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MICROBIOLOGICAL TRANSFORMATIONS OF ARTEMISINIC ACID

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Abstract—The microbial transformation of the sesquiterpene artemisinic acid **(3)** using *Mucor mucedo* and *Aspergillus flavipes* has resulted in the production of epimeric 3-hydroxyartemisinic acids **(4)** and **(5)**. The structure elucidation and full ^{1}H and ^{13}C NMR assignments for these novel metabolites were made on the basis of spectroscopic data, especially 2D NMR techniques.

INTRODUCTION

In recent years there has been intense interest in artemisinin **(1)**, a novel sesquiterpene peroxide obtained from *Artemisia annua*, because of its considerable promise for the treatment of drug resistant malaria [1, 2]. During the course of ongoing studies conducted to obtain large quantities of **(1)** two other sesquiterpenes, arteannuin B **(2)** and artemisinic acid **(3)**, have also been isolated in large amounts. The isolation and characterization of artemisinic acid **(3)** was first reported in 1982 by Liang *et al.* [3]. Recently, it was reported that *A. annua* contained *ca* eight to ten times more artemisinic acid [3] than artemisinin [1] [4]. Recent studies have also suggested that **(3)** is a possible biogenetic precursor of artemisinin **(1)** [5]. The usefulness of utilizing the microbial transformation techniques to carry out a variety of chemical conversions is well documented in the literature [6, 7]. The microbial transformations of **3** were thus undertaken in the hope of producing metabolites that might be similar in nature to the intermediates implicated in the biosynthetic pathway toward artemisinin **(1)** or also providing key intermediates that could be utilized in the current synthetic efforts aimed at converting **3**, stereospecifically, into **1** [4]. In the present study, the isolation and structure elucidation of two biotransformation products of artemisinic acid **(3)** are reported.

RESULTS AND DISCUSSION

Thirty cultures were initially examined by TLC for their ability to biotransform **3**. Several organisms converted **3** to more polar metabolites. Of these, *Mucor mucedo* (UI 4605) and *Aspergillus flavipes* (ATCC 16795) were chosen for preparative scale fermentations. *Mucor mucedo* was capable of converting **3** into one major optically active metabolite **4**. The MS of **4** showed a molecular ion which was 16 mass units higher than that of **3**. The elemental analysis data were consistent with a molecular formula of $C_{15}H_{22}O_3$ for **4**, suggesting that metabolite **4** was a monohydroxylated derivative of **3**. Absorption at 3597 cm^{-1} in the IR spectrum of **4** confirmed that the metabolite was an alcohol. To determine the position of the OH group in **4**, it was necessary to make all ^{13}C NMR assignments of artemisinic acid **3**. Even though some of the proton assignments have been made [3], none of the carbon assignments have been reported. The complete carbon assignments were confirmed by a combination of ^{1}H–^{1}H (COSY) and ^{1}H–^{13}C (HETCOR) shift correlated 2D NMR experiments and are listed in Table 1. The metabolite clearly was a monohydroxylated secondary alcohol (δC 68.1 *d*, δ ^{1}H 3.95 *d*) which confirms that hydroxylation had occurred on one of the four methylene carbons. Since C-8 and C-9 resonances did not undergo shifts (Table 1), the hydroxylation must have occurred at C-2 or C-3. Hydroxylation at C-3 was favoured because of the downfield shifts of C-2 and the upfield shifts for C-15 and C-1. An OH at C-2 would be expected to shift C-1 downfield by 5-10 ppm. The COSY spectrum confirmed this placement by showing fairly strong cross peaks between H-3 and both H-5 and Me-15. The remaining proton assignments (Experimental) and carbon assignments (Table 1) which were made from COSY and HETCOR experiments are in total agreement with structure **(4)**. The assignment of the OH as β follows after discussion of the second metabolite.

Preparative-scale incubation of **3** with *A. flavipes* gave a fermentation product which was also found to consist only of one major metabolite **5**. Metabolite **5** also showed a molecular ion at *m/z* 250, again consistent with a $C_{15}H_{22}O_3$ formula, however, it had a lower R_f than that of **(4)**. In general, the spectral features of **5** were analogous to that of **4** (Experimental, Table 1). As in **4**, the ^{13}C NMR data for **5** also showed the presence of a new secondary alcohol (δC 68.5 *d*, δ ^{1}H 4.08 *br t*) and confirms that hydroxylation had occurred at C-2 or C-3 since C-8 and C-9 resonances did not undergo any shifts. The presence of cross peaks correlating H-5 and the proton at δ4.08 (H-OH) provides evidence which also places the OH group at C-3 thus making the two metabolites

