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MICROBIAL METABOLISM STUDIES OF THE ANTIMALARIAL SESQUITERPENE ARTEMISININ

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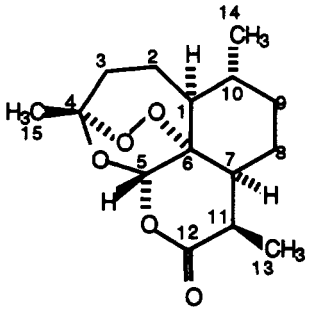
ABSTRACT.—Microbial metabolism of the sesquiterpene lactone antimalarial drug artemisinin [**1**] was studied. Screening studies have shown a number of microorganisms capable of metabolizing artemisinin [**1**]. Scale-up fermentation with *Nocardia corallina* (ATCC 19070) and *Penicillium chrysogenum* (ATCC 9480) have resulted in the production of two major microbial metabolites that have been characterized with the use of 2D-nmr techniques. These metabolites have been identified as deoxyartemisinin [**2**] and 3α-hydroxydeoxyartemisinin [**3**].

Artemisinin [**1**], a sesquiterpene lactone, is the clinically active antimalarial constituent of the Chinese medicinal herb, *Artemisia annua* L. (Compositae) (1). Its mammalian metabolism (2,3) and chemical transformation (4,5) as well as thermal rearrangement and decomposition studies (6,7) have been reported.

Metabolism studies have traditionally used model systems to predict metabolic pathways in humans. Microorganisms, particularly fungi, have recently been successfully used as in vitro models for the prediction of mammalian drug metabolites (8–14). In the present study on the microbial metabolism of artemisinin, two compounds were isolated as major microbial metabolites. Based on the chemical and spectroscopic data, especially of 2D-nmr techniques, these have been identified as deoxyartemisinin [**2**], a previously identified mammalian metabolite (2), and 3α-hydroxydeoxyartemisinin [**3**], previously prepared as a thermal rearrangement product (6). The isolation and structure elucidation of these metabolites are described herein.



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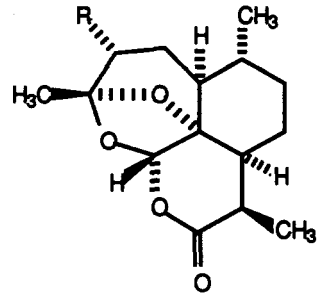

2 R=H
3 R=OH

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were determined either on a Fisher digital melting point analyzer model 355 or in open capillary tubes with Thomas-Hoover capillary melting point apparatus, and are uncorrected. Ir spectra were recorded in KBr using a Perkin-Elmer 281B infrared spectrophotometer. ^{1}H- and ^{13}C-nmr spectra were obtained in $CDCl_3$ on a Varian VXR-300 FT spectrometer operating at 300 MHz and 75 MHz, respectively. The chemical shift values are reported in ppm, and the coupling constants are in Hz. Abbreviations for nmr signals are as follows: s = singlet, d = doublet, t = triplet, q = quartet, dd = double doublet, m = multiplet, br = broad. Standard pulse sequences were used for COSY (15), HETCOR (16), DEPTGL (17), and APT (18) experiments. Electron impact mass spectra were obtained using an E.I. Finnigan model 3200 (70 eV ionization potential) with INCOS data system.

CHROMATOGRAPHIC CONDITIONS.—Tlc chromatographic analyses were carried out on precoated Silica G-25 UV_{254} plates (Macherey-Nagel Duren). The adsorbent used for cc was Si gel 60/70–270 mesh (Macherey-Nagel Duren). Developing system used for tlc was Et_2O-hexane (4:1) solution, and visualization of the tlc plates was performed using anisaldehyde/H_2SO_4 spray reagent (19).

