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1α-HYDROXYARTEETHER, A NEW MICROBIAL TRANSFORMATION PRODUCT

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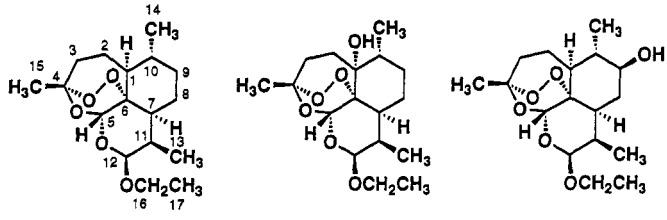
ABSTRACT.—The antimalarial drug arteether [**1**] was subjected to microbial metabolism using *Cunninghamella elegans* (ATCC 9245) and *Streptomyces lavendulae* (L-105). 1α-Hydroxyarteether [**2**], a novel microbial metabolite of **1** was isolated from the cultures of *C. elegans* and fully characterized. The stereochemistry of **2** was established by X-ray crystallographic analysis. 9β-Hydroxyarteether [**3**] was isolated from the same culture in a much higher yield (40%) than previously reported (2.2%). Compound **6**, a known metabolite of **1**, was isolated from the fermentation cultures of *S. lavendulae* for the first time.

Arteether [**1**] is the ethyl ether derivative of dihydroartemisinin, a $NaBH_4$ reduction product of artemisinin. Artemisinin is the active antimalarial constituent of the Chinese medicinal herb *Artemisia annua* L. (1). Arteether has been proven to be useful for high-risk malaria patients including those with cerebral malaria. The ^1H- and ^{13}C-nmr assignments of **1** have been reported (2), and a report on the synthesis and antimalarial properties of **1** has been published (3). Metabolism studies have been conducted in man, mammals, and by microorganisms (4–6). Microbial production represents the only source of these eleven metabolites. In order to provide quantities of these metabolites for biological evaluation and as standards for metabolism studies, the World Health Organization has funded a project to prepare the 11 known metabolites of **1**. In the course of



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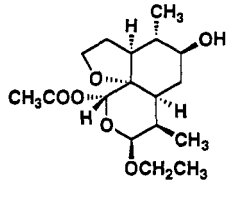

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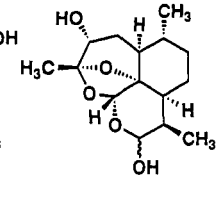

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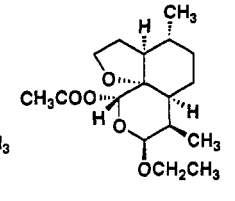

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making these metabolites, a new metabolite [**2**] was discovered, the yield of metabolite **3** was significantly increased, and metabolite **6** was isolated from *Streptomyces lavendulae* for the first time.

A preparative-scale fermentation was performed with *Cunninghamella elegans* (ATCC 9245) using arteether [**1**] as a substrate. In a previous report, fermentations with this organism were left to proceed for thirteen days (4). In the present work, the fermentation flasks were sampled after six days. Tlc analysis revealed that all the **1** was metabolized and that there were two major metabolites, one of which appeared to be new, while the other had an R_f value corresponding to the previously reported 9β-hydroxyarteether [**3**] (4).

Metabolite **2**, $C_{17}H_{28}O_6$, had one additional alcohol oxygen present when compared with **1** as determined by ir and ^{1}H-nmr (D_2O exchange) spectra. The ^{1}H- and ^{13}C-nmr spectral data of **2** showed that the alcohol was tertiary 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 (2), a comparison of ^{13}C-nmr data of **2** with those of **1** showed that, of the six possibilities (C-1, -5, -7, -10, -11, or -12), the hydroxyl group must be at C-1 (see Table 1). Especially noteworthy are downfield shifts for C-2 and C-10, and upfield shifts for C-3, C-7, and C-14. All of the ^{1}H- and ^{13}C-nmr assignments were verified by ^{1}H-^{1}H and ^{1}H-^{13}C shift-correlated 2D nmr spectroscopy. The stereochemistry of the hydroxyl group at C-1 was determined by X-ray crystallography. An ORTEP diagram of metabolite **2** (1α-hydroxyarteether) is presented in Figure 1.