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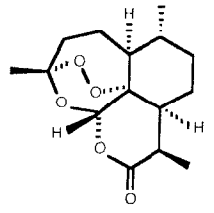
Table 1. ^{13}C NMR spectral data*† of artemisinic acid and metabolites

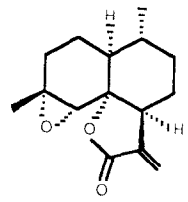
C	3	4	5
1	41.7 (1)	40.8 (1)	44.3 (1)
2	25.8 (2)	34.6 (2)	36.6 (2)
3	26.6 (2)	68.1 (1)	68.5 (1)
4	134.8 (0)	135.4 (0)	136.5 (0)
5	120.2 (1)	120.0 (1)	123.9 (1)
6	38.1 (1)	38.8 (1)	38.5 (1)
7	42.3 (1)	42.1 (1)	42.1 (1)
8	26.2 (2)	26.3 (2)	26.1 (2)
9	35.5 (2)	35.9 (2)	35.3 (2)
10	27.8 (1)	29.5 (1)	28.2 (1)
11	142.8 (0)	142.4 (0)	142.6 (0)
12	172.8 (0)	171.2 (0)	171.1 (0)
13	126.2 (2)	126.5 (2)	125.9 (2)
14	19.9 (3)	20.0 (3)	19.7 (3)
15	23.7 (3)	20.6 (3)	19.3 (3)

*The number between paranthesis indicates the number of hydrogens attached to the corresponding carbon, and was determined from DEPT experiments.

†Assignments are based on ^1H–^1H and ^1H–^{13}C shift correlated 2D NMR spectroscopy.



1



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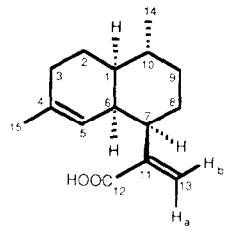

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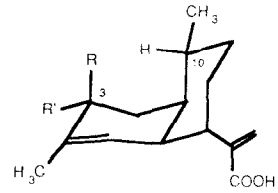

4 R = OH; R' = H

5 R = H; R' = OH

epimeric at C-3. Since the shift data and the cross peaks for H-3 and H-5 for **5** were not as definitive as for **4**, some additional selective low power heteronuclear decoupling experiments were also performed on **5**. These experiments demonstrated conclusively that the proton on the carbon containing the OH group was coupled to C-5 by long range coupling (three bond). Also, H-5 was shown to be coupled to the hydroxylated carbon. These experiments conclusively prove that the OH must also be at C-3. Complete ^1H and ^{13}C NMR assignments on **5** were also achieved from COSY and HETCOR experiments.

The only remaining question regarding the structures of the metabolites was the assignment of the stereochemistry of the OH groups in **4** and **5**. The coupling patterns for H-3 were different (**4**, *d*, J = 4.5 Hz; **5**, *br t*, *J* = 2.5 Hz), as would be expected, for epimers. Since the ring system was very flexible, it made the assignments from the dihedral angles ambiguous. To resolve this a 2D NOE experiment (NOESY) was conducted on each epimer. Both **4** and **5** showed NOE of the signals for H-5 and H-13b. Also, both showed enhancement of H-13b and H-8. This should suggest that both epimers adopt similar conformations. The major differences in the NOESY spectrum of **4** and **5** occur with the enhancements of H-3. Epimer **5** shows a strong enhancement of the signal at H-10 and a weaker one with Me-14 while epimer **4** does not show these enhancements. Both **4** and **5** showed the expected enhancements for H-2 and Me-15. These data can only be accounted for if one assigns the H at C-3 in **5** as β(αOH) which then lies in close proximity to both H-10 and Me-14. In this conformation, the coupling pattern for **5** is entirely consistent with the dihedral angles. In no other conformations can one get the other epimer's H-3α close to either H-10 or Me-14. Thus, the two metabolite structures have been shown to be epimeric with the OH assigned the β configuration in **4** and the α configuration in **5**.

