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Report

Microbial Metabolism Studies of the Antimalarial Drug Arteether

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Microbial metabolism studies of the antimalarial drug arteether (1) have shown that arteether is metabolized by a number of microorganisms. Large-scale fermentation with *Aspergillus niger* (ATCC 10549) and *Nocardia corallina* (ATCC 19070) have resulted in the isolation of four microbial metabolites which have been characterized using two-dimensional nuclear magnetic resonance (2D-NMR) techniques. These metabolites have been identified as "AEM1" (2), 3α-hydroxydeoxyarteether (3), 3α-hydroxydeoxydihydroartemisinin (4), and deoxydihydroartemisinin (5).

KEY WORDS: microbial metabolism; antimalarial; arteether; microbial metabolites; two-dimensional nuclear magnetic resonance (2D-NMR) techniques.

INTRODUCTION

Arteether (1) is the ethyl ether derivative of dihydroartemisinin, a sodium borohydride reduction product of artemisinin. Artemisinin is the active antimalarial constituent of the Chinese medicinal herb, *Artemisia annua* L. (1). Arteether has been chosen by SWG-CHEMAL (the Steering Committee of the Scientific Working Group on Malaria Chemotherapy of the World Health Organization in Geneva, Switzerland) for use in high-risk malaria patients including those with cerebral malaria. The ^{1}H- and ^{13}C-NMR assignments of arteether have been reported (2). Brossi and coworkers have recently published an article on the synthesis and antimalarial properties of arteether (3).

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 (4–10). Since there have been no previous reports on the metabolism of arteether, a comprehensive study on its metabolism has been undertaken. In the present study on the microbial metabolism of arteether, four compounds were isolated as microbial metabolites. Based on the chemical and spectroscopic data, especially two-dimensional (2D)-NMR techniques, these four metabolites have been identified as "AEM1" (*arte*ether *m*etabolite *1*) (2), the structure of which is very similar to that of a thermal rearrangement product of artemisinin (11), 3α-hydroxydeoxyarteether (3), a compound closely related to deoxyartemisinin which was obtained as a microbial and a mammalian metabolite of artemisinin (12–14), 3α-hydroxydeoxydihydroartemisinin (4), and deoxydihydroartemisinin (5), which is identical to the material prepared earlier by Brossi and co-workers (3). The isolation and structure elucidation of these metabolites are described herein.

MATERIALS AND METHODS

General Experimental Procedures

Melting points were determined either on a Fisher-digital melting point analyzer Model 355 or in open capillary tubes with a Thomas–Hoover capillary melting point apparatus and are uncorrected. Optical rotations were measured with a Perkin–Elmer 141 polarimeter. The ir spectra were recorded in KBr using a Perkin–Elmer 281B infrared spectrophotometer. The ^{1}H- and ^{13}C-NMR spectra 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, 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

The tlc chromatographic analyses were carried out on precoated Silica G-25 UV_{254} plates (Macherey-Nagel Düren). The adsorbent used for column chromatography was silica gel 60/70-270 mesh (Macherey-Nagel Düren). The developing system used for tlc was Et_2O–$CHCl_3$ (1:9) solu-

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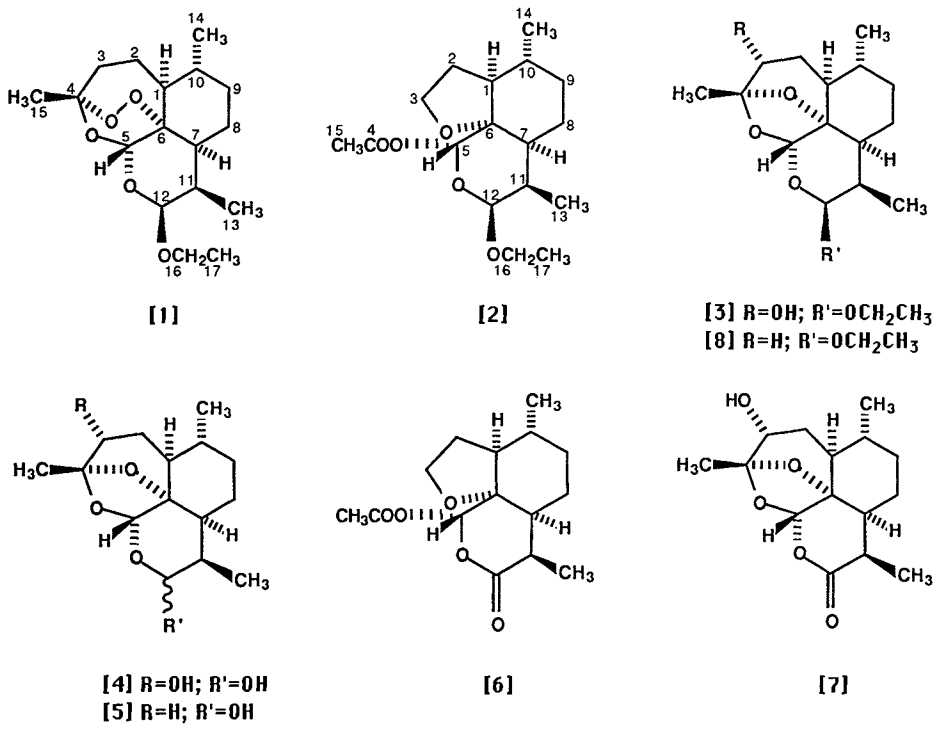

