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Microbial metabolism of artemisitene

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Abstract

Studies on the microbial transformation of the sesquiterpene endoperoxide artemisitene have revealed that artemisitene was metabolized by *Aspergillus niger* (NRRL 599) to yield 11-*epi*-artemisinin, 9β-hydroxydeoxy-11-*epi*-artemisinin and 9β-hydroxy-11-*epi*-artemisinnin. These metabolites were characterized on the basis of their spectral data.

Keywords: *Artemisia annua*; Asteraceae; Microbial metabolism; Endoperoxide sesquiterpene; Artemisitene; Artemisinin; 11-*Epi*-artemisinin; 9β-Hydroxydeoxy-11-*epi*-artemisinin; 9β-Hydroxy-11-*epi*-artemisinin

1. Introduction

Artemisitene (**1**), an endoperoxide closely related to the known antimalarial sesquiterpene artemisinin (**2**), is a minor constituent of *Artemisia annua* (Asteraceae) (Acton & Klayman, 1985). Metabolism studies have traditionally used model systems to predict metabolic fates in humans. Microorganisms, particularly fungi, have been used successfully as in vitro models for the prediction of mammalian drug metabolism (Smith & Rosazza, 1975a, 1975b, 1982; Kieslich, 1976; Rosazza & Smith, 1979; Clark & Hufford, 1979; Clark & Hufford, 1991; Clark, McChesney, & Hufford, 1985). It is anticipated that the microbial metabolism of artemisitene (**1**) would produce significant quantities of metabolites that would be difficult to obtain from either animal systems or chemical synthesis. This work may also provide some new analogs that may serve as prospective candidates for antimalarial evaluation or as starting compounds for the semi-synthesis of other derivatives. This note reports on the microbial transformation of artemisitene (**1**) and the isolation and characterization of its metabolites.

2. Results and discussion

Screening-scale studies of artemisitene (**1**) have shown that *A. niger* (NRRL 599) was the most efficient microorganism to metabolize this sesquiterpene into three metabolites. A preparative-scale fermentation was performed using artemisitene (**1**) as a substrate and compounds **3**, **4** and **5** were isolated and purified by flash chromatography.

In metabolite **3**, $C_{15}H_{22}O_5$, the reduction of the olefinic carbons, C-11 and C-13, was established from the ^{13}C NMR data. It was observed that the C-13 methyl group resonated relatively downfield, at δ 20.5, and this is consistent with an α-configuration (Acton & Klayman, 1987). Therefore, metabolite **3** was determined to be 11-*epi*-artemisinin based on its spectral data, which were indistinguishable from those previously reported (Acton & Klayman, 1987).

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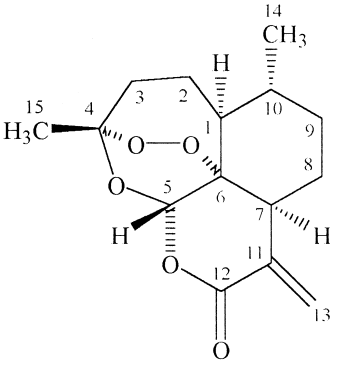

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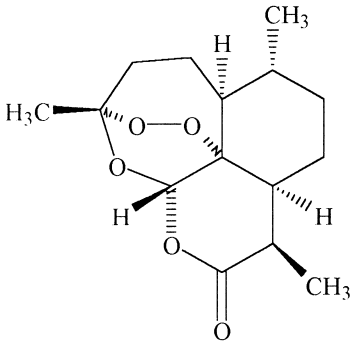

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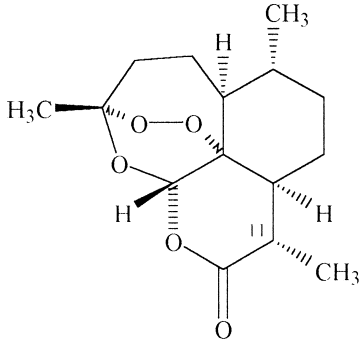

