Pharmaceutical Research, Vol. 7, No. 9, 1990

Report

Structure Elucidation and Thermospray High-Performance Liquid Chromatography/Mass Spectroscopy (HPLC/MS) of the Microbial and Mammalian Metabolites of the Antimalarial Arteether

Charles D. Hufford,[1,4] Ik-Soo Lee,[1] Hala N. ElSohly,[2] Hsien Tao Chi,[3] and John K. Baker[3]

Received December 11, 1989; accepted March 13, 1990

Microbial metabolism studies of the antimalarial drug arteether (1) have shown that arteether is metabolized to six new metabolites in addition to those previously reported (3). Large-scale fermentations with *Cunninghamella elegans* (ATCC 9245) and *Streptomyces lavendulae* (L-105) have resulted in the characterization of these metabolites primarily by two-dimensional nuclear magnetic resonance (2D-NMR) methods as 9β-hydroxyarteether (2), a ring rearrangement metabolite (3), 3α-hydroxy-11-epi-deoxydihydroartemisinin (4), 9α-hydroxyarteether (5), 2α-hydroxyarteether (6), and 14-hydroxyarteether (7). Thermospray mass spectroscopy/high-performance liquid chromatographic analyses have shown that four of these metabolites (2, 5, 6, 7) are also present in rat liver microsome preparations.

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

INTRODUCTION

Arteether (1) (Fig. 1) is a new drug candidate useful for the treatment against the erythrocytic stages of chloroquine-resistant *Plasmodium falciparum* and for cerebral malaria. Its synthesis and antimalarial properties have been reviewed (1). Recent metabolism studies using rat liver microsomes (2) and microbial enzymes (3) have identified a number of metabolites of arteether (1).

The rat liver microsome studies (2) also tentatively identified two additional metabolites as hydroxyarteethers but they could not be rigorously characterized because of the lack of sufficient material. A screening of 44 additional microbial cultures produced metabolite patterns not seen previously (3). Scale-up studies of two cultures, *Cunninghamella elegans* (ATCC 9245) and *Streptomyces lavendulae* (L-105), have resulted in the production of six additional microbial metabolites, four of which are hydroxyarteethers and do correspond to the mammalian metabolites as well. The isolation, structure elucidation, and thermospray LC/MS studies are described herein.

[1] Department of Pharmacognosy, School of Pharmacy, The University of Mississippi, University, Mississippi 38677.

[2] Research Institute of Pharmaceutical Sciences, School of Pharmacy, The University of Mississippi, University, Mississippi 38677.

[3] Department of Medicinal Chemistry, School of Pharmacy, The University of Mississippi, University, Mississippi 38677.

[4] To whom correspondence should be addressed.

MATERIALS AND METHODS

General Procedures

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-μm-particle size, octadecyl reversed-phase material (Whatman Partisil ODS-3) was utilized, with a mobile phase (1.0 ml/min) comprised of 0.1 *M* ammonium acetate in a methanol:water mixture. The methanol content of the mobile phase was linear-programmed from 56% (v/v) to 80% (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 (205°C typically) of the vaporizer was set 5°C below the takeoff temperature. The block temperature of the ion source was set to 190°C, which was 75–100°C lower than is commonly used for Model 201 thermospray unit.

The microbial metabolism studies were conducted as previously reported (3), as were the rat liver microsome

