Indo-Burma belt for biocatalytic activity using potent antimalarial compound artemisinin **1**, with an aim to synthesize novel analogues for using as scaffold for synthetic manipulation. During this study, it has been observed that fungal strain *Penicillium simplissimum*[10] converted artemisinin into C-9 acetoxylated compound **2** (20.6% yield), mp 113 °C, $[\alpha]_D$ −75 (*c* 0.5, $CHCl_3$) and C-9 hydroxylated compound **3** (31.3% yield),[11] mp 175 °C, $[\alpha]_D$ +55 (*c* 0.25, $CHCl_3$) (Scheme 1).

Fungus strains were isolated from forest soil samples collected from Tripura and Kaziranga National park, Assam, India. The strains were isolated in selective isolation medium using Rosebengal chloramphenicol agar[12] and maintained as pure culture after repeated sub-culturing in Potato Dextrose Agar (PDA) which is composed of (g/L): potato infusion (200.0), dextrose (20.0), agar (15.0). PDA was also used for routine maintenance of the fungus strains. All the strains were preserved in mineral oil as stock cultures at −4 °C with a periodic check for viability.

A panel of 40 freshly isolated fungus strains was selected to screen for bio-transformation of artemisinin using environmentally benign technique.[13] Out of the fungus strains tested, only two were able to produce metabolites polar than artemisinin in the screening. However, *P. simplissimum* produced two major metabolites and preparative TLC (1:2 EA/hexane) enabled us to obtain **2** and **3** in 20.6% and 31.3%, respectively.

The stereo chemical assignments for both the bio-transformed products have been determined using high field NMR. The COSY spectrum recorded at 300 MHz for compounds 2 and **3**, revealed

* Corresponding author. Tel.: +91 376 2372948; fax: +91 376 2370011.
E-mail address: ncbarua12@rediffmail.com (N.C. Barua).

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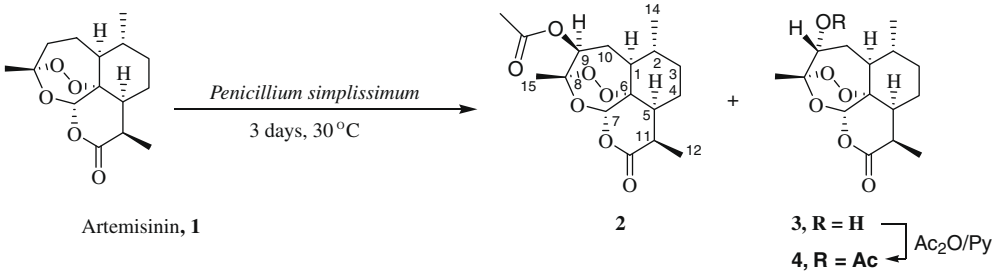

Scheme 1.

the complete coupling network of both the molecules. In the ^{1}H NMR spectrum of **2**, the signal at δ 4.21 was assigned to the proton under acetoxy group. This signal was found to be coupled with multiplets at δ 3.94, δ 1.71, and long-range ω-coupling with signal at δ 2.03. One of the protons at C-10 of compound **1** has shifted to δ 3.94 due to anisotropy of the neighboring acetoxy group and lone pair–lone pair interaction of oxygen atoms of the molecule. This long-range coupling between signal at δ 4.21 and δ 2.03 revealed that the proton under acetoxy group has same configuration as that of the H-1 (δ 2.03). Similar long-range coupling was not observed in the case of compound **3**. ^{1}H NMR recorded at 300 MHz further revealed that the acetate of compound **2** is stereochemically different from compound **4**. The ^{1}H NMR spectra of **2**, 3, and **4** with assignment by COSY are given in Table 1. The stereochemistry of compound **2** at C-9 was further confirmed by 1D NOESY experiments (Fig. 1).

When H-1 at δ 2.03 was irradiated, NOE was observed at δ 4.21 (H-9), 3.94 (H-10a), and 0.90 (H-14). However, when H-9 at δ 4.21 was irradiated, no NOE was observed at δ 1.59 (H-15) of artemisinin. Since, the absolute stereochemistry of H-1 and methyl at C-2 of natural artemisinin is α oriented,[14] therefore, stereochemistry of the acetoxy group at C-9 is assigned as β.

Antimalarial activities of the metabolites were evaluated at Regional Medical Research Centre (ICMR), Dibrugarh, Assam, India against chloroquine sensitive *P. falcipurum* 3D-7. However, none of the metabolites were found superior in antimalarial activity to that of artemisinin.

