Microbial and Mammalian Metabolism Studies of the Semisynthetic Antimalarial, Anhydrodihydroartemisinin

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Microbial metabolism studies of the semisynthetic antimalarial anhydrodihydroartemisinin (1), have shown that it is metabolized by a number of microorganisms. Large scale fermentation with *Streptomyces lavendulae* L-105 and *Rhizopogon* species (ATCC 36060) have resulted in the isolation of four microbial metabolites. These metabolites have been identified as a 14-carbon rearranged product (2), 9β-hydroxyanhydrodihydroartemisinin (3), 11-*epi*-deoxydihydroartemisinin (4), and 3α-hydroxydeoxyanhydrodihydroartemisinin (5). Microbial metabolites were completely characterized by spectral methods, including ^{1}H-NMR and ^{13}C-NMR spectroscopy. The structure and stereochemistry of metabolite 2 were unequivocally established by X-ray crystallographic analysis. Thermospray mass spectroscopy/high-performance liquid chromatographic analyses of plasma from rats used in mammalian metabolism studies of 1 have shown microbial metabolite 3 to be the major mammalian metabolite. *In vitro* antimalarial testing has shown metabolite 3 to possess antimalarial activity.

KEY WORDS: microbial and mammalian metabolism; antimalarial; anhydrodihydroartemisinin; microbial and mammalian metabolites; two-dimensional nuclear magnetic resonance (2D-NMR) techniques; thermospray liquid chromatography/mass spectroscopy (LC/MS).

INTRODUCTION

Anhydrodihydroartemisinin (1), is a semisynthetic derivative of artemisinin, the active antimalarial principle of the Chinese medicinal plant *Artemisia annua* L. (1). The synthesis and antimalarial activity of 1 have been reported by Lin and coworkers (2) while El-Feraly and coworkers have reported its synthesis by a different route (3). The ^{1}H- and ^{13}C-NMR assignments of 1 have also been reported (3,4). Metabolism studies have traditionally used model systems to predict metabolic pathways in humans. Microorganisms, particularly fungi, have been successfully used as *in vitro* models for the prediction of mammalian drug metabolism (5–12). Since there have been no previous reports on the metabolism of 1, a study of its metabolism was undertaken. Also, since some microorganisms are known to affect hydroxylation of their substrates, an active metabolite may be produced that could later be transformed into a water soluble salt. This could very well offer a solution for the water insolubility of highly potent antimalarial compounds such as 1.

In the present study, four microbial metabolites of 1, were isolated. Based on the spectroscopic data, especially two-dimensional (2D)-NMR techniques, these metabolites have been identified as the 14-carbon rearranged product 2, 9β-hydroxyanhydrodihydroartemisinin(3), 11-epi-deoxydihydroartemisinin (4), and 3α-hydroxydeoxyanhydrodihydroartemisinin (5). The isolation and structure elucidation of these metabolites are discussed herein.

MATERIALS AND METHODS

General Procedures

Melting points were determined in open capillary tubes using a Thomas-Hoover capillary melting point apparatus and are uncorrected. The IR spectra were recorded in KBr using a Perkin-Elmer 281 B infrared spectrophotometer. The ^{1}H- and ^{13}C-NMR were obtained in $CDCl_3$ on a Varian VXR-300 FT spectrometer operating at 300 and 75 MHz, respectively. The chemical shift values are reported as ppm units, and the coupling constants are as Hz. Abbreviations for NMR signals are as follows: s, singlet; d, doublet; br, broad; t, triplet; q, quartet; dd, double doublet; m, multiplet. Standard Varian pulse sequences were used for COSY, HETCOR, DEPTGL, and APT experiments. Low resolution MS were obtained using LC/MS. High resolution FAB and EI MS were carried out at the University of Kansas.

Anhydrodihydroartemisinin (1), used in this study, was prepared from artemisinin by a literature procedure (3).

Chromatographic Conditions

The TLC chromatographic analysis was carried out on precoated Silica G-25 UV_{254} plates (Macherey-Nagel Duren). The adsorbent used for column chromatography was silica gel 60 /230-400 mesh (EM Science). The visualization of the TLC plates was performed using anisaldehyde-H_2SO_4 spray reagent (13).

