Antimalarial Activity of Azafluorenone Alkaloids from the Australian Tree *Mitrephora diversifolia*

Daniela Mueller, Rohan A. Davis, Sandra Duffy, Vicky M. Avery, David Camp, and Ronald J. Quinn*

Eskitis Institute, Griffith University, Brisbane, QLD 4111, Australia

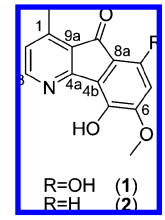
Received April 23, 2009

Mass-directed isolation of the CH_2Cl_2/MeOH extract from the roots of the Australian tree *Mitrephora diversifolia* resulted in the purification of the new azafluorenone alkaloid 5,8-dihydroxy-6-methoxyonychine (**1**) together with the known natural product 5-hydroxy-6-methoxyonychine (**2**). The structures of **1** and **2** were determined by extensive 1D and 2D NMR and MS data analyses. Both compounds were isolated during a drug discovery program aimed at the identification of new antimalarial leads from a prefractionated natural product library. When tested against two different strains of the parasite *Plasmodium falciparum* (3D7 and Dd2), **2** displayed IC_{50} values of 9.9 and 11.4 μM, respectively, while **1** showed minimal activity.

Malaria, the major parasitic infection in many tropical and subtropical regions, is still one of the largest contributors to the burden on public health expenses in more than 90, mostly underdeveloped countries.[1] Despite the presence of commercially available antimalarial drugs, the control of this ancient infection is increasingly limited by the emergence of drug-resistant strains of the malaria parasite, *Plasmodium*.[2] Annually 600 million new infections occur worldwide, and at least 1 million of these infections are fatal. Malaria is in fact among the leading causes of death worldwide from a single infectious agent.[1] Thus, new cost-effective strategies for treating malaria are urgently needed. The use of medicinal plants, such as the South American "quinine bark" *Cinchona succiruba* and the Chinese "sweet wormwood" *Artemisia annua*, has a long tradition in the treatment of malaria.[3] Identification of the major active metabolites of these plants, quinine and artemisinin, gave rise to the development of numerous antimalarial drugs.[4] Nowadays, 11 of the 15 drugs included in the WHO therapeutic schemes for malaria treatment are natural products or natural product derivatives.[5] On the basis of the developing resistance of the malaria parasite, most of these commercial drugs are rapidly losing their efficacy.[4] The re-emergence of malaria as a public health problem demonstrates the urgent need for the discovery and development of new antimalarial drugs.

During high-throughput screening of a prefractionated natural product library we discovered that two fractions derived from the roots of *Mitrephora diversifolia* (Annonaceae) showed activity in a malaria assay, with no cytotoxicity identified toward a human embryonic kidney cell line (HEK293). Chemical analysis of the active fractions from the prefractionated library identified ions in the (+)-LRESIMS at *m/z* 242 and 258, which were predicted to correspond to the bioactive natural products. Mass-directed isolation on the large-scale organic extract of *M. diversifolia* resulted in the purification of a new azafluorenone alkaloid, 5,8-dihydroxy-6-methoxyonychine (**1**), along with the previously isolated natural product 5-hydroxy-6-methoxyonychine (**2**).[6] Herein we report the isolation and structure elucidation for 5,8-dihydroxy-6-methoxyonychine (**1**), as well as the antimalarial activity of the two azafluorenone alkaloids.

The dried and ground roots of *M. diversifolia* were sequentially extracted with *n*-hexane, CH_2Cl_2, and MeOH. The CH_2Cl_2/MeOH extracts were combined and chromatographed using C_{18} bonded silica HPLC (MeOH/H_2O/0.1% TFA) to yield two relatively pure alkaloid fractions. (+)-LRESIMS indicated that fractions 29−31 contained one of the ions of interest (*m/z* 258), while the second ion of interest (*m/z* 242) was observed in fractions 32−33. Further purification of the earlier eluting fractions using C_{18} bonded silica HPLC (MeOH/H_2O/0.1% TFA) resulted in the isolation of 5,8-dihydroxy-6-methoxyonychine (**1**, 1.9 mg, 0.019% dry wt). In a similar manner the less polar fractions were purified to yield 5-hydroxy-6-methoxyonychine (**2**, 2.1 mg, 0.021% dry wt).