Fig. 1. Chemical structures of arteether and its analogues.

tion, 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 obtained 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 arteether that showed one or more metabolites by tlc are as follows: *Aspergillus niger* ATCC 10549, *Aspergillus parasiticus* ATCC 15517, *Cunninghamella echinulata* NRRL 3655, *Mucor mucedo* UI-4605, *Nocardia corallina* ATCC 19070, *Saccharomyces cerevisiae* ATCC 9763, *Sepedonium chrysospermum* ATCC 13378, *Streptomyces punipalus* UI-3529, *Streptomyces rimosus* ATCC 23955, and *Stysanus microsporus* 2833.

Media

All the preliminary screening and large-scale experiments were carried out in a medium consisting of the following composition: dextrose, 20 g; yeast extract, 5 g; peptone, 5 g; NaCl, 5 g; K_2HPO_4, 5 g; 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. The media were sterilized at 121°C and 18 psi for 15 min. Fermentations were carried out according to a standard two-stage protocol (20). In general, the substrate was prepared as a 10% solution in ethanol 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.

Arteether (1), used in this study, was prepared from dihydroartemisinin by etherification with ethyl alcohol in the presence of a Lewis acid and separated from its α isomer following the method described by Brossi *et al.* (3). Dihydroartemisinin was prepared by sodium borohydride reduction of artemisinin which was isolated from *Artemisia annua* L. grown in the Medicinal Plant Garden, School of Pharmacy, The University of Mississippi. The identity of artemisinin was established by direct comparison with an authentic sample supplied by Dr. A. Brossi of the National Institutes of Health, Bethesda, Maryland.

Microbial Metabolism of Arteether (1) by *Aspergillus niger*

Aspergillus niger (ATCC 10549) was grown in 24 1-liter culture flasks each containing 200 ml of medium. A total of 980 mg of arteether (in 9.8 ml EtOH) was evenly distributed among the 24-hr-old stage II cultures. After 15 days, the incubation mixtures were pooled and 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 (1051 mg).

Isolation and Characterization of AEM1 (2)

The residue (1051 mg) was purified by column chromatography over a silica gel column (100 *g*, 2.5 × 80 cm), using an Et_2O–$CHCl_3$ (4:1) mixture as an eluting system and 15-ml fractions were collected. Fractions 90–100, yielding a single spot with R_f = 0.69, were combined and evaporated to dryness to give 169 mg of pure **2** (17.2% yield). Crystallization from Et_2O/hexane gave colorless needles, mp 66–68°C, $[\alpha]_D^{25}$ = +92.0° (*c* = 0.030g/ml, $CHCl_3$); high-resolution eims (*m/z*) calcd for $C_{17}H_{28}O_5[M]^+$ 312.1935, found 312.1925; ir (KBr) ν_{max} (cm^{-1}), 2920, 1750, 1450, 1370, 1220, 1080, 1015, 970; ^{1}H-nmr, 0.89 (1H, m, H-9), 0.89 (3H, d, *J* = 7.2, Me-13), 0.93 (3H, d, *J* = 6.6, Me-14), 1.19 (3H, t, *J* = 7.1, Me-17), 1.32 (1H, m, H-1), 1.51 (1H, m, H-10), 1.63 (1H, ddd, *J* = 13.2, 4.8, 4.8, H-7), 1.81 (2H, m, H-2, H-8), 1.88 (1H, m, H-9), 1.95 (2H, m, H-2, H-8), 2.12 (3H, s, Me-15), 2.38 (1H, m, H-11), 3.38 (1H, dq, *J* = 9.8, 7.1, H-16), 3.91 (1H, ddd, *J* = 8.0, 8.0, 8.0, H-3), 3.92 (1H, dq, *J* = 9.8, 7.1, H-16), 4.26 (1H, ddd, *J* = 2.4, 8.0, 9.5, H-3), 4.73 (1H, d, *J* = 4.5, H-12), 6.27 (1H, s, H-5); ^{13}C-nmr (see Table I).