Microorganisms

The cultures were obtained from The University of Mississippi, Department of Pharmacognosy Culture Collection, and were originally obtained from the American Type Culture Collection (ATCC), Rockville, Maryland, or from Northern Regional Research Laboratories (NRRL), Peoria, Illinois. University of Iowa (UI) cultures were obtained from Dr. John P. Rosazza. *Mucor ramannianus* 1839 was obtained from Dr. Charles Sih at the University of Wisconsin. The cultures used for preliminary screening of anhydrodihydroartemisinin (1) that showed one or more metabolites by TLC are as follows: *Aspergillus alliaceus* NRRL 6633, *Aspergillus flavipes* ATCC 11013, *Bacillus subtilus* ATCC 6633 *Cunninghamella echinulata* NRRL 3655, *Cunninghamella*

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elegans ATCC 9245, *Cunninghamella bainieri* UI-3605, *Dactylaria haptotyla* ATCC 28924, *Mucor mucedo* UI-4605, *Nocardia restricta* ATCC 14887, *Penicillium patulum* ATCC 24550, *Rhizopogon* species ATCC 36060, *Rhizopus arrhizus* ATCC 11145, *Streptomyces griseus* L-103, *Streptomyces lavendulae* L-105, and *Streptomyces rimosus* ATCC 23955.

Media

All the preliminary screening and large-scale experiments were carried out in a medium consisting of the following composition: dextrose, 20g; yeast extract, 5g; peptone, 5g; NaCl, 5g; K_2HPO_4, 5g; distilled water, 1000 ml. Stock cultures of fungi and bacteria were stored on slants of Mycophil, (BBL, Cockeysville, MD) and Eugon agar (Difco, Detroit, MI), respectively, at 4°C.

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., NJ), operating at 250 rpm, at 25°C. Preliminary screening experiments were carried out in 125-ml stainless steel-capped Delong culture flasks containing 25 ml of medium. Fermentations were carried out according to a standard two-stage protocol (14). In general, the substrate was prepared as a 10% solution in dimethylformamide and added to the 24-hr-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.

Animal Studies

Anhydrodihydroartemisinin (1) was given by intravenous administration of an oil/water emulsion that was prepared within 24 h of the animal dosing. The procedure used for the preparation is similar to a general procedure used for the extemporaneous preparation of the oil soluble cancer chemotherapeutic agents that are given by intravenous administration (15). Under aseptic conditions, a 100.0 mg/ml solution of the test compound,1 in ethanol was slowly added dropwise (10μl/min) to a vigorously stirred commercially fat emulsion (Liposyn II®, 20%) to give a final concentration of 6.0 mg/ml. Male Wistar rats were anesthetized with sodium phenobarbital (50 mg/kg), then each animal was administered (11.6 mg/kg) of 1 by an intravenous bolus injection in the jugular vein. A blood sample (approximately 9 ml) was collected using a 20 ml syringe (containing 0.2 ml heparin solution) at 15 min after the injection. After centrifugation of the blood at 500 × *g* for 10 min, the plasma was collected and stored at −85°C for later analysis.

Plasma Extraction

A solid phase extraction procedure was adopted. The C-18 reversed phase extraction cartridges (BOND ELUT C-18®, Analytichem International, Harbor City, California) were first activated with 1.0 ml methanol, followed by 1.0 ml water; then 1.0 ml of the plasma sample was slowly drawn through using 5–10 mm Hg of vacuum in a VAC-ELUT® chamber. The cartridges were washed with 1.0 ml of water (discard), then the sample was eluted with 1.0 ml methanol directly into 2.0 ml conical evaporating tubes. After centrifugation at 500 × *g* for 5 min, the clear supernatant was transferred to a fresh conical tube, and evaporated at room temperature with a stream of nitrogen to near-dryness. The residue was taken up in 100μl of 10% methanol in water, centrifuged at 500 × *g* for 5 min, then 100ml of the sample was injected into the HPLC/MS system.

