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SCIENCE DIRECT
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 16 (2006) 1909–1912

A novel ketone derivative of artemisinin biotransformed by *Streptomyces griseus* ATCC 13273

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Received 17 October 2005; revised 5 December 2005; accepted 24 December 2005
Available online 24 January 2006

Abstract—A novel ketone derivative of artemisinin, artemisitone-9, was produced by the biotransformation of cultured *Streptomyces griseus* ATCC 13273. The structure of the ketone product was fully elucidated by various spectroscopic techniques, and the mechanism of generating such novel metabolite is also discussed.

Artemisinin (**1**, Qinghaosu) is the active component of the antimalarial herb *Qinghao* (*Artemisia annua L*, *Asteraceae*)[1]. Because of its high therapeutic values in treating malaria, especially chloroquine-resistant *Plasmodium falciparum* and the cerebral infections, tremendous efforts have been made toward structure modification and analogue synthesis with the aim of developing more potent antimalarial agents with improved in vivo stability since it was discovered in 1970s.

Thus far, most of the structural modifications took place at the lactone moiety of artemisinin, modifications and structure–activity relationship on the two saturated rings remain unexplored due mainly to the difficulty of introducing functionalities on the ring systems by conventional chemical methods. Therefore, microbial transformation comes to play an important role to overcome the inaccessibility by chemical reactions and to serve as a valuable tool to introduce hydroxy group(s) on the saturated rings. To date, a number of oxidation products of artemisinin at different positions have been reported. These transformations include conversion to 3α-hydroxy-deoxyartemisinin and deoxyartemisinin;[2,3] conversion to 9β-hydroxy-artemisinin and 3α-hydroxy-artemisinin[3–5], and conversion to 10-hydroxy-artemisinin and 9β-hydroxy-11α-artemisinin.[5] In addition, microbial transformations on some artemisinin analogues, such as artemether, arteether, artemisitene, and 12-deoxoartemisinin, have been reported to produce oxidative products by different microorganisms.[6–10]

In order to obtain novel analogues for structural modification of artemisinin, we initiated a screening of different microorganisms with a two-stage fermentation protocol,[11] and metabolite generation was monitored by analyzing the fermentation broth by HPLC over the time. A number of microorganisms were able to produce polar metabolites in the screening. However, unlike other microorganisms, *Streptomyces griseus* ATCC 13273 produced a major metabolite that was eluted between artemisinin and other three polar metabolites from the HPLC analyses. This novel phenomenon led us to pursue isolation and identification of these metabolites.

A preparative fermentation at 1.2 L scale was conducted with *S. griseus* ATCC 13273 by transformation of 720 mg artemisinin substrate to isolate and identify the products. At 84 h of the second stage of fermentation, the fermentation broth was pooled and filtered, and the filtrate (1.2 L) was extracted three times with ethyl acetate. The extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure to afford 0.84 g of dark brown residue. Then, the residue was purified by column chromatography over an 80 g silica gel column eluting with a chloroform–methanol gradient to afford artemisitone-9 (**2**) (90.1 mg, 12.5% yield), 9α-hydroxy-artemisinin (**3**) (118.7 mg, 16.5%), 9β-hydroxy-artemisinin (**4**) (115.8 mg, 16.1%), and 3α-hydroxy-deoxyartemisinin (**5**) (68.7 mg, 9.5%) as shown

Keywords: Biotransformation; *Streptomyces griseus* ATCC 13273; Artemisinin; regioselectivity; Artemisitone-9.
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doi:10.1016/j.bmcl.2005.12.076

in Scheme 1. The structures of **2**, **3**, **4**, and **5** were fully characterized by mass spectrometry and NMR spectroscopy.