0724-8741/90/0900-0923$06.00/0

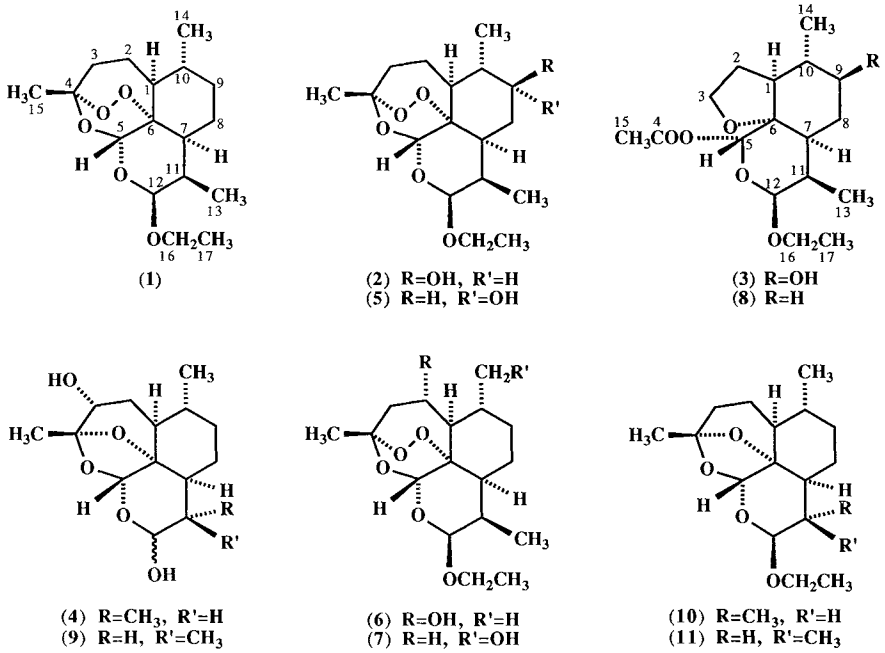

Fig. 1. Chemical structures of arteether and its analogues.

studies (2). Forty-four additional cultures were screened and those showing one or more metabolites by thin-layer chromatography (TLC) are listed here: [In parentheses are listed the metabolites present as indicated by TLC. 3α-Hydroxydeoxyarteether (3) is abbreviated AEM2.] *Acrodictys erecta* ATCC 24083 (4, 5, 6, 7, 8, 11, AEM2), *Agaricus campestris* ATCC 26815 (8, 9, 11, AEM2), *Aspergillus alliaceus* NRRL 315 (4, 8, 9, 11, AEM2), *Aspergillus flavus* NRRL 626 (8, 9, 11, AEM2), *Aspergillus fumigatus* ATCC 26934 (8, 9, 11, AEM2), *Aspergillus ochraceus* ATCC 18500 (8, 9, 11, AEM2), *Aspergillus tamarii* NRRL 8101 (2, 6, 8, 9, 11, AEM2), *Aureobasidium pullulans* ATCC 9348 (8, 9, 11, AEM2), *Beauvaria bassiana* ATCC 7159 (5, 7, 8, 9, 11, AEM2), *Calonectria decora* ATCC 14767 (8, 9, 11, AEM2), *Cellulomonas flavigena* ATCC 482 (8, 11, AEM2), *Coriolus antarcticus* ATCC 34581 (8, 9, 11, AEM2), *Cryptococcus neoformans* ATCC 32264 (8, 9, 11, AEM2), *Cunninghamella blakesleeana* UM-ATCC 8688a (2, 3, 4, 9), *Cunninghamella elegans* ATCC 9245 (2, 3, 4, 9), *Dactylaria haptotyla* ATCC 28924 (8, 9, 11, AEM2), *Fomes pinicola* ATCC 15341 (3, 8, 9, 11, AEM2), *Fusarium solani* ATCC 12823 (8, 9, 11, AEM2), *Gliocladium deliquescens* ATCC 10097 (8, 9, 11, AEM2), *Lipomyces lipofer* ATCC 10742 (8, 9, 11, AEM2), *Melanospora ornata* ATCC 26180 (8, 9, 11, AEM2), *Monilinia fructicola* ATCC 32670 (8, 9, 11, AEM2), *Mucor ramannianus* 1839 (Sih) (2, 3, 4, 9, 11, AEM2), *Neurospora africana* ATCC 18747 (8, 9, 11, AEM2), *Nocardia minima* ATCC 19150 (8, 11, AEM2), *Penicillium chrysogenum* ATCC 9480 (4, 8, 9, 11, AEM2), *Penicillium patulum* ATCC 24550 (8, 9, 11, AEM2), *Polysphondylium pallidum* 34073 (8, 9, 11, AEM2), *Pseudomonas alkanolytica* ATCC 21034 (8, 9, 11, AEM2), *Rhizopus stolonifer* ATCC 24795 (3, 8, 9, 11, AEM2), *Saccharomyces lipolytica* ATCC 16617 (8, 9, 11, AEM2), *Schizosaccharomyces pombe* ATCC 20130 (8, 9, 11, AEM2), *Septomyxa affinis* ATCC 6737 (2, 3, 6, 8, 9, 11, AEM2), *Streptomyces griseus* NRRL 5687 (9, 11, AEM2), *Streptomyces lavendulae* L-105 (5, 6, 7, 8, 9, 11, AEM2), *Streptomyces platensis* NRRL 2364 (8, 9, 11, AEM2), *Streptomyces roseochromogenus* ATCC 13400 (7, 8, 9, 11, AEM2), *Thamnidium elegans* ATCC 18191 (8, 9, 11, AEM2), *Trametes zonata* ATCC 38279 (8, 9, 11, AEM2), *Trichophyton mentagrophytes* ATCC 9972 (2, 3, 7, 9, 11, AEM2), *Debaryomyces polymorphus* ATCC 20280 (3, 8, 9, 11, AEM2), *Hansenula anomala* ATCC 20170 (3, 8, 9, 11, AEM2), *Mortierella zonata* ATCC 13309 (2, 3, 4, 5, 7, 8, 9, 11, AEM2), and *Thielavia terricola* ATCC 13807 (8, AEM2).