High Performance Liquid Chromatography and Mass Spectroscopy

The HPLC pump, pump controller software, injector, and column bypass switching system were a commercially available unit that had been specifically designed (Waters Associates Model 600-MS system) for interfacing with the Vestec Model 201 thermospray mass spectroscopy system. A 4.6-mm × 12.5 cm cartridge-type HPLC column packed with a 5-μm-particle size, octadecyl reversed-phase material (Whatman Partisil ODS-3) was utilized, with a mobile phase (1.0 ml/min) comprised of 0.1M ammonium acetate in a methanol: water mixture. The methanol content of the mobile phase was gradient programmed from 51% (v/v) to 78% (v/v) over a 10 min period.

The Vestec Model 201 mass spectrometer with a Technivent data system was operated in the filament-on mode of operation, which yields mass spectra that are more similar to chemical ionization spectra rather than electron impact spectra of more conventional mass spectrometers. Before recording any spectra, the takeoff temperature of the thermospray vaporizer was accurately determined and the tip temperature (209°C) of the vaporizer was set 5°C below the takeoff temperature. The block temperature of the ion source was set to 195°C, which was 75–100°C lower than is commonly used for model 201 thermospray unit.

Microbial Metabolism of Anhydrodihydroartemisinin (1) by *Streptomyces lavendulae* L-105

Streptomyces lavendulae L-105 was grown in 11 1-liter culture flasks each containing 200 ml of medium. A total of 440 mg of anhydrodihydroartemisinin (1) (in 4.4 ml of DMF) was evenly distributed among the 24-hr-old stage II cultures. After 14 days, the incubation mixtures were combined and filtered to remove the cells, and the filtrate (2.2 liters) was extracted three times with EtOAc. The combined extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure to afford a dark brown residue (493 mg).

Isolation and Characterization of 2

The residue (493 mg) was purified by column chromatography over a silica gel column (50g, 3.75 × 15 cm), using hexane-ether (6:4) mixture as an eluting system and 5-ml fractions were collected. Fractions 24–48 yielding a single spot with Rf = 0.37 (TLC system, hexane-ether 1:1), were combined and evaporated to dryness to give 24 mg of 2 (5.45% yield). Crystallization from hexane gave colorless

prizms, mp, 54°C, high resolution FAB MS $[M^+ - Na]^+$ 277.1453 (consistent with the formula $C_{14}H_{22}O_4 + Na^+$, calcd 277.1416); ^{1}H-NMR, δ 0.95 (3H, d, J=6.0, Me-14), 1.06 (2H, m,H-8β, H-9α), 1.16 (1H, m, H-10), 1.36 (3H, d, J=1.0, collapses to a singlet after D_2O, Me-13), 1.58 (3H, s, Me-15), 1.58 (2H, m, H-1, H-9β), 1.70 (2H, m, H-3α, H-3β), 1.82 (1H, m, H-8α), 1.95 (2H, m, H-2α, H-2β), 2.16 (1H, m, H-7), 5.21 (1H, q, J=1.0, exchangeable with D_2O), 5.66 (1H, s, H-5); ^{13}C-NMR (see Table I).

Isolation and Characterization of 9β-Hydroxyanhydrodihydroartemisinin (3)

Fractions 100–127 from the above column yielding a single spot with R_f = 0.13 (TLC system hexane-ether 1:1), were combined and evaporated to dryness to give 40 mg of metabolite 3 (9.1% yield). Crystallization from hexane-ether gave colorless needles, mp, 159–160°C. High resolution EI MS (m/z) calcd for $C_{15}H_{22}O_5$ $[M]^+$ 282.1467, found 282.1457; IR (KBr)υmax (cm^{-1}), 3240; ^{1}H-NMR, δ 1.09 (3H, d, J=6.5, Me-14), 1.28 (1H, ddd, J=11.5, 13.0, 13.5, H-8β), 1.37 (1H, m, H-10),1.43 (3H, s, Me-15), 1.56 (1H, m, H-1), 1.57 (1H, m, H-2β), 1.59 (3H, d, J=1.5, Me-13), 1.87 (1H, dd, J=4.5, 13.5, H-7), 1.91 (1H, m, H-2α), 2.07 (1H, ddd, J=4.4, 4.4, 13.0, H-3β), 2.28 (1H, ddd, J=4.5, 4.5, 13.0, H-8α), 2.40 (1H, ddd, J=4.0, 13.0, 14.5, H-3α), 3.29 (1H, ddd, J=4.5, 10.0, 11.5, H-9), 5.59 (1H, s, H-5), 6.21 (1H, q, J=1.5, H-12); ^{13}C-NMR (see Table I).