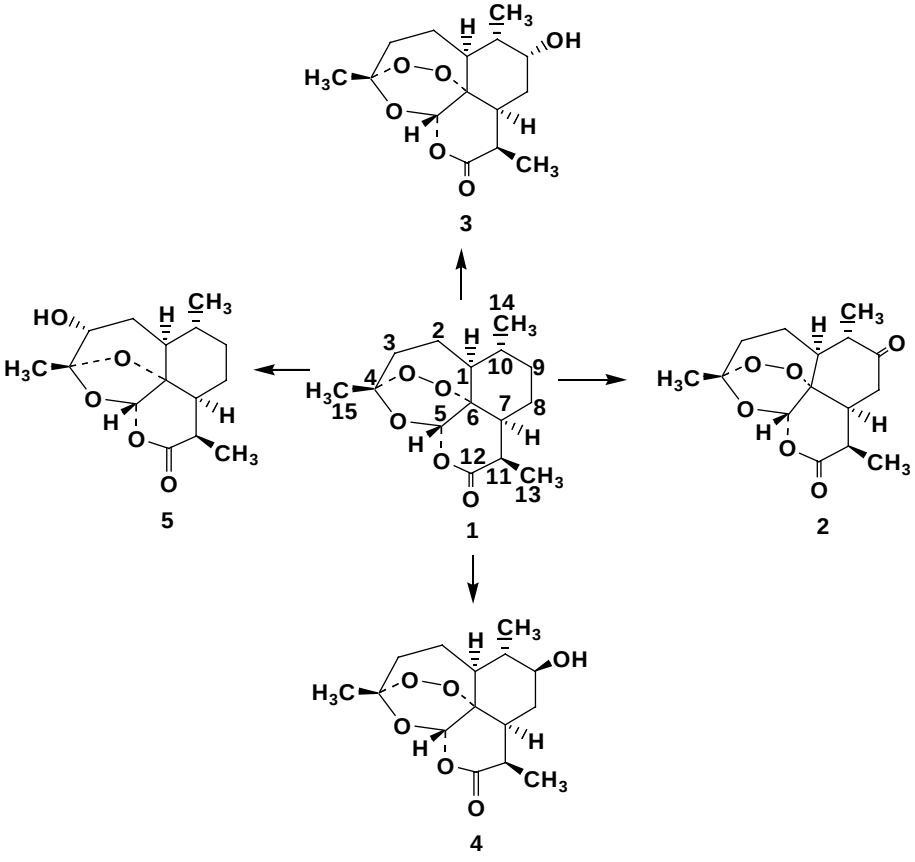
HREIMS analysis of **2** indicated its molecular formula of $C_{15}H_{20}O_6$. Peaks of IR spectra at 822, 878, 1113, and 1739 cm^{-1} suggested the existence of the endoperoxide bridge and lactone skeleton. ^{13}C NMR signal at δ205.8 and the inverted peaks at δ 26.1, 35.8, and 38.3 of DEPT spectra recorded with $\theta = 135°$ confirmed that one of the secondary carbons on the 6-membered ring of **1** was converted to a carbonyl group, and this carbonyl group is located at C-9 position according to the HMQC data. Based on the spectroscopic data compared with established ^1H and ^{13}C NMR assignments,[12] this metabolite was identified as artemisitone-9 (**2**),[13] a novel ketone derivative of **1**.

All data from IR, CIMS, NMR spectra for metabolite **3** indicated as 9α-hydroxy-artemisinin, a mammalian metabolite previously reported by Chi et al.[14]

