Microbial and Mammalian Metabolism Studies on the Semisynthetic Antimalarial, Deoxoartemisinin

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Purpose. Deoxoartemisinin is a semisynthetic antimalarial with potential for treatment of multiple drug resistant malaria. Metabolism studies were conducted to aid in future drug development.
Methods. Microbial model systems were employed which have been shown to be good predictors of mammalian drug metabolites. Metabolism studies using rats were also performed.
Results. Three microbial metabolites of deoxoartemisinin were identified (2, 3, and 4). Metabolite 3 was also found in rat plasma. HPLC/MS analyses were performed on the rat plasma using 2, 3, and 4 as standards. All metabolites were thoroughly characterized by ^{1}H and ^{13}C-NMR. An additional rat plasma metabolite was revealed and it was shown not to be 9α-hydroxyartemisinin.
Conclusions. Deoxoartemisinin was metabolized to three microbial metabolites. Metabolism by rats showed the presence of two metabolites in the plasma, one of which was the same as the microbial metabolite.

KEY WORDS: antimalarial; deoxoartemisinin; microbial; metabolites; mammalian; metabolism.

INTRODUCTION

Deoxoartemisinin 1, is a semisynthetic derivative of artemisinin, the active antimalarial principle of the Chinese medicinal plant *Artemisia annua* L (1). Artemisinin and some of its derivatives are finding clinical utility in the treatment of multiple drug resistant malaria especially in southeastern Asia (2). The synthesis and antimalarial activity of 1 have been reported by Jung and coworkers (3).

Model systems have traditionally been used to predict metabolic pathways in humans. Microorganisms, particularly fungi, have recently been successfully used as *in vitro* models for the prediction of mammalian drug metabolism and successful applications have been recently reviewed (4, 5). Some microorganisms are well known to affect hydroxylation of the artemisinin nucleus producing active metabolites. These metabolites could be transformed into water soluble salts which may well offer a method to increase the water solubility of highly potent antimalarial compounds such as 1.

Three microbial metabolites of 1 have been isolated. Based on the spectroscopic data, especially two-dimensional (2D)-NMR techniques, these metabolites have been identified as 3α-hydroxydeoxydeoxoartemisinin 2, 9β-hydroxydeoxoartemisinin 3, and a 13-carbon rearranged product 4. The isolation and structure elucidation of these metabolites as well as mammalian metabolism of 1 are discussed herein.

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MATERIALS AND METHODS

General Experimental 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 pulse sequences were used for COSY, HETCOR, DEPTGL, and APT experiments. High resolution fast atom bombardment and electron impact mass spectra were obtained at the University of Kansas. Deoxoartemisinin 1 used in this study, was prepared from artemisinin by a literature procedure (3).

Chromatographic Conditions

The TLC chromatographic analyses were 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 (6).

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. *Mucor ramannianus* 1839 was obtained from Dr. Charles Sih at the University of Wisconsin. The cultures used for preliminary screening of deoxoartemisinin 1 that showed one or more metabolites by TLC are as follows: *Aspergillus alliaceus* NRRL 6633, *Aspergillus flavipes* ATCC 11013, *Aspergillus ochraceus* ATCC 18500, *Aspergillus tamarii* NRRL 8101, *Calonectria decora* ATCC 14767, *Cunninghamella blakesleeana* ATCC 8688a, *Cunninghamella echinulata* NRRL 3655, *Cunninghamella elegans* ATCC 9245, *Mucor ramannianus* 1839, *Saccharomyces lipolytica* ATCC 16617, *Streptomyces griseus* L-103, and *Streptomyces lavendulae* L-105.

Media

All the preliminary screening and large-scale experiments were carried out in a medium consisting of the follow-

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ing 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, Yeast malt extract agar, Sabouraud dextrose agar, potato dextrose agar, and potato dextrose yeast (BBL, Cockeysville, MD or Difco, Detroit, Michigan) 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., New Jersey), operating at 250 rpm, at 25°C. Preliminary screening experiments were carried out at 125-ml stainless steel-capped Delong culture flasks containing 25 ml of medium. Fermentations were carried out according to a standard two-stage protocol (7). 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.