Microbial Metabolism of Anhydrodihydroartemisinin by *Rhizopogon* Species (ATCC 36060)

A total of 400 mg of anhydrodihydroartemisinin (1) was dissolved in 4.0 ml of DMF and distributed equally among 10, 1-liter culture flasks each containing 200 ml of 24-hr-old, *Rhizopogon* species (ATCC 36060) stage II culture. After 14 days, the entire incubation mixture was combined and filtered to remove the cells, and the filtrate (2.0 liters) was extracted three times with EtOAc. The combined extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure to afford a dark brown residue (1.0 g).

Isolation and Characterization of 11-*Epi*-Deoxydihydroartemisinin (4)

The residue (1.0 g) was purified on a silica gel column (100 g, 3.75 × 30 cm) using ethyl acetate-hexane (2:8) as an eluent, and 15 ml fractions were collected. Fractions 65–75, yielding a single spot with R_f = 0.35 (TLC system, ethyl acetate-hexane 3:7) were combined and evaporated to dryness to give 1.5 mg (0.38%) of metabolite 4 as an oil. High resolution FAB MS $[M + H]^+$ 269.1753 consistent with the formula $C_{15}H_{24}O_4 + H^+$, calcd 269.1753; ^{1}H-NMR, δ 0.89 (3H, d, J=5.8, Me-14), 1.12 (3H, d, J=7.0, Me-13), 1.57 (3H, s, Me-15), 2.76 (1H, d, J=6.0, exchangeable OH), 5.03 (1H, dd, J=5.7, 7.7, H-12), 5.30 (1H, s, H-5); ^{13}C-NMR (see Table I).

Isolation and Characterization of 3α-Hydroxydeoxyanhydrodihydroartemisinin (5)

Fractions 85–130 yielded 7.5 mg (1.9%) of pure 5 as an oil with R_f = 0.31 (TLC system, ethyl acetate-hexane 3:7). LC/MS $[M]^+$ = 266 (consistent with the formula $C_{15}H_{22}O_4$); high resolution FAB MS $[M + H^+]$ 267.1573 consistent with the formula $C_{15}H_{22}O_4 + H^+$, calcd 267.1596; ^{1}H-NMR, δ 0.91 (3H, d, J=6.2, Me-14), 1.16 (1H, m, H-9α), 1.24 (1H, m, H-8β), 1.36 (1H, m, H-10), 1.45 (1H, dd, J=5.0, 12.5, H-7), 1.5 (3H, s, Me-15), 1.59 (1H, m, H-2β), 1.63 (3H, d, J=1.7, Me-13), 1.74 (1H, dddd, J=3.0, 3.0, 3.0, 13.2, H-9β), 1.89 (2H, m, H-2α, H-8α), 1.96 (1H, m, H-1), 3.58 (1H, br d, J=8.4, H-3), 5.42 (1H, s, H-5), 6.05 (1H, q, J=1.5, H-12); ^{13}C-NMR (see Table I).

Table I. ^{13}C-NMR Chemical Shift Assignments For Compounds 1–6[a]