Microbial Metabolism of Arteether (1) by *Cunninghamella elegans*

Cunninghamella elegans (ATCC 9245) was grown in 24 1-liter culture flasks, each containing 200 ml of medium. A total of 920 mg of arteether (1) (in 9.2 ml of EtOH) was evenly distributed among the 24-hr-old stage II cultures. After 13 days, the incubation mixtures were pooled, then filtered to remove the cells, and the filtrate (4.8 liters) was extracted three 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 (960 mg).

This residue (960 mg) was purified by column chromatography over a silica gel column (100 g, 2.5 × 80 cm), using an ether–hexane (4:1) mixture as an eluting system to yield 20 mg of 2 as a single spot with R_f = 0.56, followed by 3 (25 mg) and 4 (22 mg) with R_f values 0.35 and 0.30, respectively. On recrystallization from ether–hexane, all of these compounds gave colorless needles.

Compound 2, mp 124–126°C; $[\alpha]_D^{22}$ = +99.3° (*c* = 1.4, $CHCl_3$); ir ν_{max}^{KBr} cm^{-1}, 3520 (-OH), 2960, 2920, 2880, 1450, 1380, 1175, 1105, 1025, 990, 970, 880; ^{1}H-nmr, 0.91 (3H, d, *J* = 7.2, Me-13), 1.06 (3H, d, *J* = 5.7, Me-14), 1.18 (3H, t, *J* = 7.1, Me-17), 1.2–1.4 (2H, m, H-1, H-10), 1.43 (3H, s,

Me-15), 1.6 (2H, m, H-2, H-7), 1.8–2.1 (4H, m, H-2, H-3_α, H-8_α, H-8_β), 2.36 (1H, ddd, J = 14.5, 4.6, 3.0, H-3_β), 2.59 (1H, m, H-11), 3.11 (1H, ddd, J = 9.7, 9.7, 5.1, H-9_α), 3.47 (1H, dq, J = 9.9, 7.1, H-16), 3.86 (1H, dq, J = 9.9, 7.1, H-16), 4.80 (1H, d, J = 3.3, H-12), 5.46 (1H, s, H-5); ^{13}C-nmr, see Table I.

Compound **3**, mp 118–121°C; $[\alpha]_D^{22}$ = +105° (c = 3.0, $CHCl_3$); ir ν_{max}^{KBr} cm^{-1}, 3440 (-OH), 2980, 2950, 2890, 1750 (C = O), 1460, 1410, 1370, 1225, 1170, 1080, 1020, 980, 940, 900; ^{1}H-nmr, 0.89 (3H, d, J = 7.3, Me-13), 1.05 (3H, d, J = 6.0, Me-14), 1.20 (3H, t, J = 7.1, Me-17), 1.36–2.20 (7H, m, H-1, H-2_α, H-2_β, H-7, H-8_α, H-8_β, H-10), 2.12 (3H, s, Me-15), 2.34 (1H, m, H-11), 3.24 (1H, ddd, J = 10.6, 8.7, 6.0, H-9_α), 3.39 (1H, dq, J = 9.7, 7.1, H-16), 3.93 (1H, dq, J = 9.7, 7.1, H-16), 3.98 (1H, J = ddd, 8.1, 8.1, 8.1, H-3), 4.32 (1H, ddd, J = 8.1, 8.1, 3.6, H-3), 4.73 (1H, d, J = 4.2, H-12), 6.37 (1H, s, H-5); ^{13}C-nmr, see Table I.