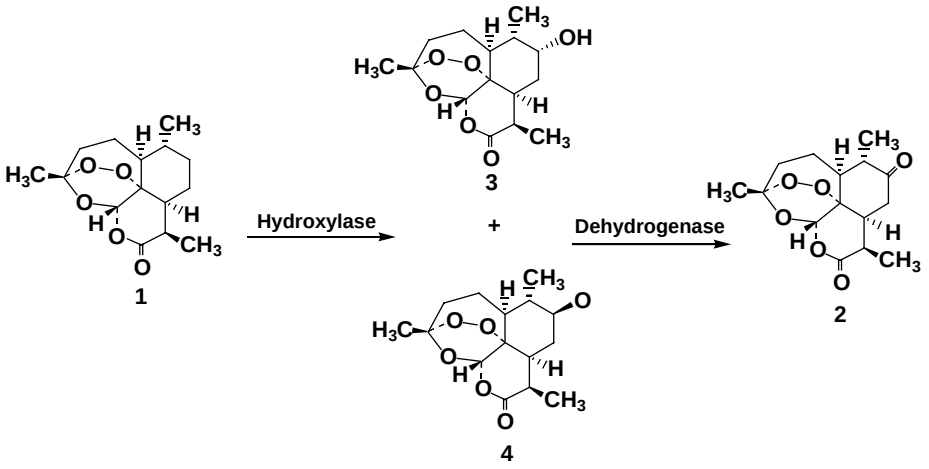
All data from IR, CIMS, NMR spectra for metabolite **4** were in excellent agreement with 9β-hydroxy-artemisinin, an epimer of metabolite **3** and previously reported in the literature.[3–5]

All data from IR, CIMS, NMR spectra for metabolite **5** were confirmed to be 3α-hydroxy-deoxyartemisinin, a compound previously reported in the literature.[2,3]

In the present work, a novel ketone derivative of **1** has been isolated and identified from microbial transformation. The regioselective introduction of a carbonyl group on the saturated ring is not possible to achieve by conventional chemical methods, and this type of transformation by an actinomycete like *S. griseus* ATCC 13273 has not been reported as well. We speculated that the formation of **2** from **1** underwent two consecutive steps by this actinomycete. First, compound **1** was hydroxylated at C-9 position by a hydroxylase to generate the monohydroxy compounds **3** and **4** with α- and β-configuration, respectively. Upon the formation of monohydroxylated compounds, an alcohol dehydrogenase in the organism catalyzed a dehydrogenation reaction, in the presence of NAD^+ or $NADP^+$ as a cofactor, to form the ketone product **2** (Scheme 2). To confirm such sequence, we used **3** and **4** as a substrate, respectively, with same biotransformation procedures. After 48 h of incubation, both compounds were indeed converted to **2** at similar rates, whereas no changes of **3** and **4** were observed in the controls without the actinomycete. This result indicated that the second step was catalyzed by a non-stereospecific alcohol dehydrogenase. Meanwhile, we measured the concentrations of products **2**, **3**, and **4** while prolonging the reaction time. Compounds **3** and **4** were produced to a peak at day 4, then gradually decreased over the period of a week, whereas product **2** was formed at day 3 and reached the peak at day 7 with conversion yield greater than 50%. Such product profiles provided additional evidence that **3**, and **4** are the intermediate metabolites for **2**.



Scheme 1. Biotransformation of artimisinin by *Streptomyces griseus* ATCC 13273.



Scheme 2. Proposed mechanism of producing **2** from **1** by *Streptomyces griseus* ATCC 13273.

The antimalarial activity of the metabolites **2**, **3**, **4** and **5** was compared with **1** by a 48-h in vitro assay against *P. falciparum* FCC-1/HN strain.[15] Not surprisingly, the metabolite without the endoperoxide bridge **5** was inactive, whereas metabolites **2**, **3**, and **4** retained the activity with IC_{50} values of 25.7, 34.6, and 29.3 ng/ml, respectively, even though they were less active than artemisinin (IC_{50} = 3.1 ng/ml). These results were similar to previous reports on the comparisons of artemisitene, arteether, anhydrodihydroartemisinin, and their microbial metabolites.[9,16,17] The decrease of activity by oxidation of **1** was expected because oxidative modifications generally are the metabolic reactions of detoxification of drugs in mammals, plants and microorganisms.