Carbon No.	Chemical shift assignments (ppm)						
	1[b]	2	3	4	5	6[b]	7[b]
C-1	51.5(1)	42.1(1)	49.1(1)	45.2(1)	40.7(1)	45.3(1)	40.8(1)
C-2	24.4(2)	23.3(2)	24.3(2)	22.01(2)	29.7(2)	22.1(2)	30.4(2)
C-3	36.2(2)	34.5(2)	36.0(2)	34.5(2)1	69.7(1)	34.5(2)[1]	69.6(1)
C-4	104.5(0)	110.8(0)	104.6(0)	107.9(0)	107.6(0)	106.9(0)	107.8(0)
C-5	89.7(1)	103.5(1)	89.3(1)	94.5(1)[2]	94.9(1)	97.3(1)	93.7(1)
C-6	79.0(0)	94.2(0)	78.2(0)	82.6(0)	84.3(0)	82.6(0)	84.2(0)
C-7	44.5(1)	49.9(1)	42.2(1)	44.2(1)	41.4(1)	44.1(1)	42.5(1)
C-8	30.0(2)	26.2(2)	38.4(2)	32.8(2)	27.5(2)	32.7(2)[1]	25.0(2)
C-9	34.1(2)	32.2(2)	73.2(1)	34.4(2)[1]	34.1(2)	34.5(2)[1]	34.8(2)
C-10	37.5(1)	35.3(1)	44.3(1)	35.1(1)	35.0(1)	35.2(1)	34.8(1)
C-11	108.1(0)	111.2(0)	107.0(0)	41.9(1)	112.4(0)	39.8(1)	30.4(1)
C-12	135.0(1)	—	135.5(1)	97.3(1)[2]	133.9(1)	100.2(1)	99.8(1)
C-13	16.2(3)	21.8(3)	16.0(3)	18.7(3)	16.4(3)	19.3(3)	12.3(3)
C-14	20.3(3)	18.5(3)	15.4(3)	19.5(3)	18.6(3)	18.7(3)	18.8(3)
C-15	25.9(3)	24.6(3)	25.8(3)	23.7(3)	20.6(3)	23.8(3)	21.0(3)

[a] The number in parentheses indicates the number of hydrogens attached to the corresponding carbon and was determined from DEPTGL experiments. Assignments are based on ^{1}H-^{1}H and ^{1}H-^{13}C chemical shift-correlated 2D-NMR spectroscopy and by comparison to other compounds. Assignments bearing the same numerical superscripts may be reversed.

[b] These data have been reported previously (4,18,19) and are listed here for comparison purposes.

X-ray Crystal Structure Analysis of Metabolite 2

Crystal data : $C_{14}H_{22}O_4$, MW = 254.33, space group = $P2_1$, cell constants, a = 11.706 (5), b = 7.714 (3), c = 16.038 (5) Å, β = 108.60(4)° (from 25 orientation reflections, θ >20°), V = 1373 Å^3, Z = 4, D_c = 1.23 g. cm^{-3}, μ = 0.95 cm^{-1}, MoKα (λ = 0.71073Å), 2119 observed [$F_o \geq 5\sigma(F_o)$] reflections measured on an Enraf-Nonius diffractometer using the ω-2θ scan technique. Crystal dimensions: 0.35 × 0.55 × 0.50 mm. Least-square refinement with isotropic thermal parameters led to R = 0.111. The geometrically constrained hydrogen atoms werre placed in calculated positions 0.95 Å from the bonded carbon atom and allowed to ride on that atom with B fixed at 5.5 Å^2. The methyl hydrogen atoms were included as a rigid group with rotational freedom at the bonded carbon atom (C-H = 0.95 Å, B = 5.5 Å^2). The alcoholic hydrogen atoms were located from a difference Fourier map and included with fixed contributions (B = 5.5 Å^2). Refinement of nonhydrogen atoms with anisotropic temperature factors led to the final values of R = 0.040 and R_w = 0.066. Crystallographic calculations were performed with computer programs SHELX (16) and SHELXS (17). Atomic coordinates, temperature factors, bond lengths, bond angles and other supplementary data are on deposit at the Cambridge Crystallographic Database.