Isolation and Characterization of 3α-Hydroxydeoxyarteether (3)

Fractions 113–140 from the above column yielded 112 mg (11.4%) of metabolite (**3**) as an oily compound (one spot with R_f = 0.40). $[\alpha]_D^{25}$ = +13.2° (*c* = 0.035g/ml, $CHCl_3$); high-resolution eims (*m/z*) calcd for $C_{17}H_{28}O_5[M]^+$ 312.1935, found 312.1916; ir (neat) ν_{max} (cm^{-1}), 3450, 2920, 1450, 1380, 1270, 1120, 1080, 1020, 980; ^{1}H-nmr, 0.85 (3H, d, *J* = 6.6, Me-13), 0.90 (3H, d, *J* = 7.5, Me-14), 0.98 (1H, m, H-9), 1.16 (3H, t, *J* = 7.0, Me-17), 1.23 (1H, m, H-9), 1.38 (1H, ddd, *J* = 5.4, 10.8, 12.9, H-7), 1.54 (3H, s, Me-15), 1.60 (1H, m, H-2), 1.70 (1H, m, H-10), 1.76–1.82 (3H, m, H-2, H-8α, H-8β), 1.85 (1H, m, H-1), 2.41 (1H, m, H-11), 3.42 (1H, dq, *J* = 9.8, 7.0, H-16), 3.55 (1H, dd, *J* = 1.7, 4.2, H-3), 3.80 (1H, dq, *J* = 9.8, 7.0, H-16), 4.74 (1H, d, *J* = 4.2, H-12), 5.24 (1H, s, H-5); ^{13}C-nmr (see Table I).

Isolation and Characterization of 3α-Hydroxydeoxydihydroartemisinin (4)

From the above column, fractions 316–330 yielded 51 mg (5.20%) of pure metabolite (**4**) (single spot with R_f = 0.16). Recrystallization from Et_2O/hexane gave colorless needles, mp 144–145°C; $[\alpha]_D^{25}$ = −72.7° (*c* = 0.030 g/ml, $CHCl_3$); high-resolution eims (*m/z*) calcd for $C_{15}H_{24}O_5[M]^+$ 284.162, found 284.162; ir (KBr)ν_{max} (cm^{-1}), 3440, 2950, 1450, 1380, 1270, 1225, 1160, 1080, 1040, 975; ^{1}H-nmr, α-form (C-12) 0.88 (3H, d, *J* = 6.3, Me-14), 0.99 (3H, d, *J* = 7.3, Me-13), 1.57 (3H, s, Me-15), 2.32 (1H, m, H-11), 3.53 (1H, dd, *J* = 1.8, 4.0, H-3), 4.80 (1H, d, *J* = 6.8, H-12), 5.28 (1H, s, H-5), β-form (C-12) 0.87 (3H, d, *J* = 6.3, Me-14), 0.96 (3H, d, *J* = 7.5, Me-13), 1.54 (3H, s, me-15), 2.44 (1H, m, H-11), 3.55 (1H, dd, *J* = 1.8, 4.4, H-3), 5.27 (1H, d, *J* = 3.7, H-12), 5.32 (1H, s, H-5); ^{13}C-nmr (see Table I).

Microbial Metabolism of Arteether (1) by *Nocardia corallina*

A total of 280 mg of arteether (**1**) was dissolved in 2.8 ml

Table I. ^{13}C-NMR Chemical Shift Assignments for Compounds 1–8[a]