Metabolite **3**, 9β-hydroxyarteether, is a known metabolite of arteether. *Cunninghamella elegans* (ATCC 9245) has been reported to produce **3** in 2.2% yield after thirteen days of fermentation (4). The production of metabolite **3** in 40% yield was achieved by stopping the fermentation process after six days. When the fermentation was allowed to run for a full 13 days, two additional metabolites were isolated, metabolites **4** and **5**. It appears that when the fermentation is stopped after six days that metabolite **3**, the likely precursor of metabolite **4**, is not allowed to be further metabolized to produce **4**. Although metabolites **4** and **5** were detected by tlc, they were not isolated because of the small amounts present. The identity of metabolite **3** was determined using mp, mixed mp, tlc, co-tlc, and nmr data.

A preparative-scale fermentation using *Streptomyces lavendulae* (L-105) led to the isolation of 9α-hydroxyarteether, 2α-hydroxyarteether, and 14-hydroxyarteether as previously reported (4). An additional metabolite [**6**] was isolated

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

Carbon	Compound	
	1[b]	**2**
C-1	52.8 (1)	76.0 (0)
C-2	24.8 (2)	28.8 (2)
C-3	36.6 (2)	33.8 (2)
C-4	104.0 (0)	103.7 (0)
C-5	87.9 (1)	87.2 (1)
C-6	81.2 (0)	82.3 (0)
C-7	44.7 (1)	39.9 (1)
C-8	24.6 (2)	23.8 (2)
C-9	34.8 (2)	33.3 (2)
C-10	37.6 (1)	40.7 (1)
C-11	31.0 (1)	31.0 (1)
C-12	101.7 (1)	102.1 (1)
C-13	13.1 (3)	13.0 (3)
C-14	20.4 (3)	15.3 (3)
C-15	26.3 (3)	26.0 (3)
C-16	63.8 (2)	63.9 (2)
C-17	15.3 (3)	15.2 (3)

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

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

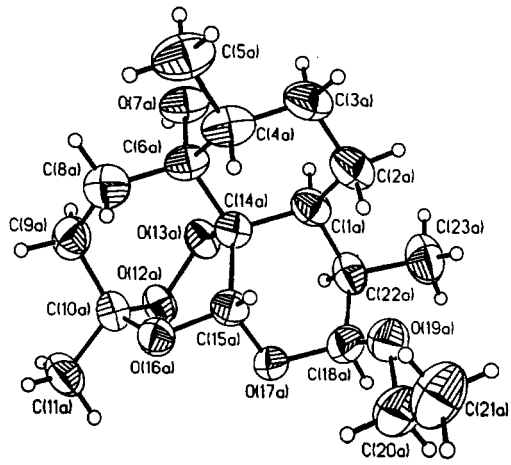

FIGURE 1. ORTEP diagram for **2**.

from the fermentation broth of *S. lavendulae* for the first time. Metabolite **6** was previously reported as a microbial metabolite of *Aspergillus niger* (ATCC 10549) (5). The identity of metabolite **6** was determined using mp, mixed mp, tlc, co-tlc, and nmr data.