MICROORGANISMS.—The cultures were obtained from the University of Mississippi, Department of Pharmacognosy Culture Collection, and were originally from the American Type Culture Collection (ATCC), Rockville, Maryland, or from Northern Regional Research Laboratories (NRRL), Peoria, Illinois. UI cultures were obtained from Dr. John P. Rosazza, University of Iowa. The cultures used for preliminary screening of artemisinin are as follows: *Aspergillus alliaceus* NRRL 315 (+), *Aspergillus flavipes* ATCC 1030 (+), *A. flavipes* ATCC 11013 (+), *A. flavipes* ATCC 16795 (+), *Aspergillus flavus* ATCC 24741 (+), *A. flavus* NRRL 501 (+), *Aspergillus niger* ATCC 10549 (+), *A. niger* ATCC 10581 (+), *A. niger* ATCC 16888 (+), *Aspergillus parasiticus* ATCC 15517 (+), *Botrytis allii* ATCC 9435, *Calonectria decora* ATCC 14767 (+), *Cryptococcus neoformans* ATCC 32264 (+), *Cunninghamella blakesleeana* ATCC 8688a (+), *Cunninghamella elegans* ATCC 9245 (+), *Fomes pinicola* ATCC 15341 (+), *Mucor mucedo* UI-4605 (+), *Nocardia corallina* ATCC 19070 (+), *Nocardia minima* ATCC 19150 (+), *Nocardia petroleophila* ATCC 15777 (+), *Penicillium chrysogenum* ATCC 9480 (+), *Rhizopus arrhizus* ATCC 11145, *Rhodotorula rubra* ATCC 20129 (+), *Saccharomyces cerevisiae* ATCC 9763 (+), *Schizosaccharomyces pombe* ATCC 20130 (+), *Sepedonium chrysospermum* ATCC 13378 (+), *Sporobolomyces pararoseus* ATCC 11386, *Streptomyces lavendulae* L-105 (+), *Streptomyces punipalus* UI-3529 (+), *Streptomyces rimosus* ATCC 23955 (+), *Streptomyces roseochromogenus* ATCC 13400, *Stysanus microsporus* 2833 (+). The "+" indicates that these microorganisms have shown one or more metabolites by tlc.

MEDIA.—All the preliminary screening and scale-up experiments were carried out in a medium consisting of dextrose 20 g, yeast extract 5 g, peptone 5 g, NaCl 5 g, K_2HPO_4 5 g, distilled H_2O 1000 ml. Stock cultures of fungi and bacteria were stored on slants of Mycophol (BBL) and Eugon (Difco) agar, respectively, at 4°.

FERMENTATION PROCEDURES.—Microbial metabolism studies were carried out by incubating the cultures with shaking on the model G-10 Gyrotory shaker (New Brunswick Scientific Co., New Jersey), operating at 250 rpm, at 25°. Preliminary screening experiments were carried out in 125-ml stainless-steel-capped DeLong culture flasks containing 25 ml of medium. The media were sterilized at 121° and 18 psi for 15 min. Fermentations were carried out according to a standard two-stage protocol (20). In general, the substrate was added to the incubation media 24 h after the inoculation of the stage II cultures as a 10% solution in EtOH at a concentration of 0.2 mg/ml of stage II 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.

Artemisinin [**1**] used in this study was isolated from *A. annua* grown in the Medicinal Plant Garden, School of Pharmacy, University of Mississippi. Its identity was established by direct comparison with an authentic sample supplied by Dr. A. Brossi of the NIH, Bethesda, Maryland. A voucher specimen is deposited at the pharmacognosy herbarium, School of Pharmacy, University of Mississippi.