Compound **4**, mp 164–165°C; $[\alpha]_D^{22}$ = −26.5° (c = 1.7, $CHCl_3$); ir ν_{max}^{KBr} cm^{-1}, 3490 (-OH), 3400 (-OH), 2930, 2890, 2880, 1460, 1390, 1270, 1225, 1160, 1090, 1050, 980, 930, 875; ^{1}H-nmr (major isomer), 0.88 (3H, d, J = 6.0, Me-14), 1.13 (3H, d, J = 7.0, Me-13), 1.55 (3H, s, Me-15), 3.55 (1H, m, H-3), 5.02 (1H, d, J = 7.9, H-12), 5.35 (1H, s, H-5); ^{13}C-nmr, see Table I.

Microbial Metabolism of Arteether (1) by *Streptomyces lavendulae*

Five hundred sixty milligrams of arteether (1) was dissolved in 5.6 ml of EtOH and distributed evenly among 14 1-liter culture flasks, each containing 200 ml of 24-hr-old, stage II cultures of *Streptomyces lavendulae* (L-105). The cultures were incubated for 14 days and were harvested by filtration. The combined aqueous filtrate (2.8 liters) was extracted three times with EtOAc (1 × 2.8 liters, 2 × 1.4 liters). The combined extracts were back-washed with H_2O, dried over anhydrous Na_2SO_4, and filtered, and the solvent was evaporated under reduced pressure to afford 720 mg of dark brown residue. The residue was purified on a silica gel column (80 g, 2.5 × 50 cm) using Et_2O–hexane (4:1) as an eluent to give 30 mg of **5** as a single spot with R_f = 0.60, followed by **6** (20 mg) and **7** (15 mg) with R_f values 0.54 and 0.45, respectively. Recrystallization from ether–hexane afforded colorless crystals for compounds **5** and **7**.

Compound **5**, mp 134–136°C; $[\alpha]_D^{22}$ = +124° (c = 2.5, $CHCl_3$); ir ν_{max}^{KBr} cm^{-1}, 3490 (-OH), 2960, 2920, 2880, 1450, 1380, 1345, 1280, 1260, 1225, 1175, 1135, 1105, 1020, 990, 960, 910; ^{1}H-nmr, 0.89 (3H, d, J = 7.3, Me-13), 1.03 (3H, d, J = 6.7, Me-14), 1.17 (3H, t, J = 7.0, Me-17), 1.43 (3H, s, Me-15), 1.5 (2H, m, H-2, H-10), 1.7–2.1 (6H, m, H-1, H-2, H-3_β, H-7, H-8_α, H-8_β), 2.39 (1H, J = ddd, 14.0, 14.0, 3.7, H-3_α), 2.64 (1H, m, H-11), 3.46 (1H, dq, J = 9.8, 7.0, H-16), 3.74 (1H, ddd, J = 2.4, 2.4, 2.4, H-9_β), 3.85 (1H, dq, J = 9.8, 7.0, H-16), 4.80 (1H, d, J = 3.2, H-12), 5.41 (1H, s, H-5); ^{13}C-nmr, see Table I.