As artemisinin derivatives and dimers continue to exhibit excellent anticancer activities[15] against different cell lines, we

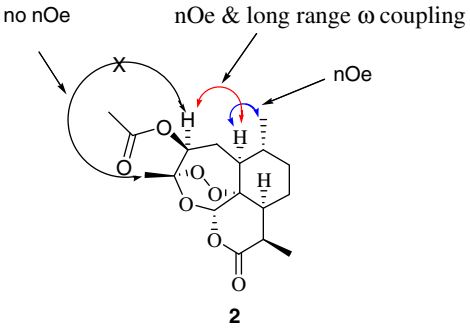

Figure 1.

were also tempted to evaluate the in vitro cytotoxicity[16]of the metabolites **2** and **3**. The study was carried out against five Human Cancer Cell lines procured from National Cancer Institute, Frederick, USA. Growth inhibitory activities indicate that both metabolite **2** and **3** are particularly inhibitory to Colon HCT-15 cell lines although active in a few other (e.g., neuroblastoma) cancer cell lines (Table 2).

In conclusion, we have reported formation of a hitherto unknown C-9β-acetoxy artemisinin along with 9α-hydroxy artemisinin from the bio-transformation of artemisinin using *P. simplissimum* which opens up further prospect of synthetic manipulation at otherwise inaccessible carbon centers. This is also the first Letter of direct C-acetoxylation using a microbial strain.

Table 1
^{1}H NMR assignments of **2**, **3**, and **4** by COSY

Compound	^{1}H NMR assignments
2	6.64 (1H, s, H-7), 4.21 (1H, ddd, *J* = 8.5, 7.8 & 2 Hz, H-9), 3.94 (1H, ddd, *J* = 15, 8.5 & 7.5 Hz, H-10a), 3.16 (1H, dq, *J* = 4.6 & 7 Hz, H-11), 2.15 (3H, s, OAc), 2.03 (1H, m, H-1), 1.90 (1H, m, H-5), 1.71 (1H, m, H-3a), 1.51 (1H, m, H-2), 1.59 (3H, s, H-15), 1.20 (3H, d, *J* = 7 Hz, H-12), 1.15 (1H, m, H-4a), 1.12 (1H, m, H-3b), 0.90 (3H, d, *J* = 6 Hz, H-14)
3	5.63 (1H, s, H-7), 3.63 (1H, m, H-9), 3.18 (1H, dq, *J* = 7 & 12 Hz, H-11), 2.07 (1H, m, H-5), 1.97 (2H, m, H-10a & H-1), 1.81 (1H, m, H-3b), 1.54 (1H, m, H-4a), 1.51 (1H, m, H-10b), 1.57 (3H, s, H-15), 1.26 (1H, m, H-2), 1.20 (3H, d, *J* = 7 Hz, H-12), 1.08 (1H, m, H-3a), 1.04 (1H, m, H-4b), 0.92 (3H, d, *J* = 6 Hz, H-14)
4	5.67 (1H, s, H-7), 4.79 (1H, dd, *J* = 2 & 1.5 Hz, H-9), 3.21 (1H, dq, *J* = 7 & 5 Hz, H-11), 2.13 (3H, s, OAc), 2.09 (1H, m, H-5), 1.99 (1H, m, H-10b), 1.98 (1H, m, H-1), 1.97 (1H, m, H-4a), 1.85 (1H, m, H-3a), 1.53 (1H, m, H-10a), 1.50 (3H, s, H-15), 1.21 (3H, d, *J* = 7, H-12), 1.14 (1H, m, H-2), 0.93 (3H, d, *J* = 6 Hz, H-14)

Table 2
In vitro cytotoxicity of the metabolites

Compound	Concn (M)	Cell line type				
		Colon HCT-15	Colon SW-620	Lung A-549	Ovary IGR-OV-1	Neuroblastoma IMR-32
2	5×10^{-5}	32	0	28	10	31
3	5×10^{-5}	28	0	18	3	20
5-FU	2×10^{-5}	49	54	–	–	–
Adriamycin	1×10^{-6}	–	–	–	–	72
Paclitaxel	1×10^{-5}	–	–	52	56	–
Artemisinin	5×10^{-5}	31	35	29	26	23