Compound **1** was isolated as an orange-brown, amorphous solid. On the basis of ^1H and ^{13}C NMR data (Table 1) in combination with the HRESIMS measurement on the $[M + H]^+$ ion (*m/z* 258.07548), the molecular formula was determined as $C_{14}H_{11}NO_4$ with 10 double-bond equivalents. The ^1H NMR spectrum (Table 1) of **1** displayed only six unique signals: one exchangeable resonance (δ_H 10.13 s), three aromatic signals [δ_H 6.40 s, 7.09 d (J = 5.4 Hz), 8.38 d (J = 5.4 Hz)], one methoxy resonance (δ_H 3.83 s), and one *C*-methyl signal (δ_H 2.53 s). All direct proton−carbon connectivities were assigned following gHSQC data analysis. Using COSY, $^1H-^1H$ coupling constants, and HMBC correlations, a trisubstituted pyridine moiety was readily established. HMBC correlations from protons of the methyl group at δ_H 2.53 (1-Me) to three carbons of the pyridine unit [δ_C 146.4 (C-1), 126.1 (C-9a), 124.9 (C-2)] indicated its *para*-position within the pyridine ring system (fragment **a**) (Figure 1). An additional weak HMBC correlation from this methyl group to a carbon at δ_C 188.6 (C-9) suggested that a carbonyl functionality was attached *ortho* to the methyl moiety.

The third aromatic proton at δ_H 6.40 (H-7) exhibited HMBC correlations to four quaternary carbons [δ_C 156.1 (C-6), 151.7 (C-8), 136.7 (C-5), 110.1 (C-8a)], three of which appeared to be attached to heteroatoms on account of their ^{13}C chemical shifts. On the basis of HMBC correlations of the methoxy protons at δ_H 3.83 (6-OMe) to carbons at δ_C 156.1 ($^3J_{CH}$) and 102.9 ($^4J_{CH}$), the methoxy group was attached to C-6. These correlations allowed the construction of fragment **b** (Figure 1). The ^{13}C NMR spectrum of **1** showed a further quaternary carbon at δ_C 123.0 (C-4b); however no HMBC correlations were observed to this carbon. At this stage all atoms of **1** had been accounted for, except for one hydrogen, which was not observed in the ^1H NMR spectrum and was postulated to be exchangeable. Literature searching using partial structures of **1**

* To whom correspondence should be addressed. Tel: +61-7-3735-6000. Fax: +61-7-3735-6001. E-mail: r.quinn@griffith.edu.au.

10.1021/np900247f CCC: $40.75
Published on Web 07/10/2009

Table 1. NMR Data of 5,8-Dihydroxy-6-methoxyonychine (**1**) and 5-Hydroxy-6-methoxyonychine (**2**)[a]

	1			2		
position	δ_H mult. (J in Hz)	δ_C	HMBC	δ_H mult. (J in Hz)	δ_C	HMBC
1		146.4			146.6	
1-Me	2.53 s	16.5	1, 2, 9, 9a	2.53 s	16.4	1, 2, 9, 9a
2	7.09 d (5.4)	124.9	1-Me, 3, 9, 9a	7.09 d (5.2)	124.6	1-Me, 3, 9, 9a
3	8.38 d (5.4)	151.1	1, 2, 4a	8.41 d (5.2)	151.8	1, 2, 4a
4a		163.1			164.0	
4b		123.0			125.2	
5		136.7			142.6	
5-OH	[b,c]			[b]		
6		156.1			154.3	
6-OMe	3.83 s	55.9	6, 7	3.90 s	56.0	6, 7
7	6.40 s	102.9	5, 6, 8, 8a, 9	6.97 d (8.2)	112.4	5, 6, 8a, 9
8		151.7		7.20 d (8.2)	116.5	4b, 6, 7, 9
8-OH	10.13[c] s					
8a		110.1			127.4	
9		188.6			190.7	
9a		126.1			125.6	

[a] Recorded in DMSO-d_6 at 30 °C. [b] Signal not observed. [c] Interchangeable signals.

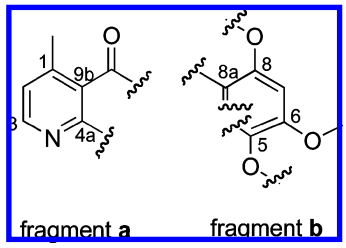


Figure 1. Partial structures of **1**.

(fragments **a** and **b**) readily identified **1** as an onychine analogue. The remaining two exchangeable hydrogens were assigned to two hydroxyl groups, substituted at C-5 and C-8. These data correlated well with compound **2**, whose structure was identified as 5-hydroxy-6-methoxyonychine following analysis of gCOSY, HSQC, and HMBC data. Comparison of the spectroscopic data of **1** and **2** with literature values of other known azafluorenones provided further proof of the assigned structures.[7−11] Hence, the structure of **1** was assigned as 5,8-dihydroxy-6-methoxyonychine. Since only the ^{1}H NMR data of 5-hydroxy-6-methoxyonychine have previously been published,[10,11] we report here both the ^{1}H and ^{13}C NMR assignments for **2** (Table 1).