Complete NMR Assignments for the Starting Material

The ^{13}C-NMR assignments of **1** are presented in Table I. The ^{1}H-NMR chemical shifts were determined to be as follows: δ 0.75 (3H, d, J = 7.0, Me-13), 0.94 (3H, d, J = 6.0, Me-14), 1.02 (1H, dddd, J = 4.0, 12.0, 12.5, 12.5, H-9α), 1.20 (1H, m, H-1), 1.28 (1H, m, H-10), 1.33 (1H, m, H-8β), 1.40 (3H, s, Me-15), 1.43 (1H, m, H-2β), 1.46 (1H, m, H-7), 1.64 (1H, dddd, J = 3.5, 3.5, 3.5, 13.0, H-8α), 1.69 (1H, dddd, J = 3.5, 3.5, 3.5, 13.0, H-9β), 1.86 (1H, dddd, J = 3.0, 4.0, 6.5, 14.0, H-2α), 2.00 (1H, ddd, J = 3.0, 5.0, 14.5, H-3β), 2.35 (1H, ddd, J = 4.0, 13.5, 14.5, H-3α), 2.62 (1H, m, H-11), 3.42 (1H, dd, J = 11.5, 11.5, H-12β), 3.70 (1H, ddd, J = 1.1, 4.5, 11.5, H-12α), 5.17 (1H, s, H-5).

Microbial Metabolism of Deoxoartemisinin 1 By *Mucor ramannianus* 1839

Mucor ramannianus 1839 was grown in 23 0.5-liter culture flasks each containing 100 ml of medium. A total of 460 mg of deoxoartemisinin **1** (in 4.6 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 (2.3 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 (1.1 g).

Isolation and Characterization of 3α-Hydroxydeoxydeoxoartemisinin

The residue (1.1 g) was purified on a silica gel column (100 g, 3.75 × 30 cm) using hexane-ether (1:1) as an eluent, and 25-ml fractions were collected. Fractions 50-68, yielding a single spot with R_f = 0.63 (TLC system, ether) were combined and evaporated to dryness to give 32.0 mg (6.96%) of metabolite **2**. Crystallization from hexane-ether gave white prisms, mp, 84-85°C. LC/MS $[M]^+$ = 268 (consistent with the formula $C_{15}H_{24}O_4$); high resolution fabms $[M + H]^+$ 269.1762 (consistent with the formula $C_{15}H_{24}O_4 + H^+$, calcd 269.1753); IR (KBr)vmax (cm^{-1}), 3530; ^{1}H-NMR, δ 0.81 (3H, d, J = 6.5, Me-14), 0.84 (3H, d, J = 7.5, Me-13), 0.92 (1H, m, H-9α), 1.17 (1H, m, H-10), 1.19 (1H, m, H-8β), 1.31 (1H, m, H-7), 1.40 (3H, s, Me-15), 1.48 (1H, ddd, J = 4.0, 12.5, 14.0, H-2β), 1.64 (1H, m, H-9β), 1.69 (1H, m, H-8α), 1.82 (1H, m, H-2α), 1.86 (1H, m, H-1), 2.0 (1H, br, ex. D_2O) 2.23 (1H, m, H-11), 3.25 (1H, dd, J = 5.0, 11.5, H-12β), 3.48 (1H, br d, J = 3.0, H-3), 3.83 (1H, dd, J = 6.5, 11.5, H-12α), 5.12 (1H, s, H-5); ^{13}C-NMR (see Table I).