EXPERIMENTAL

General. Mps: uncorr. ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Varian VXR-300 FT spectrometer operating at 300 and 75 MHz, respectively. The chemical shift values are reported in (ppm) units and the coupling constants are in Hz. Standard pulse sequences were used for homonuclear correlated spectroscopy (COSY) [8], heteronuclear correlated spectroscopy (HETCOR) [9], distorsionless enhancement by polarization transfer (DEPTGL) [10]. The phase sensitive 2D NOE (NOESY) [11] was carried out by degassing the sample prior to measuring the NOE's and a mix time of 0.8 sec was used. The proton coupled ^{13}C NMR spectrum of 5 was determined by gating the decoupler off during data acquisition. This spectrum was then compared with data obtained from the selective low power decoupling experiments ($\gamma H_2/2\pi = 200$ Hz). The experiments involved irradiation at H-3 and observing the C-5 signal and *vice versa*. Low resolution EIMS were obtained using 70 eV ionization potential. Microanalyses were performed by Scandinavian Microanalytical Laboratories, Herlev, Denmark.

Chromatography. TLC chromatographic analyses were carried out on pre-coated silica gel G-25 UV_{254} plates (0.25 mm). The adsorbent used for column chromatography was silica gel 60/70–270 mesh. Visualization of the TLC plates was done using anisaldehyde–H_2SO_4 spray reagent [12].

Organisms. The following cultures were obtained from the University of Mississippi, Department of Pharmacognosy Culture Collection and were originally from the American Type Culture Collection (ATCC), Rockville, MD or from Northern Regional Research Laboratories (NRRL), Peoria, IL, unless otherwise stated. Metabolite production denoted by (+) indicates one or more metabolites were produced, as shown by TLC. (1) *Cunninghamella echinulata* (NRRL 3655) (+); (2) *Aspergillus niger* (ATCC 16888) (+); (3) *Aspergillus flavipes* (ATCC 16795) (+); (4) *Cunninghamella blakesleeana* (ATCC 8688a) (−); (5) *Mucor mucedo* (UI 4605) (+); (6) *Streptomyces roseochromogenus* (ATCC 13400) (−); (7) *Aspergillus flavus* (ATCC 9170) (−); (8) *Chaetomium cochloides* (ATCC 10195) (−); (9) *Beauveria bassiana* (ATCC 7159) (+); (10) *Calonetria decora* (ATCC 14767) (+); (11) *Cladosporium resinae* (ATCC 22712) (+); (12) *Nocardia*

corallina (ATCC 19070 (−); (13) *Rhodotrorula rubra* (ATCC 20129) (+); (14) *Sepedonium chrysospermum* (ATCC 13378) (−); (15) *Sporobolomyces pararoseus* (ATCC 11386) (−); (16) *Stysanus microsporus* (2833) (−); (17) *Aspergillus flavipes* (ATCC 1030) (+); (18) *Aspergillus flavipes* (ATCC 11013) (+); (19) *Aspergillus flavus* (NRRL 501) (−); (20) *Aspergillus flavus* (ATCC 24741) (−); (21) *Aspergillus niger* (ATCC 10549) (+); (22) *Aspergillus niger* (ATCC 10581) (+); (23) *Aspergillus parasiticus* (ATCC 15517) (+); (24) *Cunninghamella elegans* (ATCC 9245) (+); (25) *Nocardia minima* L(ATCC 19150) (−); (26) *Nocardia petroleophila* (ATCC 15777) (−); (27) *Penicillium chrysogenum* (ATCC 9480) (+); (28) *Saccharomyces cerevisiae* (ATCC 9763) (−); (29) *Streptomyces lavandulae* (L-105) (−); (30) *Streptomyces punipalus* (UI, NRRL 3529) (−); (31) *Streptomyces rimosus* (ATCC 23955) (−).

Media. All the preliminary screening and transformation experiments were carried out in a medium consisting of (per l of H_2O) Dextrose, 20 g, and 5 g each of yeast extract, peptone, NaCl, and K_2HPO_4.

Stock cultures of fungi and bacteria were stored on slants of Mycophil and Eugon agar, respectively, at 4°.

Fermentation procedures. Microbial transformation studies were carried out by placing the cultures on rotary shakers, model G-10 gyrotory shaker operating at 250 rpm at 24–26°. Preliminary screening were carried out in 125 ml stainless steel-capped Delong culture flasks containing 25 ml of medium. The media were sterilized at 121° and 18 psi for 15 min. A standard two-stage fermentation protocol was employed in all experiments as described previously [13]. In general, the substrate was added to the incubation media 24 hr after the inoculation of stage II cultures, as a 10% soln in EtOH at a concn of 0.2 mg/ml of stage II medium.

Substrate controls were composed of sterile media to which the substrate was added and incubated without microorganisms. Culture controls consisted of fermentation blanks, in which the organisms were grown under identical conditions, but without adding the substrate.

