Hydroxylation of 10-Deoxoartemisinin by *Cunninghamella elegans*

Igor A. Parshikov, Kannoth M. Muraleedharan, Bruhaspathy Miriyala, Mitchell A. Avery, and John S. Williamson*

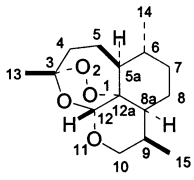
Department of Medicinal Chemistry, University of Mississippi, University, Mississippi 38677-1848

Received April 6, 2004

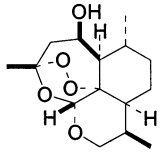
The microbial metabolism of 10-deoxoartemisinin (**1**), a derivative of the antimalarial drug artemisinin, was investigated. Various strains of fungi were investigated for their ability to transform **1**. Of these microorganisms, only *Cunninghamella elegans* was capable of transforming **1** to 5β-hydroxy-10-deoxoartemisinin (**2**), 4α-hydroxy-1,10-deoxoartemisinin (**3**), and 7β-hydroxy-10-deoxoartemisinin (**4**). The metabolites **2** and **4** retained an intact peroxide group and are therefore useful scaffolds for synthetic modification in the search for new antimalarial agents.

Malaria remains one of the deadliest diseases on earth. Emergence of drug-resistant strains of *P. falciparum*, coupled with the inefficiency of traditional drugs to combat this parasite, warrants the development of new and more effective chemotherapeutic agents to fight against malaria.

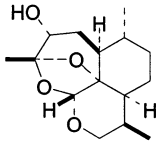
10-Deoxoartemisinin (**1**) is a derivative of the antimalarial agent artemisinin that lacks the lactone carbonyl in the 10-position. **1** has been shown to have excellent antimalarial potency and a favorable chemical stability.[1,2] Although many synthetic derivatives of artemisinin and 10-deoxyartemisinin possess promising antimalarial activities,[3−5] there remains a need for more effective drugs. This need has motivated researchers to examine new methods to generate hitherto synthetically inaccessible derivatives by biochemical means.



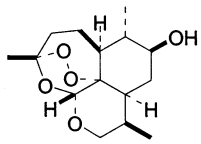
1



2



3



4

It has been reported that microbial transformations can be useful for the production of certain hydroxylated derivatives of artemisinin. Such transformations include a low-yielding conversion to 4α-hydroxyartemisinin and 7β-hydroxyartemisinin by *Mucor polymorphosporus*;[6,7] conversion to 6-hydroxyartemisinin by *Cunninghamella echinulata*;[6] conversion of 10-deoxyartemisinin to 7β-hydroxy-10-deoxyartemisinin by *Mucor rammanianus*;[8,9] conversion of artemether to 7β-hydroxyartemether by *Streptomyces lavendulae*;[10] conversion of arteether to 7β-hydroxyarteether by *C. elegans*[11] and *Beauveria sulfurescens*;[12] and conversion of artemisitene to 7β-hydroxy-9-epi-artemisinin by *Aspergillus niger* with the α orientation of the 9-methyl.[13]

The present work describes our efforts to direct the biotransforming capability of *C. elegans* to produce the desired 7β-hydroxy-10-deoxoartemisinin (**4**), which will be used in future experiments as a scaffold for synthetic manipulation.

Reverse-phase HPLC of the resulting biotransformation broth of 10-deoxoartemisinin (**1**) with *C. elegans* afforded three metabolites, 5β-hydroxy-10-deoxoartemisinin (**2**), 4α-hydroxy-1,10-deoxoartemisinin (**3**), and the major metabolite 7β-hydroxy-10-deoxoartemisinin (**4**).

Compound **2** showed absorption at 3518 cm^{-1} in the IR spectrum (OH). HRMS gave a peak at 307.1508 $[M + Na]^+$, suggesting a molecular formula of $C_{15}H_{24}O_5$, which was confirmed by elemental analysis. NMR spectra provided evidence that the hydroxylation had occurred on a secondary carbon atom, and the COSY spectrum clearly showed that this secondary carbon atom was next to C-5a, as indicated by the correlation between CH(OH) and H-5a. This was further confirmed by its NOESY correlations with H-14 and H-6. Further, NOESY correlation of H-13 to H-4 and HMBC correlation of C-13 and C-3 to H-4 confirmed that the H-4 was intact. All other NMR assignments were made on the basis of similar considerations and careful analysis of COSY, NOESY, HMBC, and HMQC spectra (see Experimental Section).