Isolation and Characterization of 9β-Hydroxydeoxoartemisinin

Fractions 208-265 from the above column yielded 50 mg (10.9% yield) of metabolite **3** with R_f = 0.45 (TLC system, ether). Crystallization from hexane-ether gave colorless prisms, mp, 132-135°C. High resolution fabms $[M + H]^+$ 285.1709 (consistent with the formula $C_{14}H_{22}O_4 + H^+$, calcd 285.1702); IR (KBr)vmax (cm^{-1}), 3520; ^{1}H-NMR, δ 0.79 (3H, d, J = 7.0, Me-13), 1.07 (3H, d, J = 6.0, Me-14), 1.35 (1H, m, H-10), 1.39 (1H, m, H-1), 1.43 (3H, s, Me-15), 1.50 (2H, m, H-8β and OH, ex. D_2O), 1.54 (1H, m, H-2β), 1.70 (1H, m, H-7), 1.88 (1H, m, H-2α), 1.90 (1H, m, H-8α), 2.04 (1H, ddd, 3.0, 4.8, 14.7, H-3β), 2.37 (1H, ddd, 4.0, 13.2, 14.6, H-3α), 2.63 (1H, m, H-11), 3.27 (1H, ddd, J = 4.0, 9.5, 10.8, H-9), 3.45 (1H, dd, J = 11.7, 11.8, H-12β), 3.75 (1H, ddd, J = 1.2, 4.3, 11.8, H-12α), 5.24 (1H, s, H-5); ^{13}C-NMR (see Table I).

Microbial Metabolism of Deoxoartemisinin by *Aspergillus ochraceus* ATCC 18500

Aspergillus ochraceus ATCC 18500 was grown in 11 1.0-L culture flasks each containing 200 ml of medium. A total of 440 mg of deoxoartemisinin **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 L) 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 4

The residue (1.0 g) was purified on a silica gel column (100 g, 3.75 × 30 cm) using hexane-ether (3:7) as an eluent. The polarity of the eluting system was later changed to 100% ether. 25-ml fractions were collected. Fractions 140-170, yielding a single spot with R_f = 0.5 (TLC system, ether) were combined and evaporated to dryness to give 80.0 mg (18.18%) of metabolite **4**. Crystallization from hexane-ether gave white needles, mp, 103-104°C. High resolution fabms $[M + H]^+$ 227.1640 (consistent with the formula $C_{13}H_{22}O_3 + H^+$, calcd 227.1647); IR (KBr)vmax (cm^{-1}), 3350; ^{1}H-NMR, δ 0.74 (3H, d, J = 7.2, Me-13), 0.86 (1H, m, H-9), 0.94 (3H, d, J = 6.5, Me-14), 1.28 (1H, m, H-1), 1.4-2.0 (1H, m, H-8), 1.66 (1H, m, H-8), 1.70-1.75 (2H, m, H-7, H-10), 1.84 (1H, m, H-2), 1.87 (1H, m, H-9), 2.18 (1H, m, H-2), 2.36 (1H,

Table I. ^{13}C-NMR Chemical Shift Assignments For Compounds 1–10[a]