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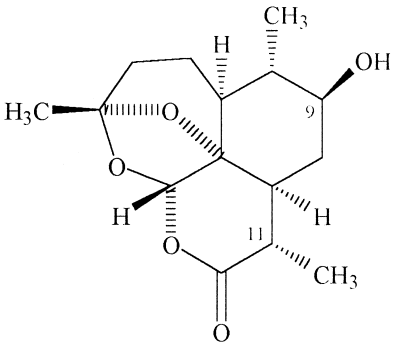
Metabolite **4**, $C_{15}H_{22}O_5$, was found to possess gross NMR spectral data that were generally similar to those of **3**. Thus, the C-13 methyl group maintained an α disposition as shown by its chemical shift value at δ 20.5 (Acton & Klayman, 1987), but the presence of two deshielded quaternary signals at δ 110.1 and 81.8 was indicative of a deoxy rather than peroxy structure (Lee, ElSohly, Croom, & Hufford, 1989). Also, the ^{13}C NMR spectrum revealed the presence of an oxygenated methine signal at δ 73.9, which was concluded to be attached to a hydroxyl group as suggested by the IR spectrum (ν_{max} 3510) and the D_2O exchangeable proton at δ 1.88 (see Table 1). This hydroxyl group was determined from COSY and GHMQC spectra to be at C-9 (Table 2). This was further supported by the expected downfield shifts for C-10 (5.4 ppm) and C-8 (9.0 ppm) and the upfield shifts for C-1 (7.7 ppm), C-7 (5.2 ppm) and C-14 (5.2 ppm), relative to those of **3**. The stereochemical assignment of the hydroxyl group as 9β was established by noting the coupling pattern of the proton at C-9. H-9 resonated at δ 3.32 as a ddd (J = 10.9, 10.0, 4.3 Hz) and hence must be axial and therefore α (Khalifa, Baker, Rogers, El-Feraly, & Hufford, 1994). These data collectively were in concordance with structure **4** for this metabolite.



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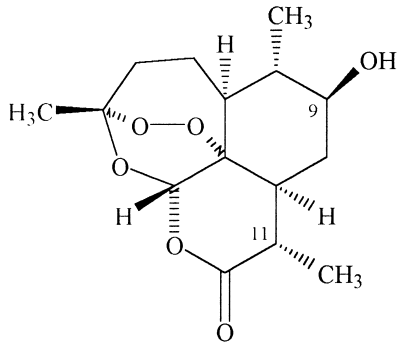

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Table 1
^{1}H NMR assignments[a] of compounds **4** and **5**

Proton No.	**4**, δH	**5**, δH
1	1.38 (1H, ddd, J = 11.8. 11.1, 4.3)	1.58 (1H, m)
2	a 1.32 (1H, m)	α 1.93 (1H, ddd, J = 12.0, 3.8, 3.8)
	b 1.89 (1H, m)	β 1.51 (1H, m)
3	α 1.57 (1H, ddd, J = 13.4, 11.5, 5.5)	α 2.38 (1H, ddd, J = 12.7, 12.7, 4.2)
	β 1.74 (1H, ddd, J = 13.4, 5.2, 1.5)	β 2.09 (1H, m)
5	5.79 (1H, s)	5.99 (1H, s)
7	2.07 (1H, dd, J = 14.0, 4.3)	1.78 (1H, dd, J = 11.3, 3.8)
8	α 2.01 (1H, ddd, J = 13.0, 4.3, 4.3)	a 2.07 (1H, m)
	β 1.42 (1H, m)	b 1.51 (1H, m)
9	3.32 (1H, ddd, J = 10.9, 10.0, 4.3)	3.29 (1H, ddd, J = 9.7, 9.7, 5.2)
10	1.22 (1H, m)	1.36 (1H, m)
11	2.37 (1H, q, J = 7.7)	2.28 (1H, q, J = 7.1)
13	1.47 (3H, d, J = 7.7)	1.46 (3H, J = 7.1)
14	1.04 (3H, d, J = 6.3)	1.09 (3H, d, J = 6.1)
15	1.51 (3H, s)	1.45 (3H, s)
OH	1.88 (1H, br s)[b]	2.17 (1H, br s)[b]

[a] Chemical shifts are reported in parts per million ($CDCl_3$), J values are in Hertz.
[b] Exchangeable proton.