Artemisinic acid (**3**) used in this study was isolated from locally grown *A. annua* and possessed physical and spectral data consistent with those reported in the literature [3].

Microbial transformation of artemisinic acid by Mucor mucedo (UI 4605). *Mucor mucedo* (UI 4605) was grown in 151 conical flasks each containing 200 ml of medium. A total of 600 mg of (**3**) (in 6 ml EtOH) were evenly distributed among the cultures. After 11 days, the incubation mixtures were pooled, filtered (3l), and the filtrate extracted 3 times with EtOAc (312 × 1.5l). The extracts were dried over dry Na_2SO_4 and evapd (*in vacuo*) to afford 1.101 g of a dark yellowish brown residue.

The residue (1 g) was applied to a silica gel column (3 × 72 cm, 100 g) and eluted with ether–hexane (4:3), and 15 ml fractions were collected. Fractions (30–100) contained a single spot (R_f 0.36) on silica gel plates developed in ether–hexane (4:3), (R_f of **3** = 0.73). These fractions were combined and evapd to dryness to given 261 mg of pure **4** (43.5%)

Recrystallization from ether-hexane afforded clusters of colorless crystals **4**), mp 156–157°; $[\alpha]_D$ +4.5° ($CHCl_3$; *c*0.45); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3597 (-OH), 3000, 2917, 2845, 1690 (>C = O), 1623, 1435, 1375, 1285, 1255, 1145, 1125, 1105, 1015, 955; ^1H NMR: 0.98 (3H, *d*, *J* = 6.0 Hz, Me-14), 1.09 (1H, *m*, H-9), 1.35–1.55 (3H, *m*, H-1, H-8) 1.71–1.79 (6H, *br m*, Me-15, H-2, H-9, H-10), 2.25 (1H, *dd*, *J* = 2.4, 14.0, H-2), 2.53 (1H, *br s*, H-6), 2.74 (1H, *m*, H-7), 3.95 (1H, *d*, *J* = 4.5, H-3), 5.16 (1H, *s*, H-5), 5.61 (1H, *s*, H-13_b), 6.49 (1H, *s*, H-13_a), 6.50 [2H, *br* COOH, OH (Disappeared after D_2O)]; ^{13}C NMR (Table 1); EIMS *m/z* (rel. int.) 250 (M^+) (9.3). (analysis: Found: C, 71.74 H, 8.84. $C_{15}H_{22}O_3$ requires: C, 71.96, H, 8.85).

Microbial transformation of artemisinic acid by Aspergillus flavipes (ATCC 16795). A total of 540 mg of **3** was distributed evenly among 24 hr old stage II cultures of *A. flavipes*. The cultures were incubated for 10 days and were harvested by filtration. The culture filtrates were extracted × 3 with EtOAc (2.2l. 2 × 1.1l). The combined extracts were back-washed with H_2O, dried over dry Na_2SO_4 and the solvent evapd under red. pres. (40°) to leave 602 mg of a dark brown residue. This residue was chromatographed on a silica gel column (60 g) and elution was carried out with ether–hexane (5:2). The earlier fractions contained a minor amount of **4**, while the subsequent fractions yielded, after further purification, 86 mg of pure **5** (18%), R_f = 0.18 using ether–hexane (4:3) as solvent system. Successive recrystallization from ether–hexane gave prism crystals of (**5**), mp 145–146°; $[\alpha]_D$ + 74.5° ($CHCl_3$ *c*0.4); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3610 (-OH), 3011, 2921, 2323, 1682 (>C = O), 1620, 1500, 1283, 1244, 1150, 1100, 1030, 1000; ^1H NMR: 0.93 (3H, *d*, *J* = 6.5, Me-14), 1.07–1.68 (7H, *m*, H-1, H-2, H-8, H-9, H-10), 1.70 (3H, *s*, Me-15), 2.38 (1H, *ddd*, *J* = 3.1, 6.0, 12.9, H-2), 2.60–2.70 (2H, *m*, H-6, H-7), 4.08 (1H, *br t*, *J* = 2.5 Hz, H-3), 5.11 (1H, *s*, H-5), 5.54 (1H, *s*, H-13_b), 6.00 [2H, *br*, COOH, OH (disappeared after D_2O)], 6.43 (1H, *s*, H-13_a) ^{13}C NMR Data (Table 1); EIMS *m/z* (rel. int.) 250 (M^+) (0.53). (analysis: Found: C, 71.87 H, 9.07. $C_{15}H_{22}O_3$ requires: C, 71.96, H, 8.85).

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