Carbon No.	Chemical shift assignments (ppm)									
	1[b]	2	3	4	5	6	7	8[b]	9[b]	10[b]
C-1	52.1(1)	39.8(1)	49.6(1)	56.2(1)	55.6(1)	51.5(1)	44.3(1)	40.8(1)	55.7(1)	54.8(1)
C-2	24.7(2)	30.3(2)	24.6(2)	27.4(2)	27.7(2)	25.7(2)	24.5(2)	30.4(2)	27.8(2)	27.7(2)
C-3	36.2(2)	69.8(1)	36.0(2)	69.2(2)	68.7(2)	35.9(2)	36.3(2)	69.6(1)	68.6(2)	69.2(2)
C-4	104.1(0)	107.4(0)	104.2(0)	—	169.2(0)	104.5(0)	104.2(0)	107.8(0)	169.3(0)	168.5(0)
C-5	92.1(1)	95.5(1)	91.8(1)	94.5(1)	92.8(1)	91.4(1)	91.5(1)	93.7(1)	88.4(1)	93.0(1)
C-6	80.7(0)	83.2(0)	80.0(0)	81.1(0)	80.4(0)	78.9(0)	80.4(0)	84.2(0)	80.6(0)	79.4(0)
C-7	44.8(1)	41.8(1)	42.5(1)	46.8(1)	47.1(1)	44.8(1)	37.6(1)	42.5(1)	47.1(1)	46.6(1)
C-8	20.7(2)	23.7(2)	29.9(2)	21.0(2)	21.0(2)	35.9(2)	28.0(2)	25.0(2)	24.7(2)	24.3(2)
C-9	34.0(2)	34.3(2)	73.9(1)	35.4(2)	35.3(2)	209.1(0)	70.0(1)	34.8(2)	35.9(2)	34.6(2)
C-10	37.2(1)	35.0(1)	44.1(1)	30.4(1)	30.4(1)	47.8(1)	40.7(1)	34.8(1)	30.6(1)	30.9(1)
C-11	27.9(1)	26.4(1)	27.6(1)	29.8(1)	29.6(1)	27.8(1)	27.2(1)	30.4(1)	33.3(1)	35.0(1)
C-12	66.1(2)	64.6(2)	66.1(2)	66.9(2)	67.7(2)	65.4(2)	66.1(2)	99.8(1)	101.7(1)	171.7(0)
C-13	13.1(3)	16.2(3)	13.1(3)	12.8(3)	12.7(3)	12.7(3)	13.0(3)	12.3(3)	12.5(3)	12.5(3)
C-14	20.2(3)	18.7(3)	15.4(3)	20.5(3)	20.6(3)	11.8(3)	16.4(3)	18.8(3)	20.5(3)	20.4(3)
C-15	26.0(3)	20.6(3)	26.0(3)	—	21.6(3)	25.9(3)	26.1(3)	21.0(3)	21.6(3)	21.2(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.

[b] These data have been reported previously (3, 10, 11) and are listed here for comparison purposes.

m, H-11), 3.43 (1H, d, J = 11.5, H-12β), 3.63 (1H, m, H-12α), 3.84 (1H, m, H-3), 4.17 (1H, ddd, J = 1.5, 8.6, 9.4, H-3), 4.99 (1H, d, J = 8.3, H-5, becomes a singlet after D_2O exchange); ^{13}C-NMR (see Table I).

Acetylation of 4 to Produce 5

Metabolite 4 (20 mg) was dissolved in a few drops of pyridine, and a few drops of acetic anhydride were added. The reaction was left to run for 2 h, after which the reaction was complete as shown by TLC. The reaction mixture was diluted to 50 ml with water and extracted with ether (50 ml × 3). The combined ether layers were washed with dilute HCl (15 ml × 3) and finally with H_2O (15 ml × 3). After drying over anhydrous Na_2SO_4 and evaporation, the sample was purified by flash chromatography on silica gel using toluene-ether (6:4) as an eluent. 17 mg (85% yield) of the acetate, 5 in the form of an oil were obtained, LC/MS $[M]^+$ = 268 (consistent with the formula $C_{15}H_{24}O_4$); high resolution fabms $[M]^+$ 268.1677 (consistent with the formula $C_{15}H_{24}O_4$, calcd 268.1675); ^{1}H-NMR, δ 0.77 (3H, d, J = 7.0, Me-13), 0.92 (3H, d, J = 6.3, Me-14), 0.93 (1H, m, H-9), 1.33 (1H, m, H-1), 1.53 (1H, m, H-10), 1.71 (2H, m, H-8α, H-8β), 1.75 (1H, m, H-7), 1.80 (1H, m, H-2), 1.93 (1H, m, H-9), 1.95 (1H, m, H-2), 2.11 (3H, s, Me-15), 2.45 (1H, m, H-11), 3.53 (1H, dd, J = 11.8, 11.8, H-12b), 3.71 (1H, dd, J = 5.2, 11.7, H-12a), 3.91 (1H, ddd, J = 8.0, 8.0, 8.0, H-3), 4.25 (1H, ddd, J = 2.0, 7.7, 9.8, H-3), 5.98, (1H, s, H-5); ^{13}C-NMR (see Table I).