Metabolite **5**, $C_{15}H_{22}O_6$, has one additional oxygen when compared with metabolite **3** and this was clearly present as a hydroxyl group as determined by the ^{1}H NMR (D_2O exchange) spectrum. The ^{1}H and ^{13}C NMR spectral data showed that the alcohol was secondary and also revealed that the peroxide function was intact, as in 11-*epi*-artemisinin (**3**) (see Tables 1 and 2). Again, this hydroxyl group was assigned to C-9, with β-stereochemistry, based on the NMR data as described for **4**. As in **3** and **4**, the C-13 methyl group resonated relatively downfield at δ 20.8, suggesting an α-configuration. Thus, **5** was established as 9β-hydroxy-11-*epi*-artemisinin.

During the in vitro antimalarial activity test, only metabolite **5**, with an intact endoperoxide moiety, was shown to possess some activity; IC_{50} = 640 and 400 ng ml^{-1} against African (D6) and Indochina (W2) clones of *Plasmodium falciparum*, respectively. However, metabolite **4** was inactive. These results are consistent with the fact that an intact endoperoxide moiety is necessary for antimalarial activity (Lee & Hufford, 1990).

Table 2
^{13}C NMR assignments[a] of compounds **4** and **5**

Carbon No.	**4**, δC	**5**, δC
1	42.8 d	48.6 d
2	22.5 t	25.0 t
3	33.8 t	36.2 t
4	110.1 s	105.8 s
5	100.2 d	94.3 d
6	81.8 s	80.5 s
7	40.3 d	43.2 d
8	40.1 t	39.7 t
9	73.9 d	73.3 d
10	43.0 d	44.8 d
11	39.5 d	39.6 d
12	172.9 s	172.7 s
13	20.5 q	20.8 q
14	14.7 q	15.9 q
15	24.4 q	25.9 q

[a] Spectra recorded in $CDCl_3$ with TMS as internal standard.

3. Experimental

3.1. General

M.p.'s were determined in open capillary tubes using an Electrothermal 9100 capillary melting-point apparatus and are uncorr. IR spectra were recorded in KBr using a PYE Unicam infrared spectrophotometer and specific rotations were obtained at amb. temp. on a Perkin-Elmer digital polarimeter model 241MC. The ^{1}H and ^{13}C NMR spectra were obtained in $CDCl_3$ on a Bruker DRX-500 NMR spectrometer operating at 500 and 125 MHz, respectively. The chemical shift values are reported as ppm referenced to TMS and the coupling constants are in Hz.

HRMS was carried out using a Bruker Bioapex FTMS with electrospray ionization spectrometer. Thin layer chromatographic (TLC) analyses were carried out on precoated silica gel 60 F_{254} (Merck) using 10% hexane in ether as the solvent system and visualized by spraying with *p*-anisaldehyde spray reagent (El-Feraly & Hufford, 1982). The adsorbent used for column chromatography was silica gel 60/230–400 mesh (EM Science). Artemisitene (**1**), the substrate used in this project, was synthesized as previously

reported (El-Feraly, Ayalp, Al-Yahya, McPhail, & McPhail, 1990).

3.2. Microorganisms

Twenty microorganisms, obtained from the American Type Culture Collection (ATCC), Rockville, MD or Northern Regional Research Laboratories (NRRL), Peoria, IL, were used for initial screening. The microorganisms used were, *Aspergillus alliaceous* NRRL 315, *A. flavipes* ATCC 11013, *A. niger* NRRL 599, *A. niger* 2295, *A. ochraceous* NRRL 398, *A. ochraceous* NRRL 405, *Candida albicans*, lab isolate, *Cunninghamella blackesleeana* MR 198, *C. echinulata* NRRL 1382, *C. elegans* NRRL 1392, *Gymnascella citrina* NRRL 6050, *Lindera pinnespora* NRRL 2237, *Penicillium chrysogenum* ATCC 10002, *P. chrysogenum* ATCC 10002-K, *P. purpureus* UI 193, *P. uermiculatum* NRRL 1009, *Rhizopus nigricans* NRRL 1477, *Rhodotorula rubra* NRRL Y1592, *Saccharomyces cerevisiae* (Baker's yeast) and *Streptomyces fulrissimus* NRRL 1453B.