To date, less than 40 alkaloids possessing an azafluorenone skeleton have been isolated.[12] Their occurrence is restricted to Annonaceae, a plant family well known as a source of various alkaloids belonging to different structural classes.[12] The co-occurrence of azafluorenones with oxoaporphines, diazafluoranthenes, and azaanthraquinones gave rise to the proposal of a common oxoaporphine precursor for these alkaloid classes.[13] To date, minimal bioactivity data have been provided for azafluorenone alkaloids. Onychine has been reported to possess anticandidal activity.[14] When tested in cytotoxicity assays, darienine, polyfothine, and isocondine have been shown to be inactive,[8] while cyathocaline has been found to be moderately active in a mechanism-based yeast bioassay for DNA-modifying agents.[9]

Compounds **1** and **2** were tested against both a chloroquine-sensitive (3D7) and chloroquine-resistant (Dd2) *Plasmodium falciparum* strain. Preliminary toxicity toward human cells was investigated using a human embryonic kidney cell line (HEK293). 5,8-Dihydroxy-6-methoxyonychine (**1**) was shown to display inhibition values of 87% and 80% at 120 μM against the 3D7 and Dd2 strains, respectively. Compound **1** was inactive against the HEK293 cell line at all concentrations tested up to and including 120 μM. In contrast, 5-hydroxy-6-methoxyonychine (**2**) was shown to be more active when tested against the *P. falciparum* strains (3D7 and Dd2), with IC_{50} values of 9.9 and 11.4 μM, respectively. 5-Hydroxy-6-methoxyonychine displayed some cytotoxicity toward HEK293 cells with 96% growth inhibition observed at 120 μM.

Experimental Section

General Experimental Procedures. NMR spectra were recorded at 30 °C on either a Varian 500 or 600 MHz Unity INOVA spectrometer (Varian, Walnut Creek, CA). The latter spectrometer was equipped with a triple resonance cold probe. The ^{1}H and ^{13}C NMR chemical shifts were referenced to the solvent peak for DMSO-d_6 at δ_H 2.49 and δ_C 39.5. LRESIMS were recorded on a Waters ZQ mass spectrometer (Waters, Milford, MA). HRESIMS were recorded on a Bruker Daltronics Apex III 4.7e Fourier-transform mass spectrometer (Bruker, Karlsruhe, Germany). IR and UV spectra were recorded on a Bruker Tensor 27 spectrometer (Bruker, Karlsruhe, Germany) and a Jasco V650 UV/vis spectrophotometer (Jasco, Tokyo, Japan), respectively. A BIOLINE orbital shaker (Edwards Instrument Company, Narellan, NSW, Australia) was used for the large-scale extraction of plant material. Machery Nagel Polyamide CC6 (0.05−0.016 mm) was used for tannin/polyphenolic removal (Machery Nagel, Düren, Germany). Alltech Davisil 40−60 μm 60 Å C_{18} bonded silica was used for preadsorption work (Alltech, Deerfield, IL). A Waters 600 pump equipped with a Waters 996 PDA detector and a Waters 717 autosampler (Waters, Milford, MA) were used for HPLC. A Thermo-Electron C_{18} Betasil 5 μm 143 Å column (21.2 mm × 150 mm) (Thermo Scientific, Los Angeles, CA) and a Phenomenex ONYX Monolithic C_{18} column (10 mm × 100 mm) (Phenomenex, Torrance, CA) were used for semipreparative HPLC separations. All solvents used for chromatography, UV, and MS were Lab-Scan HPLC grade (RCI Lab-Scan, Bangkok, Thailand), and the H_2O was Millipore Milli-Q PF filtered (Millipore, Billerica, MA). Parasite strains 3D7 and Dd2 were from the Queensland Institute of Medical Research. O+ erythrocytes were obtained from the Australian Red Cross Blood Service. Cell Carrier polylysine-coated imaging plates were from PerkinElmer (PerkinElmer, Waltham, MA). 4′,6-Diamidino-2-phenylindole (DAPI) stain and Alamar Blue were from Invitrogen (Invitrogen, Carlsbad, CA). Triton-X, saponin, puromycin, and artemisinin were all from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). HEK293 cells were purchased from the American Tissue Culture Collection (ATCC, Manassa, VA). The 384-well Falcon sterile tissue culture treated plates were from BD (BD, Franklin Lakes, NJ).