The IR spectrum of compound **3** also indicated the presence of a hydroxyl (3379 cm^{-1}). LCMS analysis of **3** yielded a mass of 291.03 $[M + Na]^+$. DEPT-135 experiments showed that in comparison to the starting material (**1**) the total number of tertiary carbons increased from five to six, while the number of secondary carbons decreased from five to four, suggesting the addition of a hydroxyl group to a secondary carbon. Significant NOESY correlation of C-*H*(OH) with H-5β indicated the hydroxyl group to have been introduced at C-4 in the α orientation. NMR spectral assignments of **3** were made using COSY, NOESY, HMQC, and HMBC experiments (see Experimental Section).

Compound **4** also showed the presence of a hydroxyl (3526 cm^{-1}). HRMS analysis revealed a peak at 307.1522 $[M + Na]^+$, which corresponded to a molecular formula of $C_{15}H_{24}O_5$. This molecular formula was confirmed by elemental analysis. The position of hydroxylation was evident from an examination of the COSY spectrum, which indicated a distinct correlation of C*H*(OH) with H-6 and H-8.

* Corresponding author. Tel: + 1 662 915 7142. Fax: + 1 662 915 5638. E-mail: mcjsw@olemiss.edu.

10.1021/np040089c CCC: $27.50
Published on Web 07/30/2004

Experimental Section

General Experimental Procedures. All fermentation extracts were analyzed by high-performance liquid chromatography (HPLC), using a Waters 2690 (Milford, MA) separation module with a Waters XTerra RP_{18} 5 μm column (7.8 × 100 mm) coupled to an evaporative light-scattering (PL-ELS 1000) detector (Amherst, MA). The nebulizer temperature was maintained at 70 °C with the evaporator's temperature at 80 °C, the gas flow rate of 1.0 L/min, and the time constant of 1 s. Mobile phase components consisted of solvent A (H_2O) and solvent B (MeOH). The mobile phase was set at a flow rate of 0.7 mL/min with a gradient that was increased from 40% to 90% solvent B over 20 min and then decreased to 40% for 5 min. All melting points were noted on a FP62 Mettler Toledo apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DPX 300 operating at 400 and 100 MHz, respectively. Chemical shifts were reported in parts per million (δ) downfield from tetramethylsilane, and *J*-values are in Hz. IR spectra were recorded using a Thermo Nicolet IR 300 FT/IR spectrometer on a germanium crystal plate as neat solids. High-resolution mass spectra (HRMS) were recorded using a Micromass Q-Tof Micro with a lock spray source. Optical rotations were determined using a Autopol IV (Rudolph, NJ) polarimeter with a sample tube of 0.05 mL and a length of 10 mm (λ = 589 nm). Elemental analysis data were obtained using a Perkin-Elmer Series II 2400 CHNS/O analyzer.

Chromatographic Conditions. Metabolites were purified by column flash-chromatography on silica gel 60 (Scientific Adsorbents Inc.), particle size 32−63 μm, pore size 60 Å, using an EtOAc/hexanes mobile phase in a gradient mode, eluting with 10 to 40% EtOAc at a flow rate of 30 mL/min. Fractions were collected in 50 mL aliquots. TLC was performed using precoated silica gel G and GP Uniplates (Analtech, Inc.) and a mobile phase of EtOAc/hexane (40:50). The plates were visualized in an iodine chamber or by charring with acid.

Microorganisms. Strains of mycelial fungi were obtained from collections of microorganisms in the United States and Russia. *Penicillium adametzi* ATCC 10407, *Cunninghamella elegans* ATCC 9245, *Mucor rammanianus* ATCC 9624, and *Mucor rammanianus* ATCC MYA-883 were from American Typical Cultures Collection. *Mucor rammanianus* 1839 was from University of Mississippi. *Trichoderma viride* T-58, *Pestalotiopsis guepini* P-8, and *Penicillium purpurescens* P-10 were collected from a forest in Jefferson County, AR. *Cunninghamella verticillata* VKPM F-430 was from the All-Russian Collection of Industrial Microorganisms (Moscow, Russia), and *Penicillium simplicissimum* KM-16 was from the Biological Faculty of Moscow State University.