3.3. Media

All fermentation experiments were carried out in a medium of the following composition: 10 g dextrose, 10 ml glycerol, 5 g yeast extract, 5 g peptone, 5 g K_2HPO_4, 5 g NaCl and 1000 ml distilled water. Stock cultures of fungi were stored on slants of Sabourad dextrose agar (Oxoid) at 4°C.

3.4. Fermentation procedures

Microbial metabolism studies were carried out by incubating the cultures with shaking on an Orbit Floor Model shaker (Lab-line Instruments, IL), operating at 250 rpm at 25°C. Fermentations were carried out according to a standard two-stage protocol (El-Marakby, Clark, Baker, & Hufford, 1986). In general, the substrate was prepared as a 4% solution in acetone and added to the 24-h-old stage II culture medium of the microorganism at a concentration of 0.2 mg/ml of medium. 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 the substrate addition.

3.5. Microbial metabolism of artemisitene (*1*) by *A. niger*

A. niger (NRRL 599) was grown in 100 250-ml culture flasks each containing 50 ml of medium. A total of 1000 mg of artemisitene (**1**) (in 25 ml of acetone) was evenly distributed among the 24-h-old stage II cultures. After 10 days, the incubation mixtures were checked by TLC. TLC revealed that most of compound **1** was transformed and three major metabolites were produced.

The incubation mixtures were combined and filtered to remove the cells and the filtrate (5 l) was extracted 3× with $CHCl_3$ (5 l). The combined extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure to afford 1.2 g of brownish residue.

3.6. Isolation and characterization of 11-epi-artemisinin (*3*)

The residue (1.2 g) was purified by column chromatography over a silica gel column using 13% hexane in ether as an eluent. Fractions 13–23 yielded metabolite **3** with R_f 0.38. These fractions were combined and evaporated to give 89 mg of **3** (8.9% yield). Crystallization from hexane–EtOAc gave colorless tiny prisms; m.p. 152–155°C, with NMR and MS data indistinguishable from those previously reported (Acton & Klayman, 1987).

3.7. Isolation and characterization of 9β-hydroxydeoxy-11-epi-artemisinin (*4*)

Fractions 30–36 yielded metabolite **4** with R_f 0.21 and were combined and evaporated to dryness to give 55 mg of **4** (5.5% yield). Crystallization from hexane–CH_2Cl_2 gave colorless prisms, m.p. 218–219°C, $[\alpha]_D$ −124.4° (*c* 0.078; $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3510 br (OH), 1740 (C0); ^1H NMR and ^{13}C NMR: Tables 1 and 2; High resolution ESIMS $[M + H]^+$ 283.1617 (calc. for $C_{15}H_{23}O_5$, 283.3446).

3.8. Isolation and characterization of 9β-hydroxy-11-epi-artemisinin (*5*)

Fractions 43–54 yielded metabolite **5** with R_f 0.12 and were combined and evaporated to afford 71 mg of **5** (7.1% yield). Crystallization from hexane–CH_2Cl_2 gave colorless needles, m.p. 161°C, $[\alpha]_D$ +90.1° (*c* 0.085; $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3460 br (OH), 1720 (CO), ^1H NMR and ^{13}C NMR: Tables 1 and 2; High resolution ESIMS $[M + H]^+$ 299.1571 (calc. for $C_{15}H_{23}O_6$, 299.3440).

In addition to the above-mentioned metabolites, artemisinin (**2**) was isolated as a minor metabolite and identified based on chromatographic analysis with no further investigation.

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