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10. Fungus strain was isolated from forest soil samples collected from Tripura and Kaziranga National park, India. The strains were isolated in selective isolation medium using Rosebengal chloramphenicol agar and maintained as pure culture, after repeated sub-culturing, in Potato Dextrose agar (PDA) medium (Hi Media Laboratories Pvt. Ltd, Mumbai, India). After detailed study on the vegetative hyphae, sporangium and spore characteristics such as color, shape, size, position, and texture, including biochemical characterizations, the strain capable of carrying out bio-transformation of the substrate was identified as *Penicillium simplissimum* by MTCC & Gene Bank, IMT, Chandigarh, India, also an International Depository Authority.
11. 9β-Acetoxy artemisinin (**2**): Colorless needles; mp 111–113 °C; $[\alpha]_D^{20}$ −75 (*c* 0.5, $CHCl_3$); IR ($CHCl_3$) *ν* 2926, 1757, 1459, 1222, 1155 cm^{-1}; ^{13}C NMR (75 MHz, $CDCl_3$) *δ* 171.43, 168.21, 92.68, 79.04, 68.98, 54.44, 46.28, 34.66, 34.25, 32.78, 30.58, 29.5, 27.36, 23.96, 20.96, 20.09, 12.20 ESIMS (*m/z*): 338.8 (M^+-1) Anal. Calcd for $C_{17}H_{24}O_7$: C, 59.99; H, 7.11. Found: C, 59.96; H, 7.23. 9α-Hydroxy artemisinin (**3**): colorless needles; mp 173–175 °C; $[\alpha]_D^{20}$ +55 (*c* 0.25, $CHCl_3$); IR ($CHCl_3$) *ν* 3430, 2922, 1746, 1145, 1016 cm^{-1}; ^{13}C NMR (75 MHz, $CDCl_3$) *δ* 171.27, 98.65, 82.61, 68.67, 41.68, 40.17, 34.79, 33.02, 32.39, 29.96, 29.39, 23.19, 20.21, 18.10, 12.27 ESIMS (*m/z*): 300 (M^++2) Anal. Calcd for $C_{15}H_{22}O_6$: C, 60.39; H, 7.43. Found: C, 60.44; H, 7.29. Compound (**4**) colorless needles; mp 133 °C; $[\alpha]_D^{20}$ −60.8 (*c* 0.4, $CHCl_3$); IR ($CHCl_3$) *ν* 2925, 1751, 1219 cm^{-1}; ^{13}C NMR (75 MHz, $CDCl_3$) *δ* 171.31, 98.69, 82.69, 68.71, 41.6, 40.27, 34.67, 32.29, 32.49, 29.84, 29.57, 23.31, 20.25, 18.17, 12.62. ESIMS (*m/z*): 338.9 (M^+-1).
12. Rosebengal chloramphenicol agar is composed of (g/L): mycological peptone (5.0), dextrose (10.0), mono-potassium phosphate (1.0), magnesium sulfate (0.50), rosebengal (0.05), chloramphenicol (0.10), agar (15.0).
13. All the fungus strains were previously grown in PDA slants for 6 days and a spore suspension was prepared by transferring spores from the sporulating slants. A loopful of spores (2×10^6 spore ml^{-1}) from selected strains were used to inoculate PDA broth (50 ml) contained in 250 ml Erlenmeyer flasks which were then incubated at 30 °C for 3 days in a gyratory shaker (3.3 Hz at 200 rev min^{-1}; Clim-O-Shake, Adolf Kuhener AG). In the meantime, PDA broth was amended with substrate artemisinin (600 mg L^{-1}). After 3 days of incubation, the fermented broth was filtered through glass wool to separate the mycelial mat. The culture filtrate was extracted three times with equivalent amount of ethylacetate.
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16. Stock solutions of 2×10^{-2} M of test materials were prepared in DMSO. The stock solutions were serially diluted with complete growth medium containing 50 μg/ml of gentamycin to obtain working test solution of 2×10^{-5} M so that a final concentration of 5×10^{-5} M could be achieved. The 100 μl of cell suspension was added to each well of the 96-well tissue culture plate. The cells were incubated for 24 h (at 37 °C in an atmosphere of 5% CO_2 and 90% relative humidity in a carbon dioxide incubator). Test materials in complete growth medium (100 μl) were added after 24 h incubation to the wells containing cells. The plates were further incubated for 48 h in a carbon dioxide incubator after addition of test materials. The cell growth was stopped by gently layering trichloroacetic acid (50 μl, 50% w/v) on top of the medium in all the wells. The plates were incubated at 4 °C for 1 h to fix the cells attached to the bottom of the wells. The liquid of all the wells was then gently decanted and discarded. The plates were washed five times with distilled water and air-dried. Cell growth was measured by staining with sulforhodamine B dye.[17]The adsorbed dye was dissolved in Tris-Buffer (100 μl, 0.01 M, pH 10.4) and plates were gently stirred for 10 min on a mechanical stirrer. The optical density (OD) was recorded on ELISA reader at 540 nm. The cell growth was calculated by subtracting mean OD value of respective blank from the mean OD value of experimental set. Percent growth in presence of test material was calculated considering the growth in absence of any test material as 100% and in turn percent growth inhibition in presence of test material was calculated.
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