Carbon No.	Chemical shift assignment (ppm)							
	1[b]	2	3	4[c]	5[c,d]	6[d]	7[b]	8[d]
1	52.8 (1)	55.7 (1)	40.8 (1)	41.2 (1)[1]	45.6 (1)	54.8 (1)	40.5 (1)	46.8 (1)
2	24.8 (2)	27.8 (2)	30.4 (2)	30.3 (2)	22.0 (2)	27.7 (2)	30.3 (2)	22.2 (2)
3	36.6 (2)	68.6 (2)	69.6 (1)	69.6 (1)	34.2 (2)	69.2 (2)	69.0 (1)	34.9 (2)
4	104.0 (0)	169.3 (0)	107.8 (0)	108.1 (0)	108.1 (0)	168.5 (0)	108.9 (0)	107.9 (0)
5	87.9 (1)	88.4 (1)	93.7 (1)	95.4 (1)	96.1 (1)	93.0 (1)	99.0 (1)	94.7 (1)
6	81.2 (0)	80.6 (0)	84.2 (0)	83.1 (0)	82.4 (0)	79.4 (0)	82.9 (0)	83.4 (0)
7	44.7 (1)	47.1 (1)	42.5 (1)	41.5 (1)[1]	41.3 (1)	46.6 (1)	42.0 (1)	41.1 (1)
8	24.6 (2)	24.7 (2)	25.0 (2)	22.8 (2)	22.8 (2)	24.3 (2)	23.5 (2)	25.0 (2)
9	34.8 (2)	35.9 (2)	34.8 (2)	34.2 (2)	34.6 (2)	34.6 (2)	33.4 (2)	34.6 (2)
10	37.6 (1)	30.6 (1)	34.8 (1)	35.0 (1)	35.3 (1)	30.9 (1)	35.1 (1)	35.2 (1)
11	31.0 (1)	33.3 (1)	30.4 (1)	33.5 (1)	34.0 (1)	35.0 (1)	32.7 (1)	30.6 (1)
12	101.7 (1)	101.7 (1)	99.8 (1)	96.6 (1)	97.0 (1)	171.7 (0)	171.5 (0)	99.3 (1)
13	13.1 (3)	12.5 (3)	12.3 (3)	14.6 (3)	15.0 (3)	12.5 (3)	12.6 (3)	12.2 (3)
14	20.4 (3)	20.5 (3)	18.8 (3)	18.6 (3)	18.8 (3)	20.4 (3)	18.4 (3)	19.0 (3)
15	26.3 (3)	21.6 (3)	21.0 (3)	20.8 (3)	24.4 (3)	21.2 (3)	20.5 (3)	24.5 (3)
16	63.8 (2)	63.7 (2)	64.0 (2)					63.9 (2)
17	15.3 (3)	15.0 (3)	15.2 (3)					15.2 (3)

[a] The number in parentheses indicates the number of hydrogens attached to the corresponding carbon and was determined from DEPT experiments. 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. Assignments bearing the same numerical superscript may be reversed.

[b] These data have been reported previously (2,12) and are listed here for comparison purposes.

[c] For compounds 4 and 5, chemical shift assignments are shown for major isomers only.

[d] These compounds have been reported previously (3,11) but without ^{13}C-nmr assignments. A sample of 6 was prepared as described in the literature (11), whereas 8 was obtained as an authentic sample.

of EtOH and distributed equally among seven, 1-liter culture flasks each containing 200 ml of 24-hr-old, *Nocardia corallina* (ATCC 19070) stage II culture. After 15 days, the entire incubation mixture was combined and filtered, and the cells and fermentation solids were washed with water. The combined aqueous filtrate (1.4 liters) was extracted three times with EtOAc (1 × 1.4 liters, and 2 × 0.7 liters). The organic layer was dried over anhydrous Na_2SO_4 and filtered, and the solvent was evaporated under reduced pressure to afford 380 mg of dark brown residue. The residue was purified on a silica gel column (35*g*, 2.5 × 30 cm) using Et_2O–hexane(4:1) as an eluent, and 20-ml fractions were collected. The tlc of fractions 62–64 showed a single spot, and these fractions were combined and evaporated to dryness to give 14.1 mg of pure metabolite (2) (5.0% yield). Fractions 65–73 were combined to give 18 mg of 3 (6.4%).

Isolation and Characterization of Deoxydihydroartemisinin (5)

The tlc of fractions 75–80 from the above column showed a single spot with R_f = 0.24. These fractions were combined and evaporated to dryness to give 6.3 mg of 5. Recrystallization from Et_2O/hexane afforded colorless needles of deoxydihydroartemisinin (2.8 mg, 1%), which was directly compared with an authentic sample of deoxydihydroartemisinin (mp, mmp, tlc, ir, ^{1}H-nmr) (3); ^{13}C-nmr (see Table I).

RESULTS AND DISCUSSION

Screening-scale studies of arteether have shown that a number of fungi are capable of metabolizing this sesquiterpene to a number of metabolites. Of the 10 microorganisms screened, *Aspergillus niger* (ATCC 10549) and *Nocardia corallina* (ATCC 19070) were selected for preparative scale fermentations.