Plant Material. The roots of *M. diversifolia* (Annonaceae) were collected at Leo Creek and Nesbit River confluence, Silver Plains, Queensland, Australia, in July 1997. A voucher sample (AQ 604343) has been lodged at the Queensland Herbarium, Brisbane, Australia.

Extraction and Isolation. The dried and ground roots of *M. diversifolia* (10 g) were transferred to a conical flask (1 L), *n*-hexane (250 mL) was added, and the flask was shaken at 200 rpm for 2 h. The *n*-hexane extract was filtered under gravity, then discarded. CH_2Cl_2 (250 mL) was added to the defatted plant material in the conical flask and shaken at 200 rpm for 2 h. The resulting extract was filtered under gravity and set aside. 100% MeOH (250 mL) was added, and the MeOH/plant mixture was shaken for a further 2 h at 200 rpm. Following gravity filtration the plant material was extracted with another volume of 100% MeOH (250 mL) while being shaken at 200 rpm for 16 h. All CH_2Cl_2/MeOH extractions were combined and dried under reduced pressure to yield a dark brown solid (0.56 g). This material was resuspended in MeOH (150 mL), loaded onto a MeOH-conditioned polyamide gel (30 g) in a sintered glass column, and washed with MeOH (300 mL). The resulting extract (0.49 g) was preadsorbed on C_{18}-bonded silica, then packed into a stainless steel cartridge (10 × 30 mm) that was subsequently attached to a C_{18} Betasil HPLC column. Isocratic HPLC conditions of 90% H_2O (0.1% TFA)/10% MeOH (0.1% TFA) were initially employed for the first 10 min, then a linear gradient to 100% MeOH (0.1% TFA) was run over 40 min, followed by isocratic conditions of 100% MeOH (0.1% TFA) for a further 10 min, all at a flow rate of 9 mL/min. Sixty fractions (60 × 1 min) were collected from time = 0 min, then analyzed by (+)-LRESIMS. Fractions 29−31 (16.55 mg) contained one of the ions of interest (*m*/*z* 258), while fractions 32−33 (13.77 mg) were shown to contain the other desired ion (*m*/*z* 242). Fractions 29−31 (16.55 mg) were further purified by HPLC using a C_{18} ONYX column. Initially isocratic conditions of 85% H_2O (0.1% TFA)/15% MeOH (0.1% TFA) at 9 mL/min for the first 5 min were employed, then a linear gradient to 30% MeOH (0.1% TFA)

was run over 45 min. For the next 5 min a steep linear gradient to 100% MeOH (0.1% TFA) was used to flush the column, followed by isocratic conditions of 100% MeOH (0.1% TFA) for a further 5 min, all at a flow rate of 9 mL/min. (+)-LRESIMS analysis of all resulting fractions and subsequent lyophilization of the fraction containing the ion at *m*/*z* 258 yielded 5,8-dihydroxy-6-methoxyonychine (**1**, 1.9 mg, 0.019% dry wt). Further purification of fractions 32−33 (13.77 mg), from the first C_{18} fractionation step, was undertaken using the following HPLC conditions: C_{18} ONYX column, flow rate 9 mL/min, isocratic conditions of 80% H_2O (0.1% TFA)/20% MeOH (0.1% TFA) for 5 min, then a linear gradient to 60% H_2O (0.1% TFA)/40% MeOH (0.1% TFA) run over 40 min followed by a 5 min gradient to 100% MeOH (0.1% TFA), isocratic conditions of 100% MeOH (0.1% TFA) for the last 10 min. (+)-LRESIMS analysis of all resulting fractions and subsequent lyophilization of the fraction containing the ion at *m*/*z* 242 yielded pure 5-hydroxy-6-methoxyonychine (**2**, 2.1 mg, 0.021% dry wt).

5,8-Dihydroxy-6-methoxyonychine (1): orange-brown solid; UV (MeOH) λ_{max} (log ε) 251 (5.36), 293 (4.90), 415 nm (4.67); IR ν_{max} (KBr) 1690, 1680, 1650, 1205, 1138 cm^{-1}; ^1H and ^{13}C NMR data (DMSO-d_6) see Table 1; (+)-LRESIMS (rel int) *m*/*z* 258 (100) $[M + H]^+$, (+)-HRESIMS *m*/*z* 258.07548 ($C_{14}H_{12}NO_4$ $[M + H]^+$ requires 258.07608).