Media for Transformation. All microorganisms were examined for the ability to biotransform **1** in a medium consisting of 20.0 g of malt extract, 15.0 g of sucrose, 10.0 g of peptone, and 1000 mL of deionized H_2O. The pH was adjusted to 6.5 with 0.1 N NaOH. Stock cultures of fungi were stored on slants containing similar media and (Difco) bacto-agar (3%) at 4 °C.

Fermentation Procedures. Fungal mycelia were scraped from the surface of the agar, suspended in 5 mL of sterilized H_2O,[14] and used to inoculate 125 mL stainless steel capped DeLong culture flasks that contained 50 mL of medium. Cultures were grown for 24 h on an Innova 4430 gyrotary shaker (New Brunswick Scientific Co., NJ) at 28 °C while shaking at 180 rpm. The 24 h old cultures were used to inoculate 2500 mL shake flasks, which contained 500 mL of culture medium. The cultures contained in the 2500 mL flasks were then incubated using the same conditions previously employed with the cultures in the 125 mL flask for 48 h, at which time they were then dosed with **1**.

Compound **1** was synthesized from artemisinin (Mediplantex, Vietnam) using standard methodology.[2] Stock solutions with a concentration of 2.5% of **1** in MeOH were prepared and filter-sterilized. After 48 h each culture flask received 10.0 mL of stock solution to bring the final concentration of **1** to 500 mg/L. After the addition of substrate, cultures were incubated for 14 days (16 total) at 28 °C while shaking at 180 rpm. In previous biotransformation experiments, a daily analysis of the culture broth by HPLC showed the maximal yields of the desired product to occur at approximately 14 days. Therefore, a 14-day incubation was used as a harvest time for all of the transformation cultures. In addition, culture controls containing only the fermentation media and the microorganism (without substrate) were employed to help distinguish biotransformation products from naturally occurring products.

The fungal mycelia were harvested by filtration through a paper in a Büchner funnel. Solids collected on the filter paper were discarded since previous exhaustive extraction of these materials yielded no discernible starting material or metabolites. Extraction was accomplished by employing three, one to one extractions of the broth with EtOAc. Further extraction with EtOAc provided no supplementary substrate or products as determined by TLC. The EtOAc extract was evaporated in vacuo, and the resulting residues were redissolved in a minimal volume of MeOH for analysis.

Microbial Metabolism of 10-Deoxoartemisinin (1). Of the fungal strains examined only *C. elegans* transformed 10-deoxoartemisinin to three dissimilar metabolites. HPLC analysis of the EtOAc extract from cultures treated with 10-deoxoartemisinin showed residual 10-deoxoartemisinin to elute at 21.2 min, while the three metabolites 5β-hydroxy-10-deoxoartemisinin (**2**, 5.1% of the total peak area), 4α-hydroxy-10-deoxoartemisinin (**3**, 4.4% of the total peak area), and 7β-dihydroxy-10-deoxoartemisinin (**4**, 88.7% of the total peak area) eluted at 16.4, 14.4, and 12.8 min, respectively. Other metabolic products were observed, but none that were not also detected in the control broth extracts. It was determined that after 14 days only 1.8% (total peak area) of the original **1** remained in the culture broth.