In summary, this study reports on the isolation and characterization of a novel microbial metabolite of **1**, 1α-hydroxyarteether [**2**], which retains the endoperoxide moiety, a feature essential for the antimalarial activity (7). The known metabolite, 9β-hydroxyarteether [**3**], was also isolated but in a much greater yield than previously reported (4). This metabolite [**3**] is of special value due to its antimalarial activity, which was found to be comparable to **1** (7). Metabolite **6** was isolated from a different organism, *Streptomyces lavendulae*, for the first time.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Mps were determined in open capillary tubes using a Thomas-Hoover capillary melting-point apparatus and are uncorrected. Ir spectra were recorded in KBr using a Perkin-Elmer 281 B infrared spectrophotometer and specific rotations were obtained on a Jasco digital polarimeter model DIP-370. 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 referenced to TMS, and the coupling constants are in Hz. Standard pulse sequences were used for COSY, HETCOR, DEPTGL, and APT nmr experiments. Low-resolution ms were obtained using lc-ms (Vestec Model 201 thermospray mass spectroscopy system). Hrfabms (Fisons/VG Autospec Q) was carried out at the University of Kansas. Arteether [**1**] used in this study was provided by the World Health Organization. The tlc chromatographic analyses were carried out on precoated Silica G-25 UV_{254} plates (Macherey-Nagel Duren). The adsorbent used for cc was Si gel 60/230–400 mesh (EM Science). The visualization of the tlc plates was performed using anisaldehyde-H_2SO_4 spray reagent (8).

MICROORGANISMS.—The cultures *C. elegans* (ATCC 9245) and *S. lavendulae* (L-105) 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.

MEDIA.—All the fermentation experiments were carried out in a medium of the following composition: 20 g dextrose, 5 g yeast extract, 5 g peptone, 5 g NaCl, 5 g K_2HPO_4, and 1000 ml distilled H_2O. Stock cultures of fungi were stored on slants of Mycophil (BBL, Cockeysville, MD) and medium #5 (Difco, Detroit, MI) at 4°.

FERMENTATION PROCEDURES.—Microbial metabolism studies were carried out by incubating the cultures with shaking on the model G-10 Gyrotory shaker (New Brunswick Scientific Co., NJ), operating at 250 rpm, at 25°. Fermentations were carried out according to a standard two-stage protocol (9). In general, the substrate was prepared as a 10% solution in EtOH and added to the 24-h-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.

MICROBIAL METABOLISM OF ARTEETHER [**1**] BY *CUNNINGHAMELLA ELEGANS*.—*Cunninghamella elegans* (ATCC 9245) was grown in 12 2-liter culture flasks each containing 400 ml of medium. A total of 960 mg of arteether [**1**] (in 9.6 ml of EtOH) was evenly distributed among the 24-h-old stage II cultures. After 6 days, the incubation mixtures were checked by tlc. Tlc revealed that all the **1** was transformed, and two major metabolites were produced. The incubation mixtures were combined and filtered to remove the cells, and the filtrate (4.8 liters) was extracted three times with EtOAc (4.8 liters each). The combined extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure to afford a dark-brown residue (1.438 g).

ISOLATION AND CHARACTERIZATION OF 1α-HYDROXYARTEETHER [**2**].—The residue (1.438 g) was purified by cc over a Si gel column (5×2 in.) using Et_2O-hexane (6:4) mixture as an eluent, and 10-ml fractions were collected. Fractions 16–36 yielded a new metabolite with R_f 0.8 (tlc system, Et_2O-hexane, 7:3). These fractions were combined and evaporated to dryness to give 35 mg of **2** (3.6% yield). Crystallization from hexane gave opaque prisms: mp 128°; $[\alpha]^{25}D$ +1.8° (c=0.022, MeOH); ir (KBr) ν max 3480 cm^{-1}; lc-ms $[M+18]^+$ 346 (consistent with the formula $C_{17}H_{28}O_6+NH_4^+$); hrfabms $[M+1]^+$ 329.1942 (consistent with the formula $C_{17}H_{28}O_6+H^+$, calcd 329.1964); ^1H nmr ($CDCl_3$) δ 5.53 (1H, s, H-5), 4.88 (1H, d, J=3.6 Hz, H-12), 3.94 (1H, dq, J=9.8 and 7.1 Hz, H-16), 3.54 (1H, dq, J=9.8 and 7.1 Hz, H-16), 2.58 (1H, m, H-11), 2.42 (1H, ddd, J=8.0, 9.7, and 15.2 Hz, H-3α), 2.08 (1H, ddd, J=4.0, 4.0, and 14.9 Hz, H-3β), 1.55–1.94 (6H, m, H-7, H-8α, H-8β, H-9α, H-9β, H-10), 1.53 (3H, s, Me-15), 1.42 (2H, m, H-2α, H-2β), 1.26 (3H, t, J=7.0 Hz, Me-17), 1.09 (3H, d, J=6.4 Hz, Me-14), 0.97 (3H, d, J=7.4 Hz, Me-13); ^{13}C-nmr data, see Table 1.

