

An Antimalarial Benzyloquinoline Alkaloid from the Rainforest Tree, *Doryphora sassafras*

*Malcolm S. Buchanan, Rohan A. Davis, Sandra Duffy, Vicky M. Avery, and Ronald J. Quinn**

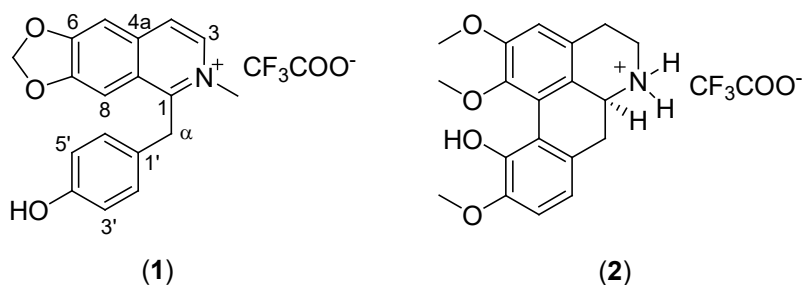
Eskitis Institute, Griffith University, Nathan, Queensland 4111, Australia

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

Mass-directed isolation of the CH₂Cl₂/MeOH extract of *Doryphora sassafras* resulted in the purification of a new benzylisoquinoline alkaloid, 1-(4-hydroxybenzyl)-6,7-methylenedioxy-2-methylisoquinolinium (**1**) and the known aporphine alkaloid (*S*)-isocorydine (**2**). The structures of **1** and **2** were determined by 1D and 2D NMR, and MS data analyses. The compounds were isolated during a drug discovery program aimed at identifying new antimalarial leads from a prefractionated natural product library. When tested against two different strains of the parasite *Plasmodium falciparum* (3D7 and Dd2), **1** displayed IC₅₀ values of 3.0 and 4.4 μM, respectively. Compound **1** was tested for cytotoxicity towards the human embryonal kidney cell line (HEK293) and displayed no activity at 120 μM.

Malaria is a major infectious disease caused by the protozoan parasite *Plasmodium falciparum*. Each year greater than 250 million clinical cases of malaria are reported with over 1 million of these cases resulting in death.¹ Although several drugs are currently available for the treatment of malaria, the emergence of drug-resistant *Plasmodium* strains means that new therapies are urgently needed to treat this devastating disease.¹

High-throughput screening (HTS) of a prefractionated natural product library has recently been undertaken at the Eskitis Institute in order to discover new antimalarial compounds. Two fractions derived from a leave extract of *Doryphora sassafras* (Monimiaceae), showed inhibitory activity in the malaria HTS assay, with no cytotoxicity identified towards the human embryonal kidney cell line (HEK293). MS analysis of the two active fractions identified ions in the (+)-LRESIMS at m/z 294 and 328, that were predicted to correspond to the bioactive natural products. Mass-directed isolation of the large-scale $\text{CH}_2\text{Cl}_2/\text{MeOH}$ extract of *D. sassafras* afforded the new benzyloisoquinoline alkaloid, 1-(4-hydroxybenzyl)-6,7-methylenedioxy-2-methyloisoquinolinium trifluoroacetate (**1**) and the TFA salt of the known aporphine alkaloid (*S*)-isocorydine (**2**).²⁻³ The genus *Doryphora* has previously yielded aporphines, isoquinolines, benzyloisoquinolines, and bisbenzyloisoquinolines.⁴⁻⁶ There have been reports of both synthetic and natural isoquinoline alkaloids with antimalarial activity.⁷⁻⁸ This paper reports the isolation, structure elucidation, and antimalarial activity of **1**.



The $\text{CH}_2\text{Cl}_2/\text{MeOH}$ extract from the dried and ground leaves of *D. sassafras* was initially chromatographed through polyamide gel. The resulting MeOH eluent was then purified by three steps of reversed-phase C_{18} HPLC to give the benzyloisoquinoline alkaloid **1** and the aporphine alkaloid **2**.