5β-Hydroxy-10-Deoxoartemisinin (2). 2 (95 mg, 8.8%) was isolated from 2.0 L of fermentation solid (1 g of starting material **1** was used) by flash column chromatography: mp 112−114 °C; $[\alpha]^{27}_D$ +62° (*c* 1.0, MeOH); IR (neat) ν_{max} 3518, 2946, 1053, 1025 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.18 (1H, s, H-12), 4.00 (1H, ddd, *J* = 4.7, 7.7 9.0, Hz, H-5α), 3.74 (1H, dd, *J* = 4.3, 11.7, Hz, H-10α), 3.48 (1H, dd, H-10β), 2.67 (1H, m, H-9), 2.54 (1H, dd, *J* = 10.7, 14.3 Hz, H-4α), 2.49 (1H, dd, *J* = 7.2, 14.8 Hz, H-4β), 1.74 (1H, ddd, *J* = 3.3 6.7, 13.4 Hz, H-7α), 1.68 (1H, ddd, *J* = 4.8, 8.2, 12.6 Hz, H-8α), 1.62 (1H, ddd, *J* = 4.4, 8.7, 8.8 Hz, H-8a), 1.55 (1H, m, H-6), 1.53 (1H, m, H-8β), 1.45 (3H, s, CH_3-13), 1.31 (1H, dd, *J* = 8.1, 11.4 Hz, H-5a), 1.17 (3H, d, *J* = 6.4 Hz, CH_3-14), 1.15 (1H, m, H-7β), 0.80 (3H, d, *J* = 7.2 Hz, CH_3-15); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 102.5 (C, C-3), 91.8 (CH, C-12), 80.1 (C, C-12a), 69.4 (CH, C-5), 66.3 (CH_2, C-10), 59.4 (CH, C-5a), 46.2 (CH_2, C-4), 44.7 (CH, C-8a), 36.9 (CH, C-6), 34.9 (CH_2, C-7), 27.9 (CH, C-9), 25.9 (CH_3, C-13) 21.3 (CH_3, C-14), 20.7 (CH_2, C-8), 13.1 (CH_3, C-15); HRMS (ESI): 307.1508 $[M + Na]^+$ (calcd for $C_{15}H_{24}O_5$-Na, 307.1521); *anal*. C 63.17%, H 8.61%, calcd for $C_{15}H_{24}O_5$, C 63.36%, H 8.51%.

4α-Hydroxy-1,10-Deoxoartemisinin (3). 3 (45 mg, 4.6%) was isolated from 2.0 L of fermentation solid (1 g of starting material **1** was used) by flash column chromatography: $[\alpha]^{27}_D$ −32° (*c* 1.0, MeOH); IR (neat) ν_{max} 3379, 2925, 1021, 980 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.15 (1H, s, H-12), 3.90 (1H, dd, *J* = 6.4, 11.6, Hz, H-10α), 3.55 (1H, brs, H-4β), 3.32 (1H, dd, *J* = 5.2, 11.6, Hz, H-10β), 2.30 (1H, m, H-9), 1.96 (1H, m, H-8a), 1.88 (1H, m, H-5α), 1.78 (1H, m, H-8α), 1.74 (1H, m, H-7α), 1.57 (3H, s, CH_3-13), 1.54 (1H, m, H-5β), 1.39 (1H, m, H-5a), 1.31 (1H, m, H-8β), 1.21 (1H, m, H-6), 1.04 (1H, m, H-7β), 0.92 (3H, d, *J* = 7.2 Hz, CH_3-14), 0.88 (3H, d, *J* = 6.4 Hz, CH_3-15); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 107.4 (C, C-3), 95.6 (CH, C-12), 83.3 (C, C-12a), 69.8 (CH, C-4), 64.7 (CH_2, C-10), 41.8 (CH, C-5a), 39.8 (CH, C-8a), 35.0 (CH, C-6), 34.4 (CH_2, C-7), 30.3 (CH_2, C-5), 26.4 (CH, C-9), 23.8 (CH_2, C-8) 20.6 (CH_3, C-13), 18.7 (CH_3, C-14), 16.3(CH_3, C-15); LCMS (ESI) 291 $[M + Na]^+$.

7β-Hydroxy-10-deoxoartemisinin (4): 4 (889 mg, 83.9%) was isolated from 2.0 L of fermentation solid (1 g of starting material **1** was used) by flash column chromatography: mp 131−133 °C; $[\alpha]^{27}_D$ +44° (*c* 0.5, MeOH); IR (neat) ν_{max} 3526,