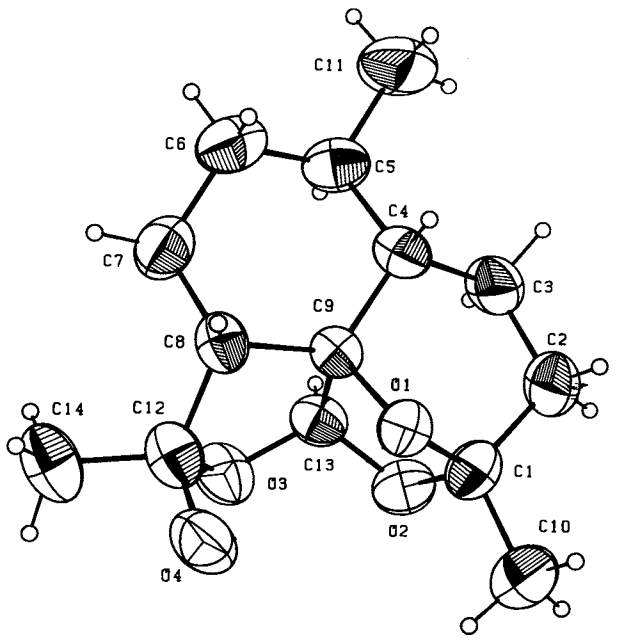

Fig. 1. ORTEP diagram for metabolite 2.

RESULTS AND DISCUSSION

Screening-scale studies of anhydrodihydroartemisinin (1) have shown that a number of microorganisms are capable of metabolizing this sesquiterpene to a number of metabolites. Of the organisms screened, *Streptomyces lavendulae* L-105 and *Rhizopogon* species (ATCC 36060) were selected for preparative-scale fermentation.

A preparative-scale fermentation was performed with *Streptomyces lavendulae* L-105 using anhydrodihydroartemisinin (1) as a substrate, and compounds 2 and 3 were isolated and purified as microbial metabolites. The ^{13}C-NMR spectrum of 2 showed 14 carbon resonances indicating loss of one carbon atom from the substrate (1). Interpretation of the combined spectroscopic data suggested that it contained a new carbon system. Thus, single crystal X-ray analysis was performed and led to the structure of metabolite 2 with all of the stereochemistries defined. An ORTEP diagram of the solid-state conformation is provided in Figure 1.

Metabolite 3, $C_{15}H_{22}O_5$, had one additional oxygen present when compared with the starting material (1), and this was clearly present as a hydroxyl group as determined by IR and ^{1}H-NMR (D_2O exchange) spectra. The ^{1}H-NMR and ^{13}C-NMR spectral data showed that the alcohol was secondary and also revealed that the peroxide function was intact. Thus, 3 represented a hydroxyanhydrodihydroartemisinin with only the position and the stereochemistry of the hydroxyl group to be determined. Since complete ^{13}C-NMR assignments of 1 have been established (4), a comparison of ^{13}C-NMR data of 3 with those of 1 showed that, of the four possibilities (C-2,-3,-8, or -9), the hydroxyl group must be at carbon 9 (see Table I). Especially noteworthy are downfield shifts for C-10 (6.8 ppm) and upfield shifts for C-1, C-14, and C-7. The assignment of the hydroxyl group as 9β was established by noting the coupling pattern of the proton at C-9. The C-9 proton resonated at δ 3.29 as a ddd (J = 4.5, 10.0, 11.5 Hz), and therefore must be axial.

A preparative-scale fermentation of 1 with *Rhizopogon* species (ATCC 36060) led to the isolation and purification of metabolites 4 and 5. Metabolite 4, $C_{15}H_{24}O_4$, had spectroscopic data showing that there was a secondary hydroxyl group present, and that it was of the deoxy series. The key to solving the structure for 4 came by noting that when the methyl group at C-11 has the alpha configuration as is seen in