MICROBIAL METABOLISM OF ARTEMISININ [**1**] TO DEOXYARTEMISININ [**2**].—A total of 280 mg of artemisinin [**1**] was dissolved in 2.8 ml of EtOH and distributed equally among seven 1-liter culture flasks each containing 200 ml of 24-h-old *N. corallina* (ATCC 19070) stage II culture. After 14 days, the entire incubation mixtures were combined and filtered, and the cells and fermentation solids were washed with H_2O. The combined aqueous filtrates (1.4 liter) were extracted 3 times with EtOAc (1 × 1.4 liter, 2 × 0.7 liter). The organic layer was dried over anhydrous Na_2SO_4 and filtered, and the solvent was evaporated under reduced pressure to afford 290 mg of dark brown residue. The residue was purified on a Si gel column (30 g, 2.5 × 30 cm) using Et_2O-hexane (4:1) as an eluent, and 10-ml fractions were collected. Tlc of fractions 6–9 showed a single spot with R_f 0.89. These fractions were combined and evaporated to dryness to give 68 mg of deoxyartemisinin [**2**]. Recrystallization from isopropyl ether afforded colorless crystals of **2** (42 mg, 15% yield): mp 111–112° [lit. (22) mp 110–111°]; eims *m/z* $[M]^+$ 266; ir (KBr) 2935, 1740, 1385, 1140, 1030, 1010 cm^{-1}; ^1H nmr 0.91 (3H, d, J = 5.1 Hz, Me-14), 0.97 (1H, m, H-8), 1.11 (1H, m, H-9), 1.17 (3H, d, J = 7.4 Hz, Me-13), 1.22 (1H, m, H-2), 1.25 (1H, m, H-10), 1.26 (1H, m, H-1), 1.50 (3H, s, Me-15), 1.59 (1H, m, H-3), 1.75–1.77 (2H, m, H-3, H-9), 1.88 (2H, m, H-2, H-8), 1.98 (1H, dt, J = 12.8, 4.5 Hz, H-7), 3.16 (1H, dq, J = 4.5, 7.4 Hz, H-11), 5.67 (1H, s, H-5); ^{13}C nmr see Table 1.

MICROBIAL METABOLISM OF ARTEMISININ [**1**] TO 3α-HYDROXYDEOXYARTEMISININ [**3**].—*P. chrysogenum* (ATCC 9480) was grown in 24 1-liter culture flasks each containing 200 ml of medium. A total of 960 mg of artemisinin (in 9.6 ml EtOH) was evenly distributed among the 24-h-old stage II cultures.

TABLE 1. ^{13}C-nmr Chemical Shift Assignments for Compounds **2** and **3**.[a]

Carbon	Chemical Shift Assignments (ppm)[b]	
	2	**3**
1	44.7 (1)	40.5 (1)
2	22.1 (2)	30.3 (2)
3	34.0 (2)	69.0 (1)
4	109.0 (0)	108.9 (0)
5	99.5 (1)	99.0 (1)
6	82.4 (0)	82.9 (0)
7	42.5 (1)	42.0 (1)
8	23.5 (2)	23.5 (2)
9	33.6 (2)	33.4 (2)
10	35.4 (1)	35.1 (1)
11	32.8 (1)	32.7 (1)
12	171.3 (0)	171.5 (0)
13	12.6 (3)	12.6 (3)
14	18.5 (3)	18.4 (3)
15	24.0 (3)	20.5 (3)

[a]Assignments are based on ^{1}H-^{1}H and ^{1}H-^{13}C chemical shift correlated 2D-nmr spectroscopy. ^{13}C-nmr chemical shift assignment data for artemisinin and its several derivatives including 3α-hydroxydeoxyartemisinin [**3**] were also reported recently by Wang *et al.* (21), in which assignments for C-1 and C-7 were reversed.

[b]The number in parentheses indicates the number of hydrogens attached to the corresponding carbon and was determined from DEPT experiments.