2958, 1053, 1041 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.24 (1H, s, H-12), 3.74 (1H, ddd, J = 1.1, 3.6, 12.6 Hz, H-10α), 3.45 (1H, dd, J = 11.7, 11.8 Hz, H-10β), 3.26 (1H, ddd, J = 4.0, 10.0, 10.2 Hz, H-7α), 2.62 (1H, m, H-9), 2.37 (1H, ddd, J = 3.9, 14.1, 13.9 Hz, H-4α), 2.05 (1H, ddd, J = 3.2, 5.2, 14.6 Hz, H-4β), 1.90 (1H, m, H-8α), 1.88 (1H, m, H-5α), 1.69 (1H, m, H-8a), 1.53 (1H, m, H-5β), 1.48 (1H, m, H-8β), 1.43 (3H, s, Me-13), 1.38 (1H, m, H-5a), 1.35 (1H, m, H-6), 1.07 (3H, d, J = 5.6 Hz, CH_3-14), 0.79 (3H, d, J = 7.2 Hz, CH_3-15); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 104.2 (C, C-3), 91.7 (CH, C-12), 80.0 (C, C-12a), 73.3 (CH, C-7), 65.9 (CH_2, C-10), 49.6 (CH, C-5a), 43.9 (CH, C-8a), 42.4 (CH, C-6), 35.9 (CH_2, C-4), 29.5 (CH, C-9), 27.5 (CH_2, C-8), 25.7 (CH_3, C-13) 24.5 (CH_2, C-5), 15.3 (CH_3, C-14), 12.8(CH_3, C-15); HRMS (ESI): 307.1522 $[M + Na]^+$ (calcd for $C_{15}H_{24}O_5Na$, 307.1521); *anal*. C 63.37%, H 8.80%, calcd for $C_{15}H_{24}O_5$, C 63.36%, H 8.51%.

Note Added after ASAP: There was an error in the ^1H NMR data for **3** in the version posted on July 30, 2004. The correct data appear in the version posted on August 10, 2004.

Acknowledgment. This work was supported by Centers for Disease Control cooperative agreements U50/CCU418839 and UR3/CCU418652.

References and Notes

(1) Jung, M. *Curr. Med. Chem*. **1994**, *1*, 35−49.
(2) Jung, M.; Li X.; Bustos D. A.; ElSohly, H. N.; McChesney, J. D.; Mihous, W. K. *J. Med. Chem*. **1990**, *33*, 1516−1518.
(3) Jung, M.; Freitas, A. C. C.; McChesney, J. D.; ElSohly, H. N. *Heterocycles* **1994**, *39*, 23−29.
(4) Posner, G. H.; Parker, M. H.; Northrop, J.; Elias, J. S.; Ploypradith, P.; Xie, S.; Shapiro, T. A. *J. Med. Chem*. **1999**, *42*, 300−304.
(5) Lee, S.; Oh, S. *Tetrahedron Lett.* **2002**, *43*, 2891−2894.
(6) Zhan, J.; Guo, H.; Dai, J.; Zhang, Y.; Guo, D. *Tetrahedron Lett*. **2002**, *43*, 4519−4521.
(7) Zhan, J.; Zhang, Y.; Guo, H.; Han, J.; Ning, L.; Guo D. *J. Nat. Prod*. **2002**, *65*, 1693−1695.
(8) Khalifa, S. I.; Baker, J. K.; Jung, M.; McChesney, J. D.; Hufford, C. D. *Pharm. Res.* **1995**, *12*, 1493−1498.
(9) Fiaux de Medeiros, S.; Avery, M. A.; Avery, B.; Leite, S. G. F.; Freitas, A. C.C.; Williamson, J. S. *Biotechnol. Lett.* **2002**, *24*, 937−941.
(10) Abourashed, E. A.; Hufford, C. D. *J. Nat. Prod*. **1996**, *59*, 251−253.
(11) Hufford, C. D.; Khalifa, S. I.; Orabi, K. Y.; Wiggers, F. T. *J. Nat. Prod*. **1995**, *58*, 751−755.
(12) Ziffer, H.; Hu, Y.; Pu, Y. *NATO ASI Series, Ser. C: Math. Phys. Sci*. **1992**, *381*, 361−373.
(13) Orabi, K. Y.; Galal, A. M.; Ibrahim, A. R. S.; El-Feraly, F. S.; Khalifa, S. I.; El-Sohly, H. N. *Phytochemistry* **1999**, *51*, 257−261.
(14) Parshikov, I. A.; Freeman, J. P.; Lay, J. O.; Beger, R. D.; Williams, A. J.; Sutherland, J. B. *FEMS Microbiol. Lett.* **1999**, *177*, 131−135.

NP040089C