Antimalarial Assays. Compounds were incubated in the presence of 2% or 3% parasitemia (3D7 or Dd2) and 0.3% hematocrit in a total assay volume of 50 μL, for 72 h at 37 °C and 5% CO_2, in poly-D-lysine-coated CellCarrier Imaging plates. After incubation the plates were stained with DAPI in the presence of saponin and Triton X-100 and incubated for a further 5 h at rt in the dark before imaging on the Evotec OPERA HCS confocal imaging system (PerkinElmer, Waltham, MA). The digital images obtained were then analyzed using the Perkin-Elmer Acapella spot detection software, where fluorescent spots that fulfilled the criteria established for a stained parasite were counted. The percent inhibition of parasite replication was calculated using DMSO and 2 μM artemisinin control data.

Cytotoxicity Assays. Compounds were added to Falcon 384-well black/clear tissue-treated assay plates containing 3000 adherent cells/well (HEK293) in an assay volume of 45 μL. The plates were incubated for 72 h at 37 °C and 5% CO_2. After incubation the supernatant was aspirated from the wells and 40 μL of 10% Alamar Blue added per well. Plates were incubated for a further 5−6 h and measured for fluorescence at 535 nm excitation and 590 nm emission using a VICTOR II (PerkinElmer, Waltham, MA). The percent inhibition of cell proliferation was calculated using DMSO and 10 μM puromycin control data. IC_{50} values were obtained by plotting percent inhibition against log dose using the Prizm4 graphing package and nonlinear regression with variable slope plot.

Acknowledgment. The authors thank C. Lewis and K. Watts from the Molecular Libraries group (Eskitis Institute), for their assistance in the preparation of the screening library. We thank H. T. Vu from Griffith University for acquiring the HRESIMS measurements. We are indebted to P. Forster and G. Guymer of the Queensland Herbarium, Brisbane, Australia, for collection and identification of the plant material. The authors would like to acknowledge Medicines for Malaria Venture (MMV) for financial support. We also acknowledge the Australian Red Cross Blood Service for the provision of type O+ erythrocytes and K. Andrews QIMR for *P. falciparum* strains.

Supporting Information Available: NMR spectra for 5,8-dihydroxy-6-methoxyonychine (**1**) and 5-hydroxy-6-methoxyonychine (**2**). This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Arrow, K. J.; Panosian, C.; Gelband, H. *Saving Lives, Buying Time: Economics of Malaria Drugs in an Age of Resistance*; The National Academies Press: Washington, D.C., 2004; p 388.
(2) Craft, J. C. *Curr. Opin. Microbiol.* **2008**, *11*, 428–33.
(3) Schwikkard, S.; van Heerden, F. R. *Nat. Prod. Rep.* **2002**, *19*, 675–92.
(4) Kayser, O.; Kiderlen, A. F.; Croft, S. L. *Stud. Nat. Prod. Chem.* **2002**, *26*, 779–848.
(5) Bourdy, G.; Willcox, M. L.; Ginsburg, H.; Rasoanaivo, P.; Graz, B.; Deharo, E. *Int. J. Parasitol.* **2008**, *38*, 33–41.
(6) El-Shanawany, M. A. *Bull. Pharm. Sci.* **1985**, *8*, 127–43.
(7) Arango, G. J.; Cortes, D.; Cassels, B. K.; Cave, A.; Merienne, C. *Phytochemistry* **1987**, *26*, 2093–8.
(8) Wu, Y. C.; Duh, C. Y.; Wang, S. K.; Chen, K. S.; Yang, T. H. *J. Nat. Prod.* **1990**, *53*, 1327–31.
(9) Wijeratne, E. M. K.; De Silva, L. B.; Kikuchi, T.; Tezuka, Y.; Gunatilaka, A. A. L.; Kingston, D. G. I. *J. Nat. Prod.* **1995**, *58*, 459–62.
(10) Zhang, J.; El-Shabrawy, A. R. O.; El-Shanawany, M. A.; Schiff, P. L., Jr.; Slatkin, D. J. *J. Nat. Prod.* **1987**, *50*, 800–6.
(11) Laprevote, O.; Roblot, F.; Hocquemiller, R.; Cave, A. *J. Nat. Prod.* **1988**, *51*, 555–61.
(12) *Dictionary of Natural Products on CD-Rom*, version 17.2; Chapman and Hall/CRC Press: London, UK, 2009.
(13) Tadic, D.; Cassels, B. K.; Leboeuf, M.; Cave, A. *Phytochemistry* **1987**, *26*, 537–41.
(14) Hufford, C. D.; Liu, S.; Clark, A. M.; Oguntimein, B. O. *J. Nat. Prod.* **1987**, *50*, 961–4.

NP900247F