Compound **6**, amorphous solid; $[\alpha]_D^{22}$ = +144° (c = 1.0, $CHCl_3$); ir ν_{max}^{KBr} cm^{-1}, 3460 (-OH), 2960, 2930, 2880, 1450, 1380, 1245, 1195, 1175, 1120, 1105, 1020, 990, 970; ^{1}H-nmr, 0.91 (3H, d, J = 7.4, Me-13), 1.00 (1H, m, H-9_α), 1.15 (3H, d, J = 6.5, Me-14), 1.18 (3H, t, J = 7.1, Me-17), 1.28 (1H, dd, J = 8.1, 11.1, H-1), 1.45 (3H, s, Me-15), 1.52 (2H, m, H-7, H-10), 1.64 (1H, dddd, J = 13.0, 3.2, 3.2, 3.2, H-9_β), 1.75 (1H, dddd, J = 13.7, 3.9, 3.9, 3.9, H-8_α), 1.92 (1H, dddd, J = 13.7, 13.7, 13.7, 3.8, H-8_β), 2.50 (2H, m, H-3_α, H-3_β), 2.62

Table I. ^{13}C-nmr Chemical Shift Assignments for Compounds **1–11**[a]

Carbon no.	Chemical shift assignment (ppm)										
	1[b]	**2**	**3**	**4**[c]	**5**	**6**	**7**	**8**[b]	**9**[b,c]	**10**[d]	**11**[b]
1	52.8 (1)	50.1 (1)	52.7 (1)	43.7 (1)	44.7 (1)	59.9 (1)	47.0 (1)	55.7 (1)	41.2 (1)[1]	45.3 (1)	46.8 (1)
2	24.8 (2)	24.6 (2)	27.2 (2)	30.2 (2)	24.6 (2)	69.5 (1)	24.2 (2)[1]	27.8 (2)	30.3 (2)	22.1 (2)	22.2 (2)
3	36.6 (2)	36.3 (2)	69.8 (2)	69.6 (1)	36.6 (2)	46.6 (2)	36.4 (2)	68.6 (2)	69.6 (1)	34.5 (2)[1]	34.9 (2)
4	104.0 (0)	104.1 (0)	169.1 (0)	107.0 (0)	104.1 (0)	102.4 (0)	104.0 (0)	169.3 (0)	108.2 (0)	106.9 (0)	107.9 (0)
5	87.9 (1)	87.6 (1)	88.1 (1)	96.8 (1)	87.3 (1)	87.7 (1)	87.8 (1)	88.4 (1)	95.4 (1)	97.3 (1)	94.7 (1)
6	81.2 (0)	80.4 (0)	80.2 (0)	83.1 (0)	80.9 (0)	80.6 (0)	81.2 (0)	80.6 (0)	83.1 (0)	82.6 (0)	83.4 (0)
7	44.7 (1)	42.0 (1)	45.1 (1)	41.7 (1)	37.1 (1)	44.6 (1)	44.4 (1)[2]	47.1 (1)	41.5 (1)[1]	44.1 (1)	41.1 (1)
8	24.6 (2)	33.6 (2)	34.4 (2)	32.6 (2)[1]	31.8 (2)	24.5 (2)	24.1 (2)[1]	24.7 (2)	22.8 (2)	32.7 (2)[1]	25.0 (2)
9	34.8 (2)	74.2 (1)	77.6 (1)	34.3 (2)[1]	70.1 (1)	35.6 (2)	28.7 (2)	35.9 (2)	34.2 (2)	34.5 (2)[1]	34.6 (2)
10	37.6 (1)	44.2 (1)	37.6 (1)	34.9 (1)	40.7 (1)	37.2 (1)	44.4 (1)[2]	30.6 (1)	35.0 (1)	35.2 (1)	35.2 (1)
11	31.0 (1)	30.5 (1)	33.0 (1)	40.9 (1)	30.3 (1)	30.9 (1)	30.8 (1)	33.3 (1)	33.5 (1)	39.8 (1)	30.6 (1)
12	101.7 (1)	101.6 (1)	101.6 (1)	94.6 (1)	101.9 (1)	101.8 (1)	101.7 (1)	101.7 (1)	96.6 (1)	100.2 (1)	99.3 (1)
13	13.1 (3)	12.9 (3)	12.4 (3)	19.4 (3)	13.0 (3)	13.0 (3)	13.0 (3)	12.5 (3)	14.6 (3)	19.3 (3)	12.2 (3)
14	20.4 (3)	15.4 (3)	16.4 (3)	18.4 (3)	16.6 (3)	21.4 (3)	65.0 (2)	20.5 (3)	18.6 (3)	18.7 (3)	19.0 (3)
15	26.3 (3)	26.1 (3)	21.5 (3)	20.3 (3)	26.3 (3)	26.1 (3)	26.1 (3)	21.6 (3)	20.8 (3)	23.8 (3)	24.5 (3)
16	63.8 (2)	64.0 (2)	63.9 (2)		63.9 (2)	64.0 (2)	63.8 (2)	63.7 (2)		64.7 (2)	63.9 (2)
17	15.3 (3)	15.2 (3)	14.9 (3)		15.3 (3)	15.3 (3)	15.2 (3)	15.0 (3)		15.1 (3)	15.2 (3)