Oxidation of 3 to the Ketone 6

Metabolite 3 (22 mg) was dissolved in acetone (3 ml) and the temperature was kept between 10-15°C. In an ice bath, chromium trioxide (6.7 g) was dissolved in H_2O (12.5 ml) and concentrated H_2SO_4 (5.8 ml). The chromium trioxide reagent (4 drops) was added to the starting material. The reaction was stirred for 5 min, after which it was complete as shown by TLC. The reaction was diluted to 50 ml with H_2O and extracted with ether (50 ml × 3). The combined ether layers were washed with H_2O (10 ml × 3). After drying over anhydrous Na_2SO_4 and evaporation, the sample was purified by flash chromatography on silica gel using toluene-ethyl acetate (7.5:2.5) as an eluent. Crystallization from hexane-ether gave white needles (14.5 mg, 66% yield), mp 153-155°C; LC/MS $[M]^+$ = 282 (consistent with the formula $C_{15}H_{22}O_5$); high resolution fabms $[M + H]^+$ 283.1536 (consistent with the formula $C_{15}H_{22}O_5 + H^+$, calcd 283.1545); ^{1}H-NMR δ 0.76 (3H, d, J = 7.3, Me-13), 1.08 (3H, d, J = 6.5, Me-14), 1.46 (3H, s, Me-15), 1.68 (1H, m, H-2), 1.69 (1H, m, H-1), 1.90 (1H, m, H-2), 2.06 (1H, m, H-7), 2.09 (1H, m, H-3β), 2.30-2.53 (4H, m, H-3α, H-8α, H-8β, H-10), 2.69 (1H, m, H-11), 3.50 (1H, dd, J = 12.0, H-12β), 3.83 (1H, ddd, J = 1.2, 12.0, H-12α), 5.49 (1H, s, H-5); ^{13}C-NMR (see Table I).

Reduction of 6 to 7

The ketone, 6 (14.5 mg) was dissolved in methanol (2 ml). The temperature was kept between 0-5°C. $NaBH_4$ (8 mg) was placed in a vial in an ice bath to which a few drops of methanol were added. The reducing agent was added to the ketone dropwise with vigorous stirring over a period of 30 min after which the reaction was complete as shown by TLC. At that point, the mixture was neutralized with acetic acid (0.5 ml). The reaction mixture was dried under nitrogen and then subjected to flash chromatography on silica gel using toluene-ethyl acetate (1:1) as an eluent. The alcohol, 7 was obtained as a white solid (6.5 mg, 45% yield), mp 136-138°C; LC/MS $[M]^+$ = 284 (consistent with the formula $C_{15}H_{24}O_5$); high resolution fabms $[M + H]^+$ 285.1705 (consistent with the formula $C_{15}H_{22}O_5 + H^+$, calcd 285.1702);

^{1}H-NMR, δ 0.78 (3H, d, J = 7.2, Me-13), 1.05 (3H, d, J = 6.7, Me-14), 1.43 (3H, s, Me-15), 1.51 (2H, m, H-2, H-10), 1.63 (1H, ddd, J = 2.6, 14.1, 14.1, H-8β), 1.75-1.86 (3H, m, H-1, H-2, H-8α), 2.04 (1H, m, H-3β), 2.07 (1H, m, H-7), 2.4 (1H, ddd, J = 3.9, 13.5, 14.7, H-3α), 2.68 (1H, m, H-11), 3.42 (1H, dd, J = 11.8, 11.8, H-12β), 3.75 (1H, ddd, J = 1.4, 4.2, 11.8, H-12α), 3.84 (1H, ddd, J = 2.6, 2.6, 2.6, H-9β), 5.18 (1H, s, H-5); ^{13}C-NMR (see Table I).