After 13 days, the incubation mixtures were pooled, filtered to remove the cells (4.8 liters), then extracted 3 times with EtOAc (1 × 4.8 liters, 2 × 2.4 liters). The combined extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure to afford a dark brown residue (1026 mg). This residue was purified by cc over a Si gel column (100 g, 2.5 × 80 cm), using Et_2O-hexane (4:1) as eluting system, and 10-ml fractions were collected. Fractions 92–120, yielding a single spot with R_f 0.40, were combined and evaporated to dryness to give 35 mg of pure **3** (3.6% yield). From fractions 21–30, 10 mg of **2** was also obtained. Crystallization from isopropyl ether gave colorless needles: mp 192–193° [lit. (6) mp 190–191°]; eims *m/z* $[M]^+$ 282; ir (KBr), 3490, 2920, 1740, 1390, 1140, 1030 cm^{-1}; ^{1}H nmr 0.92 (3H, d, J = 6.3 Hz, Me-14), 0.97 (1H, m, H-8), 1.14 (1H, m, H-9), 1.18 (3H, d, J = 7.2 Hz, Me-13), 1.26 (1H, m, H-10), 1.47 (1H, m, H-2), 1.52 (1H, m, H-1), 1.55 (3H, s, Me-15), 1.81 (1H, m, H-9), 1.92 (1H, m, H-8), 1.97 (1H, m, H-2), 2.06 (1H, dt, J = 12.9, 4.5 Hz, H-7), 2.20 (1H, br, *HO*-3), 3.18 (1H, dq, J = 4.5, 7.2 Hz, H-11), 3.60 (1H, br s, H-3), 5.62 (1H, s, H-5); ^{13}C nmr see Table 1.

RESULTS AND DISCUSSION

A total of 33 microorganisms were screened for their ability to biotransform artemisinin [**1**]. *N. corallina* and *P. chrysogenum* were selected for preparative scale transformation with **1** because no artemisinin was detected and they produced different metabolites.

A preparative scale fermentation was performed with *N. corallina*, and the major metabolite **2** was isolated and purified by chromatography. The mass spectra of this metabolite ($[M]^+$ at *m/z* 266) clearly indicated that a single oxygen atom had been removed from the substrate molecule. A comparison of the mp and the ^{13}C-nmr spectral data of **2** with those of deoxyartemisinin indicated that the metabolite was deoxyartemisinin. A direct comparison of this metabolite with deoxyartemisinin (mp, mmp, tlc, ir, ^{1}H nmr) confirmed the identity of **2** as deoxyartemisinin.

A preparative scale fermentation of artemisinin with *P. chrysogenum* led to the isolation and purification of two major metabolites, one of which proved to be the same compound as metabolite **2**. Mass spectral data of metabolite **3** indicated that there was no change in the mol wt (*m/z* $[M]^+$ 282). The ir spectrum shows a broad peak at 3490 cm^{-1}, which suggests the presence of a hydroxyl group. The ^{1}H-nmr spectrum shows a new broad singlet at 2.20 ppm, which turned out to be exchangeable on treatment with D_2O, and a broad singlet at 3.60 ppm. The ^{13}C-nmr spectrum has a new doublet at 69.0 ppm. All of these data indicated that the new metabolite was a hydroxylated deoxyartemisinin. Comparison of the ^{13}C-nmr spectral data of **1**, **2**, and metabolite **3** supported C-3 as the most likely position of hydroxylation. The stereochemistry of the hydroxyl group was established as alpha from the ^{1}H-nmr data. All of the evidence suggested that metabolite **3** was 3α-hydroxydeoxyartemisinin, previously prepared as a thermal rearrangement product of **1**. A sample of 3α-hydroxydeoxyartemisinin was prepared, and direct comparison (mp, mmp, tlc, ir, ^{1}H nmr) established that **3** was 3α-hydroxydeoxyartemisinin.

The microbial metabolism study on artemisinin has resulted in the isolation and characterization of two major metabolites. These have been identified as deoxyartemisinin and 3α-hydroxydeoxyartemisinin. Dihydroartemisinin, a previously reported mammalian metabolite (2,3), was not detected as a microbial metabolite for any of the microorganisms used in this study.

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