[a] The numbers in parentheses represent the number of attached hydrogens as determined by the DEPTGL pulse sequence. Assignments are based on ^{1}H–^{1}H and ^{1}H–^{13}C chemical shift-correlated 2D-nmr spectroscopy and by comparisons to the other compounds. Signals bearing the same numerical superscript may have interchangeable assignments.

[b] These data have been published before (3, 4) and are listed here for comparison purposes.

[c] For compounds 4 and **9**, chemical shift assignments are shown for the major isomers only.

[d] This compound has been reported previously (5) but without ^{13}C-nmr assignments. A sample of **10** was obtained as an authentic sample.

(1H, ddq, J = 7.4, 7.4, 3.6, H-11), 3.45 (1H, dq, J = 9.9, 7.1, H-16), 3.84 (1H, dq, J = 9.9, 7.1, H-16), 3.99 (1H, ddd, J = 8.1, 8.1, 8.1, H-2_β), 4.79 (1H, d, J = 3.6, H-12), 5.37 (1H, s, H-5); ^{13}C-nmr, see Table I.

Compound 7, mp 108–110°C; $[\alpha]_D^{22}$ = +53.5° (c = 2.0, $CHCl_3$); ir ν_{max}^{KBr} cm^{-1}, 3480 (-OH), 2960, 2920, 2880, 1450, 1385, 1340, 1190, 1170, 1120, 1090, 1050, 1025, 990, 940; ^{1}H-nmr, 0.91 (3H, d, J = 7.3, Me-13), 1.05 (1H, m, H-9_α), 1.18 (3H, t, J = 7.1, Me-17), 1.44 (3H, s, Me-15), 1.5–1.7 (4H, m, H-1, H-2, H-7, H-10), 1.7–1.9 (4H, m, H-2, H-8_α, H-8_β, H-9_β), 2.04 (1H, m, H-3), 2.39 (1H, m, H-3), 2.62 (1H, m, H-11), 3.46 (1H, dq, J = 9.8, 7.1, H-16), 3.59 (1H, dd, J = 10.7, 5.7, H-14), 3.74 (1H, J = dd, 10.7, 3.0, H-14), 3.86 (1H, dq, J = 9.8, 7.1, H-16), 4.80 (1H, d, J = 3.2, H-12), 5.41 (1H, s, H-5); ^{13}C-nmr, see Table I.

Jones Oxidation of Metabolites 2 and 5

A solution of 1 mg of metabolite 2 in 1 ml of acetone (analytical reagent grade) was cooled to 0–5°C in a 5-ml Erlenmeyer flask immersed in an ice bath. Two-tenths milliliter of cooled Jones reagent was added to the flask, and the reaction mixture was filtered after 1 min. The filtered solution was directly subjected to LC/MS analysis. Metabolite 5 was oxidized using the same procedure as described above. The LC/MS analysis showed one major peak with the same retention time, with no starting material present from each reaction. Each peak had identical mass spectra with $[M + NH_4]^+$ = 344. TLC analysis showed no starting material and one less polar spot. TLC cospotting of the two showed only one spot [R_f = 0.66; ether–hexane (4:1)]. Metabolites 2 and 5 show $[M + NH_4]^+$ = 346.

RESULTS AND DISCUSSION

A preparative-scale fermentation was performed with *Cunninghamella elegans* (ATCC 9245) using arteether (1) as substrate, and metabolites 2 (20 mg), 3 (25 mg), and 4 (22 mg) were isolated.