Animal Studies

Deoxoartemisinin 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 (8). Under aseptic conditions, a 100.0 mg/ml solution of the test compound, 1 in ethanol was slowly added dropwise (10 μl/min) to a vigorously stirred commercially available fat emulsion (Liposyn II®, 20%) to give a final concentration of 6.0 mg/ml. Male Wistar rats were anesthetized with sodium pentobarbital (60 mg/kg), then each animal was administered 1 (11.6 mg/kg) by an intravenous bolus injection in the jugular vein. A blood sample (approximately 8 ml) was collected using a 20 ml syringe (containing 0.2 ml heparin solution) at 15 or 45 min. after the injection. A blank experiment was carried out where no drug was administered to the animal.

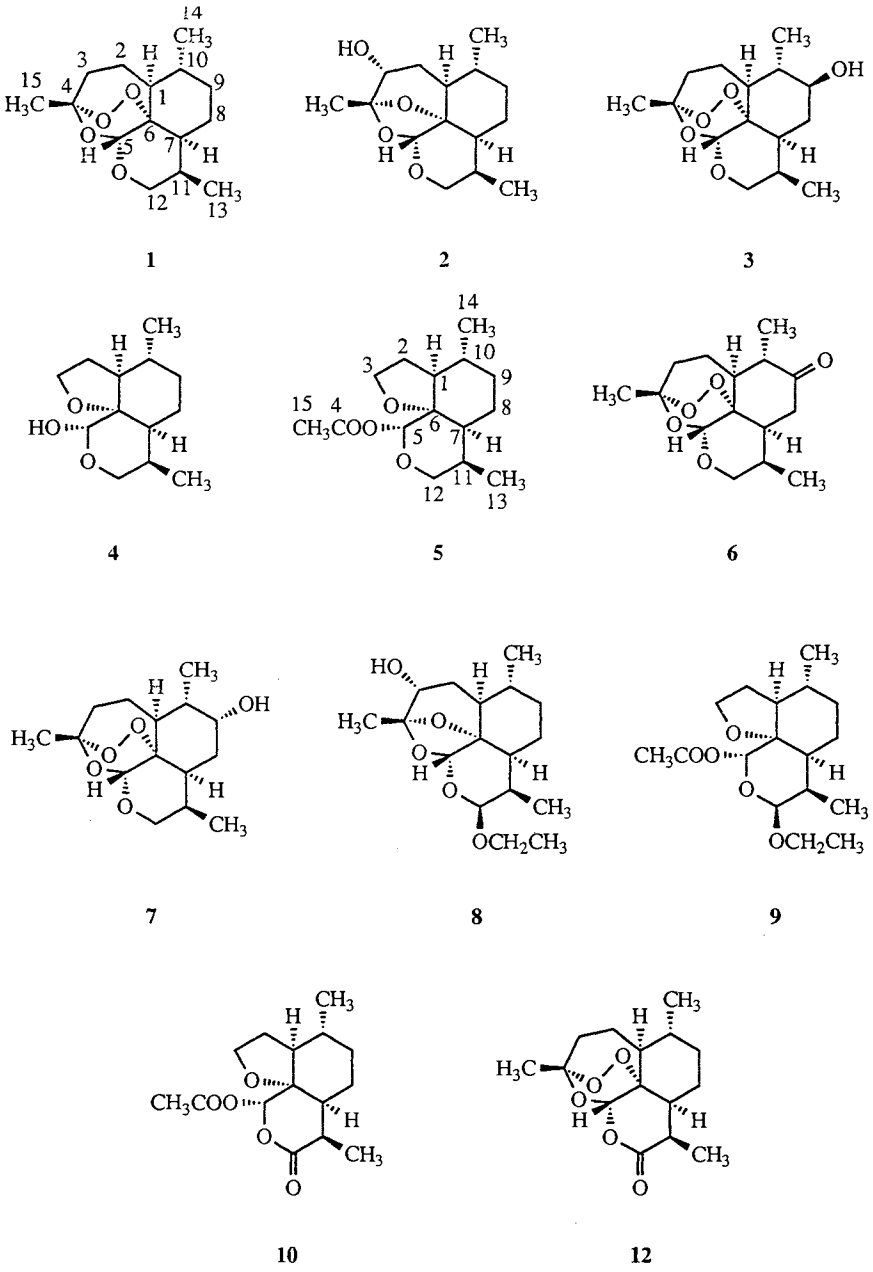

Fig. 1. Structures for deoxoartemisinin and metabolites.

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, then 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 the supernatant 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 × 25 cm cartridge-type HPLC column packed with a 5-mm-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 51.5% (v/v) to 82% (v/v) over a 10 min. period following an initial 5 min. hold.

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 (typically 215°C) of the vaporizer was set 5°C below the takeoff temperature. The block temperature of the ion source was set to 193°C, which was 75-100°C lower than is commonly used for model 201 thermospray unit.

RESULTS AND DISCUSSION

Screening-scale studies of deoxoartemisinin **1** have shown that a number of microorganisms are capable of metabolizing this sesquiterpene to a number of metabolites. Of the organisms screened, *Mucor ramannianus* 1839 and *Aspergillus ochraceus* ATCC 18500 were selected for preparative-scale fermentation because these organisms showed complete conversions to several different metabolites as judged by TLC.