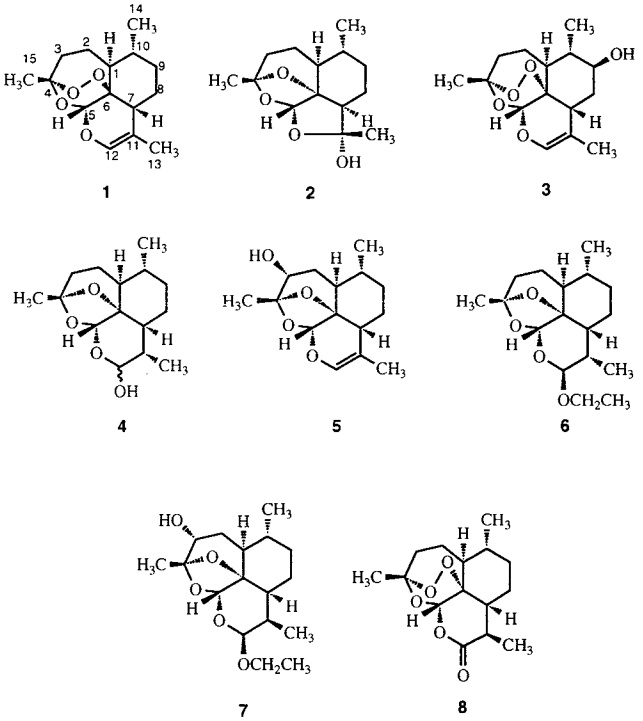

Table II. Characteristic thermospray mass spectral peaks for anhydrodihydroartemisinin and metabolites[a]

Compound	M^+	MH^+	$[M + NH4]^+$	$[M - OH]^+$
(1)		267 (23%)	284 (100%)	
(2)	254 (7%)			237 (100%)
(3)		283 (40%)	300 (100%)	
(4)	268 (32%)		286 (100%)	251 (54%)
(5)		267 (62%)	284 (100%)	

[a]The values listed represent key ions and % relative abundance in parentheses.

C-(11)-*epi*-deoxyarteether (6) (18,19), substantial changes in the carbon chemical shifts at C-8, C-11, and C-13 were observed (see Table I). It should be noted that the structure for 6 was solved by X-ray analysis (20). A careful comparison of the ^{13}C-NMR data for 4 with 6 and other similar arteether metabolites in reference (18) led us to conclude that metabolite 4 can be represented as 11-*epi*-deoxydihydroartemisinin.

Metabolite 5, $C_{15}H_{22}O_4$ had a molecular weight similar to that of anhydrodihydroartemisinin (1). The presence of one exchangeable hydroxyl group was established by ^{1}H-NMR (D_2O exchange). Comparison of the ^{13}C-NMR spectral data of 1 and metabolite 5 indicated that there was a carbon signal at δ 69.7. DEPTGL data proved that the carbon at δ 69.7 had one attached proton, which strongly indicated the presence of a carbon atom directly attached to a hydroxyl group. ^{13}C-NMR spectral analysis showed that, of the four possibilities (C-2, -3, -8, and -9), the hydroxyl group must be at carbon 3 (see Table I). Especially noteworthy are downfield shifts for C-2 and C-4 and upfield shifts for C-1. The assignment of the hydroxyl group as alpha (α) came about by direct comparison of the ^{13}C-NMR data of 5 with 3α-hydroxydeoxyarteether (7) (19).

Mammalian metabolism studies of anhydrodihydroartemisinin found that all of the metabolites obtained from the microbial fermentation isolates had thermospray mass spectra that were closely related to that of 1. The prominent ions are listed in Table II. Using these ions to monitor the thermospray HPLC/MS, a synthetic mixture of 2, 3, 4, and 5 obtained from the individual isolates of the microbial fermentations was found to give a well-resolved chromatogram (2, 9.3 min; 3, 4.5 min; 4, 11.6 min; 5, 7.3 min). Using the same thermospray HPLC/MS procedure on the rat plasma, 9β-hydroxyanhydrodihydroartemisinin (3) was found to be the major metabolite in the rat. Metabolites 2, 4, and 5 were not detected.

Table III. Antimalarial Testing Results[a]

	Indochina Clone W2		African Clone D6	
Compound	IC_{50} (ng/ml)	IC_{90} (ng/ml)	IC_{50} (ng/ml)	IC_{90} (ng/ml)
Artemisinin (8)	0.93		2.65	
(1)	0.35	0.61	1.07	3.21
(3)	6.28	12.9	12.41	67.53

[a]Antimalarial testing was done by Dr. Wilbur K. Milhous of Walter Reed Army Medical Center, Washington, DC. Artemisinin is provided here as a standard for comparison.

In vitro antimalarial testing was limited to metabolite 3 since it is the only one retaining the endoperoxide intact, a feature that has been shown to be necessary for antimalarial activity (21). Table III shows that metabolite 3 possesses *in vitro* antimalarial activity, but is less active than anhydrodihydroartemisinin (1).

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