Metabolite 2, $C_{17}H_{28}O_6$, had one additional oxygen present when compared with arteether (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 ethyl ether and peroxide functions were intact. Thus 2 represented a hydroxyarteether with only the position and stereochemistry of the hydroxyl group to be determined. Since the complete ^{13}C-nmr assignments for 1 have been established (4), a comparison of ^{13}C-nmr data of 2 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 (6.6 ppm) and upfield shifts for C-1, C-14, and C-7. All of the ^{1}H-nmr and ^{13}C-nmr assignments were verified by ^{1}H–^{1}H and ^{1}H–^{13}C shift-correlated 2D-nmr spectroscopy. The assignment of the hydroxyl group as β is discussed below where metabolite 5 is presented.

Metabolite 3 had the same molecular formula as 2 but clearly had substantially different spectroscopic data. Metabolite 3 had a carbonyl band at 1750 cm^{-1} in the ir spectrum and a signal at δ_c 169.1 in the ^{13}C-nmr spectrum. These signals are characteristic for the skeletal rearranged products noted previously (3) and represented by structure 8. Metabolite 3 was also hydroxylated (secondary OH) and the position and stereochemistry were established as 9β for the same reasons as discussed for 2 (see Table I).

Metabolite 4, $C_{15}H_{24}O_5$, clearly had spectroscopic data showing that the ethyl ether function was not present, that there was a secondary hydroxyl group present, and that it was of the deoxy series. In fact, the conclusions from interpretation of the ^{1}H-nmr and ^{13}C-nmr spectral data led to the postulation of a structure similar to that previously seen before as a metabolite (3) (9). However, there were chemical shifts of carbon atoms that were difficult to rationalize into a proposed structure. 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 C-(11)-epi-deoxyarteether (10) (5), substantial changes in the carbon chemical shifts in C-8, C-11, and C-13 are noted (see Table I). It should be noted that the structure for 10 was solved by X-ray analysis. Also, the ^{13}C-nmr data for 10 were not reported (5), but a sample of 10 was obtained and the ^{13}C-nmr assignments were verified as was done for deoxyarteether (11) (3). A careful comparison of the ^{13}C-nmr data for 10 and 11 with those for 4 and 9 led us to conclude that metabolite 4 can be represented as 3α-hydroxy-11-epi-deoxydihydroartmisinin.

A preparative-scale fermentation using *Streptomyces lavendulae* (L-105) led to the isolation of three additional metabolites, 5 (30 mg), 6 (20 mg), and 7 (15 mg). Metabolites 5, 6, and 7 had the same molecular formula ($C_{17}H_{28}O_6$). The ir, ^{1}H-nmr, and ^{13}C-nmr spectral data clearly showed the presence of a hydroxyl group, the ethyl ether function, and the peroxide group, leading to the conclusion that all three were hydroxyarteethers.

An analysis of the ^{1}H-nmr and ^{13}C-nmr data for metabolite 5 similar to that done for 2 led to the conclusion that the hydroxyl group was also at C-9 and therefore metabolites 5 and 2 were isomers at C-9. This was proven by oxidation of 5 and 2 with Jones reagent to produce the same ketone. The assignment of the hydroxyl group to 9β in 2 and 9α in 5 was established by noting the coupling patterns of the proton at C-9. The C-9 proton resonated at δ 3.11 as a ddd (J = 9.7, 9.7, 5.1 Hz) in 2, and therefore must be axial, and at δ 3.74 as a ddd (J = 2.4, 2.4, 2.4 Hz) in 5, and therefore must be equatorial.

The hydroxyl group in metabolite 6 was established as secondary from ^{1}H-nmr and ^{13}C-nmr data and therefore could be located only at C-2, C-3, or C-8. An analysis of ^{13}C-nmr data (compared to 1) clearly showed a large downfield shift of C-1 (7.1 ppm) and smaller upfield shifts for C-4, C-6, and C-10, leading to the conclusion that the hydroxyl group was at C-2. The ^{1}H-nmr data showed the proton attached to the hydroxyl group as an apparent ddd with equal couplings (J = 8 Hz). It should also be noted that both protons at C-3 resonated as a complex non-first-order pattern. Based on these data it was difficult to be certain of the configuration of the hydroxyl group. Spin simulation analyses (7) of the two potential α,β-hydroxyl isomers using the coupling constants [$J_{1,2\beta}$ = 12.7, $J_{2\beta,3\alpha}$ = 13.5, $J_{2\beta,3\beta}$ = 4.4 for the α-OH isomer and $J_{1,2\alpha}$ = 6.4, $J_{2\alpha,3\alpha}$ = 3.9, $J_{2\alpha,3\beta}$ = 3.0 for the β-OH isomer; values previously reported for arteether (6)] duplicated the pattern for 6 when the hydroxyl group was placed alpha. A NOESY experiment clearly