A preparative-scale fermentation was performed with *Mucor ramannianus* 1839 using deoxoartemisinin **1** as a substrate, and compounds **2** and **3** were isolated and purified as major microbial metabolites. Metabolites **2**, $C_{15}H_{24}O_4$ had a molecular weight identical to that of deoxoartemisinin **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 **2** indicated that there was an absence of a methylene and a new carbon signal at δ 69.8. DEPTGL data proved that the carbon at δ 69.8 had one attached proton which strongly indicated the presence of a carbon atom directly attached to a hydroxyl group. A comparison of ^{13}C-NMR data of **2** with those of **1** showed that, of the five possibilities (C-2, -3, -8, -9, and -12), 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 stereochemistry of the hydroxyl group was established as alpha (α) by direct comparison of the ^{13}C-NMR data of metabolite **2** with those of 3α-hydroxydeoxyarteether **8** (7).

Metabolite **3**, $C_{15}H_{24}O_5$, had one additional oxygen present when compared with deoxoartemisinin **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 hydroxydeoxoartemisinin with only the position and the stereochemistry of the hydroxyl group to be determined. A comparison of ^{13}C-NMR data of **3** with those of **1** showed that, of the five possibilities (C-2, -3, -8, -9, and -12), the hydroxyl group must be at carbon 9 (see Table I). Especially noteworthy are downfield shifts for C-10 (6.9 ppm) and upfield shifts for C-1, C-7, and C-14. The assignment of the hydroxyl group as 9β was established by noting the coupling patterns of the proton at C-9. The C-9 proton resonated at δ 3.27 as a ddd (J = 4.0, 9.5, 10.8 Hz), and therefore must be axial.