A preparative-scale fermentation was performed with *Aspergillus niger* (ATCC 10549) using arteether (1) as a substrate, and compounds 2–4 were isolated and purified as major metabolites. The mass spectrum of 2 indicated that the molecular weight (*m/z* 312; M^+) was the same as that of 1. The ir spectrum showed a strong absorption at 1750 cm^{-1}, which suggested the presence of either an ester or six-membered ring lactone. The ^{13}C-nmr spectrum showed 17 carbon resonances, including a carbonyl (δ 169.3); the ethyl ether linkage as evidenced by the peaks at δ 101.7 (C-12), δ 63.7 (C-16), and δ 15.0 (C-17), had two carbons attached to one oxygen (δ 68.6 and 80.6, CH_2 and quaternary, respectively), and one carbon attached to two oxygens (δ 88.4, CH). The ^{1}H-nmr spectrum showed two proton resonances at δ 3.91 and δ 4.26 which were characteristic of the α protons of a tetrahydrofuran ring and that ethyl ether linkage of arteether was retained (δ 3.38 and 3.92). A check of the literature revealed that compound 6 prepared from artemisinin had almost identical properties (11). The structure of this product was established by X ray. A sample of 6 was prepared according to the literature procedure. Compound 2 had ^{13}C-nmr data nearly identical to those of 6 (see Table I).

The mass spectrum of compound 3 showed the molecular weight to be the same as that of arteether (*m/z* 312; M^+). The ir spectrum showed a strong absorption at 3450 cm^{-1}. Comparison of the ^{13}C-nmr spectral data of 1 and metabolite 3 indicated that there was no cleavage in ethyl ether linkage and that there was a carbon signal at δ 69.6. DEPTGL data proved that the carbon at δ 69.6 had one attached proton which strongly indicated the presence of a carbon atom directly attached to a hydroxyl group. In the ^{1}H-nmr, D_2O treatment proved the presence of one exchangeable hydroxyl group. ^{13}C-nmr spectral analyses supported C-3 as the most likely position of hydroxylation (see Table I; cf. 3, 4, 7). The stereochemistry of the hydroxyl group was established as alpha (α) from the ^{1}H-nmr data (H-3, dd, two small *J*'s). All the spectroscopic data of 3, compared with those of 3α-hydroxydeoxyartemisinin (7), previously obtained as a microbial metabolite of artemisinin (12), suggested that metabolite 3 was 3α-hydroxydeoxyarteether.

The ^{13}C-nmr spectrum of compound 4 showed 15 carbon atoms, suggesting the cleavage of ethyl ether linkage. It also showed a new doublet at δ 69.6 (CH attached to secondary OH). The ir spectrum showed a strong absorption at 3440 cm^{-1}, indicating the presence of a hydroxyl group. Comparison of the spectral data of 4 with those of 3 and 5 led to the conclusion that metabolite 4 was a hydroxylated derivative of deoxyarteether with the ethyl ether linkage cleaved. ^{13}C-nmr spectral comparison of 4 and 1 again supported C-3 as the most likely position of hydroxylation. In the same manner as with 3, the stereochemistry of the hydroxyl group was established as alpha (α) from the ^{1}H-nmr data. All of the evidence suggested that metabolite 4 was 3α-hydroxydeoxydihydroartemisinin. The nmr spectra clearly indicated that this compound (4) was a mixture of α and β isomers (C-12).

A preparative-scale fermentation of arteether with *Nocardia corallina* (ATCC 19070) led to the isolation and purification of three compounds, two of which proved to be the same as the above-mentioned metabolites 2 and 3. The ^{13}C-nmr spectrum of compound 5 indicated 15 carbon atoms and cleavage of ethyl ether linkage of arteether. The ^{13}C-nmr data also suggested that it was related to the deoxy series. A comparison of the mp and the ^{1}H-nmr spectral data of 5 with those of deoxydihydroartemisinin as reported in the literature (3) indicated that the two were identical. A direct comparison of this metabolite with an authentic sample of deoxydihydroartemisinin (mp, mmp, tlc, ir, ^{1}H, ^{13}C-nmr) confirmed the identity of 5 as deoxydihydroartemisinin. The ^{1}H-nmr spectral data indicated that compound 5 exists as a mixture of α and β isomers (C-12).

These four microbial metabolites (2–5) have been provided as reference standards for metabolism studies in rat liver microsomes, the results of which have been published (21). Compounds 3–5 were also isolated as *in vitro* metabolites of the rat liver microsomes, but 2 was not. In addition, deoxyarteether 8 was not found either as a microbial metabolite or as an *in vitro* metabolic product using the rat liver microsomes. Dihydroartemisinin, the major metabolite in the rat liver microsome (21), was not detected in any of our microbial cultures. Further microbial and mammalian metabolism studies on arteether (1) are in progress.

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