ISOLATION AND CHARACTERIZATION OF 9β-HYDROXYARTEETHER [**3**].—Fractions 37–65 yielded metabolite **3** with R_f 0.53 (tlc system, Et_2O-hexane, 7:3) and were combined, and evaporated to dryness to give 380 mg of **3** (40% yield). Crystallization from hexane/Et_2O gave white prisms: mp 124–126°. The isolated metabolite [**3**] was compared to a standard sample of 9β-hydroxyarteether from our laboratory. Mp, mmp, tlc, co-tlc, nmr, and thermospray lc/ms analyses were identical to those of the standard (4).

MICROBIAL METABOLISM OF ARTEETHER [**1**] BY *STREPTOMYCES LAVENDULAE*.—*Streptomyces lavendulae* (L-105) was grown in 12 2-liter culture flasks, each containing 400 ml of medium. A total of 960 mg of arteether [**1**] (in 9.6 ml of EtOH) was evenly distributed among the 24-h-old stage II cultures. After 14 days, the incubation mixtures were combined and filtered to remove the cells, and the filtrate (4.8 liters) was extracted three times with EtOAc (4.8 liters each). The combined extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure to afford a dark-brown residue (1.466 g).

ISOLATION AND CHARACTERIZATION OF **6**.—The residue (1.466 g) was purified by cc over a Si gel column (150, 2.5×21 in.), using EtOAc-toluene (1:20) mixture as an eluent to give metabolite **6** with R_f 0.29. Fractions containing metabolite **6** were combined and evaporated to dryness to give 18 mg of **6** (1.9% yield). Crystallization from hexane afforded colorless needles: mp 66–68°. The identity and purity of metabolite **6** were confirmed by direct comparison with an authentic standard from our laboratory. Mp, mmp, tlc, co-tlc, nmr, and thermospray lc/ms analyses were identical to those of the standard (5). A total of 102 mg of **1** was also recovered.

X-RAY CRYSTAL STRUCTURE ANALYSIS OF 1α-HYDROXYARTEETHER [**2**].[1]—Crystal data: $C_{17}H_{28}O_6$, mol wt 328, triclinic space group P1, a=10.734 (2), b=11.602 (2), c=15.142 (3) Å, α=90.27 (3), β=109.41 (3), γ=98.25 (3), Z=4, Dc=1.241 $g\cdot cm^{-3}$, μ(CuKα radiation, λ=1.54178 Å)=0.767 mm^{-1}. Crystal dimensions 0.20×0.25×0.30 mm, 4616 reflections were collected and used in the refinement based on F^2. The final R value based on the $I>2\sigma$ (I) data was 0.039 and 0.039 and ωR=0.101 based on all data (10).

Structure solution was achieved in the unusual space group P1 with four independent molecules. The four molecules are nearly identical and exhibit an average r.m.s. fix of 0.12Å (based on fitting the C and O atoms only).

ACKNOWLEDGMENTS

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[1]Hydrogen coordinates, thermal parameters, bond distances and angles, and observed and calculated structure factors have been deposited with the Cambridge Crystallographic Data Centre and can be obtained upon request from Dr. Olga Kennard, University Chemical Laboratory, 12 Union Road, Cambridge CB2 1EZ, UK.

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