A preparative-scale fermentation of deoxoartemisinin with *Aspergillus ochraceus* ATCC 18500 led to the isolation and purification of metabolite **4** that possessed the molecular formula, $C_{13}H_{22}O_3$ and a broad IR band at 3350 cm^{-1}. The loss of two carbon units immediately indicated the possibility that the 7-membered endoperoxide ring might have rearranged into a 5-membered tetrahydrofuran ring. This type of rearrangement has been reported in similar compounds (9, 10, 11). This is further evidenced by the ^{1}H-NMR spectral data that showed two proton resonances at δ 3.84 and δ 4.17 which are characteristic of the α protons of a tetrahydrofuran ring. A careful comparison of the ^{13}C-NMR data for **4** (9) with those for a similarly rearranged arteether metabolite **9** suggested that this metabolite could be represented by **4**. It should be noted that the structure of compound **9** was established through a ^{13}C-NMR comparison of **9** with a similarly rearranged artemisinin derivative **10**. The structure of compound **10** had been established by X-ray crystallography (11). To further confirm the structure of metabolite **4**, the acetate of **4** was prepared **5**. The acetylation reaction afforded a single product with the acetate function in the α orientation. The stereochemistry of the acetate was proved by comparison to the arteether metabolite **9** which was in turn compared to the artemisinin derivative **10**. In order to further confirm the stereochemistry at C-5 a 2D-NOESY experiment was conducted on **4**, **5**, and **9**. In the case of **4** there were two strong NOE interactions between H-5 and

protons H-10 and H-8(β) and a weak NOE interaction between H-5 and H-12 (β). The same interactions were observed in **5**. In the case of **9** NOE interactions between H-5 and protons H-10 and H-8 (β) were observed.

In preparation for mammalian metabolism studies of deoxoartemisinin, it was found that all of the metabolites obtained from the microbial fermentation isolates had thermospray mass spectra that were closely related to that of **1**. Using these ions to monitor the thermospray HPLC/MS, a synthetic mixture of **2**, **3**, and **4** obtained from the individual isolates of the microbial fermentations was found to give a well-resolved chromatogram (**2**, 12.8 min; **3**, 8.0 min; **4**, 16.0 min). Using the same thermospray HPLC/MS procedure on the rat plasma, 9β-hydroxydeoxoartemisinin **3** was found to be the major metabolite of deoxoartemisinin in the rat. Metabolites **2** and **4** were not detected. In the mammalian metabolism there appeared to be a second major metabolite **11**. Using the selected ion monitoring mode, this metabolite was picked up as an isomer of **3** possessing the same molecular weight (retention time, 10.8 min.). Since the mammalian metabolism of arteether produced both the 9β-hydroxyarteether and the 9α-hydroxyarteether (9), it seemed likely that the 9α-hydroxy metabolite would be a mammalian metabolite of deoxoartemisinin. Since the 9α-hydroxy derivative of deoxoartemisinin was not being produced as a microbial metabolite, it had to be chemically synthesized in order for a comparison with the metabolite from the mammalian system. Oxidation of **3** using Jones reagent produced the corresponding ketone **6**. LC/MS and high resolution fabms indicated a loss of two mass units when compared to **3**, a finding that is consistent with the oxidation of secondary alcohols. Also the shift of the C-9 signal from δ 73.9 in **3** to δ 209.1 in **6** is consistent with the formation of a carbonyl group. The ketone, **6** was reduced using sodium borohydride. The reaction produced two products, one of which was identified by TLC analysis as **3**. The second compound was presumably the 9α-hydroxydeoxoartemisinin **7**. Upon separation and purification of **7**, LC/MS and high resolution fabms revealed a molecular ion at 284 mass units indicating that it is indeed an isomer of **3**. An additional finding that further confirms the α orientation of the hydroxyl group at C-9 is the coupling pattern of the proton at C-9. The C-9 proton resonated at δ 3.84 as a ddd (J = 2.6, 2.6, 2.6 Hz), and therefore must be equatorial. Upon examination of plasma, **7** was found to have a different retention time that **11**. Thus, metabolite **11** is not the 9α isomer and is apparently another hydroxylated isomer of **1**.

In vitro antimalarial testing was limited to metabolite **3** since it is the only metabolite retaining the endoperoxide intact, a feature that has been shown to be necessary for antimalarial activity (12). Metabolite **3** possesses *in vitro* antimalarial activity, but is less active than deoxoartemisinin **1**.

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