Streptomyces griseus ATCC 13273 has been known and utilized as a biocatalyst to generate oxidative products, especially hydroxylation products,[18,19] however, this is the first successful conversion of **1** to **2**, which should be very difficult, if not impossible, to conduct through organic synthesis. Such finding will inevitably increase the value and utility of this actinomycete in the preparation of ketone derivatives for a variety of natural products, particularly for terpenoids that typically are difficult to introduce functional groups by chemical methods. Ultimately the enzymes involved in this transformation will be very useful as biocatalysts for the development of various biocatalytic processes after cloning and expression. Moreover, introduction of an amino group on the 6-membered ring will be relatively easy by reductive amination of the ketone derivative due to the presence of carbonyl group, and it would be very interesting to compare different nitrogen-containing analogues of artemisinin for their structure–activity relationship since there have been no reports on studying activity and property of amino derived analogues. Hopefully, such efforts will result in more potent analogues with favorable in vivo properties for developing new antimalarial agents.

Acknowledgments

This work was supported by grants from National Natural Science Foundation of China (No. 2997256) and grants for Outstanding Young Scholars at Universities in Jiangsu Province, PR China, awarded to B-Y. Yu.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2005.12.076.

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11. (a) Betts, R. E.; Walters, D. E.; Rosazza, J. P. *J. Med. Chem.* **1974**, *17*, 599; (b) Two-stage fermentations were performed in 125-ml stainless steel-capped culture flasks containing 25 ml of medium on rotary shakers operating at 250 rpm at 28 °C. The medium consists of (per liter of H_2O) dextrose, 20 g; yeast extract, 5 g; soybean meal, 5 g; NaCl, 5 g; K_2HPO_4, 5 g, adjusting to pH 7.0 with 6N HCl, and was sterilized at 121 °C and 18 psi for 20 min. Artemisinin (240 mg/ml solution in DMF) was added to the cultures at 24 h after the inoculation of stage II cultures at a final

concentration of 0.6 mg/ml. Substrate controls were composed of sterile medium to which the substrate was added and incubated without microorganisms. Culture controls consisted of fermentation blanks in which the microorganisms were grown under identical conditions but without addition of the substrate. After 4 days of incubation, the fermentation broth was pooled and extracted twice with equal volume of ethyl acetate. Metabolite production was then analyzed by TLC and HPLC.

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13. Identification of Artemisitone-9 (**2**): white needles ($CHCl_3$); mp, 164–165 °C; IR (KBr) ν_{max}, 1739, 1714 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ6.17 (1H, s, H-5),3.41 (1H, dt, J = 4.8, 7.2 Hz, H-7), 2.60 (1H, m, H-2b), 2.50 (1H, m, H-3b), 2.20 (1H, m, H-2a), 2.19 (1H, m, H-10), 2.15 (1H, m, H-3a), 2.07 (1H, d, J = 4.8 Hz, H-8a), 1.84 (1H, m, H-11), 1.82 (1H, m, H-1), 1.66 (1H, d, J = 5.2 Hz, H-8b), 1.48 (3H, s, H-15), 1.18 (3H, d, J = 7.2 Hz, H-13), 1.13 (3H, d, J = 6.4 Hz, H-14); ^{13}C NMR (400 MHz, $CDCl_3$) δ205.8 (s, C-9), 170.7 (s, C-12), 105.8 (s, C-4), 92.8 (d, C-5), 77.9 (s, C-6), 49.5 (d, C-11), 48.6 (d, C-1), 43.7 (d, C-10), 38.3 (t, C-2), 35.8 (t, C-3), 33.0 (d, C-7), 26.1 (t, C-8), 25.1 (q, C-15), 12.4 (q, C-14), 11.9 (q, C-13); CIMS m/z 297 [M^+ + 1] (9), 233 (34), 222 (28), 206 (63), 91 (67), 55(60), 43 (100); HREIS m/z 296.1359 [M^+] (calculated for $C_{15}H_{20}O_6$ 296.1260).
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