showed NOE enhancements between H-5 and H-8_β, between H-5 and H-10, and between H-5 and H-2_β. The H-5/H-2 enhancement clearly allowed placement of the hydroxyl group as alpha (**6**).

The hydroxyl group in metabolite **7** was established as primary, thereby suggesting that one of the four methyl groups in **1** had been hydroxylated. ^{13}C-nmr data showed that the hydroxyl group was located at C-14 (see Table I).

With regard to the mammalian metabolism of arteether, this laboratory has previously reported (2) the conversion of arteether to dihydroartemisinin, deoxydihydroartemisinin, 3α-hydroxydeoxydihydroartemisinin (**9**), 3α-hydroxydexoyarteether, and several isomers of hydroxylated arteether with unknown stereochemistry. In the present study it was found that all of the isomers of hydroxyarteether (**2, 5, 6, 7**) obtained from the microbial fermentation isolations had thermospray mass spectra that were essentially identical to that for arteether except that all of the lines were displaced upward by 16 *m/z* units (giving 265, 283, 300, and 346 *m/z* as prominent ions). Using these four ions to monitor the thermospray HPLC/MS, a synthetic mixture of **2, 5, 6,** and **7** obtained from the individual isolates of the microbial fermentations was found to give a well-resolved chromatogram (**2**, 9.6 min; **7**, 11.0 min; **5**, 11.7 min; **6**, 17.2 min). Using the same thermospray HPLC/MS procedure, the rat liver microsome incubations of arteether were also found to contain **2, 5, 6,** and **7**.

In summary of the present and previous studies (2) on the metabolism of arteether in a rat liver microsome preparation, the major mammalian metabolite of arteether was dihydroartemisinin, which resulted from the oxidative *O*-dealkylation of arteether. Oxidation of the ring system of arteether gave 9α-hydroxyarteether (**2**), 9β-hydroxyarteether (**5**), 2α-hydroxyarteether (**6**), and 14-hydroxyarteether (**7**) as mammalian metabolites. Other minor metabolites arising from multistep transformation found to be present included deoxydihydroartemisinin, 3α-hydroxydeoxydihydroartemisinin (**9**), and 3α-hydroxydeoxyarteether.

ACKNOWLEDGMENTS

This research was supported in part by a grant from UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases. The authors wish to express their thanks to Dr. Arnold Brossi, National Institutes of Health, Bethesda, MD, for the authentic samples of 11-epi-deoxyarteether (**10**) and deoxyarteether (**11**).

REFERENCES

1. A. Brossi, B. Venugopalan, L. D. Gerpe, H. J. C. Yeh, J. L. Flippen-Anderson, P. Buchs, X. D. Luo, W. Milhous, and W. Peters. *J. Med. Chem.* **31**:645–650 (1988).
2. J. K. Baker, R. H. Yarber, C. D. Hufford, I.-S. Lee, H. N. ElSohly, and J. D. McChesney. *Biomed. Environ. Mass Spectrom.* **18**:337–351 (1989).
3. I.-S. Lee, H. N. ElSohly, and C. D. Hufford. *Pharm. Res.* 7:199–203 (1990).
4. C. D. Hufford and H. N. ElSohly. *Spectrosc. Lett.* **20**:439–444 (1987).
5. L. D. Gerpe, H. J. C. Yeh, Q.-S. Yu, A. Brossi, and J. L. Flippen-Anderson. *Heterocycles* **27**:897–901 (1988).
6. J. K. Baker, H. N. ElSohly, and C. D. Hufford. *Spectrosc. Lett.* **23**:111–122 (1990).
7. The computer software is derived from a previous program titled "LAOCOON": A. A. Bothner-By and S. Castellano. *J. Chem. Phys.* **41**:3863–3869 (1964).