Compound **1** was obtained as an optically inactive gum. The molecular formula for the free base of **1** was determined to be $C_{18}H_{16}NO_3^+$ by (+)-HRESIMS on the $[M-CF_3COO]^-$ ion at m/z 294.1111. IR (3434, 1682, 1469, 1205, 1133 cm^{-1}), UV (strong absorption at 253 nm), and NMR data (Table 1) indicated that **1** was a benzyloisoquinoline derivative. Analysis of the 1H NMR data suggested that the molecule contained a *p*-oxygenated phenyl group [δ_H 6.90 d ($J = 8.4$ Hz); 6.70 d ($J = 8.4$ Hz)], α and β pyridine protons [δ_H 8.52 d ($J = 7.2$ Hz); 8.18 d ($J = 7.2$ Hz)], an *N*-methyl moiety (δ_H 4.25), a methylenedioxy group (δ_H 6.41), an α -methylene moiety (δ_H 4.84), two isolated aromatic protons (δ_H 8.08 and 7.70) and a phenolic hydroxy group (δ_H 9.44). The correlations observed in the gHSQC and gHMBC spectra indicated that the molecule contained 16 unique carbons, which included two sets of coincident carbons that were associated with the *p*-oxygenated phenyl group. The gHSQC correlation between the two-proton singlet at δ_H 6.41 and the methylene carbon δ_C 103.9 support the presence of a methylenedioxy group. The methylenedioxy singlet at δ_H 6.41 showed gHMBC correlations to two downfield oxygenated aromatic carbons at δ_C 151.8 and 155.2. Correlations from the two aromatic singlets (δ_H 8.08 and 7.70) to the same oxygenated aromatic carbons indicated that these protons were *ortho* to the methylenedioxy group and *para* to each other. The singlet at δ_H 7.70 also showed a correlation to the β -pyridine carbon (δ_C 122.3), thus indicating that an isoquinoline moiety was present in the compound. This was expanded to an *N*-methyloisoquinolinium unit by gHMBC correlations from the *N*-methyl protons at δ_H 4.25 to the α -pyridine carbons at δ_C 155.7 and 136.3. A two-proton singlet at δ_H 4.84 was assigned to the benzylic protons and showed gHMBC correlations to the carbons at δ_C 128.8 (2C) and 124.3 of the *p*-oxygenated phenyl group. These benzylic protons showed further gHMBC correlations to the α and β -pyridine carbons at δ_C 155.7 and 125.3 revealing the *p*-oxygenated benzyl group was attached to the isoquinoline moiety at C-1. In order to satisfy the molecular formula of **1** a *p*-hydroxy group was attached to C-4' (δ_C 156.4) of the benzyl moiety. This was supported by gHMBC correlations from the exchangeable proton at δ_H 9.44 to δ_C 156.4 and 115.5 (2C). Therefore, compound **1** was assigned as 1-(4-hydroxybenzyl)-6,7-methylenedioxy-2-methyloisoquinolinium trifluoroacetate.

The related natural product, 2-methyl-1-(*p*-methoxybenzyl)-6,7-methylenedioxyisoquinolinium chloride, has been previously isolated from the leaves of a separate *D. sassafras* that was also collected in Queensland, Australia.⁶ This 4-*O*-methyl analog of **1** had been previously reported to display weak activity in a κ opioid receptor binding assay. Comparison of the NMR data of both these benzyloisoquinoline alkaloids showed only minor differences.⁶

Compound **2** was identified as the TFA salt of the known aporphine alkaloid, (*S*)-isocorydine following comparison of the spectroscopic data with literature values.²⁻³

Table 2 shows the antimalarial activity for compounds **1** and **2** when tested against a chloroquine-sensitive (3D7) and a chloroquine-resistant (Dd2) *Plasmodium falciparum* strain. Preliminary toxicity towards human cells was also investigated for **1** and **2** using a human embryonal kidney cell line (HEK293). Neither compounds **1** or **2** showed any cytotoxicity towards the HEK293 cells up to 120 μ M.

Table 2. Antimalarial and Cytotoxic Activity for Compounds **1** and **2**.

Compound	IC ₅₀ (μ M)			Selectivity Index
	Dd2	3D7	HEK293	
1	4.4	3.0	0% @ 120 μ M	>27 (Dd2), >40 (3D7)
2	19.0	17.0	0% @ 120 μ M	>6 (Dd2/3D7)
Artemisinin ^a	0.021	0.021	0% @ 2 μ M	>100 (Dd2/3D7)
Chloroquine ^a	0.130	0.025	0% @ 2 μ M	>16 (Dd2), >80 (3D7)

^a If artemisinin and chloroquine were screened at a higher concentration the selectivity index would be much larger.⁹

Experimental Section

General Experimental Procedures. UV and IR spectra were recorded on an Agilent 8453 UV/vis spectrophotometer (Agilent, Santa Clara, CA, USA) and a Bruker Tensor 27 FTIR spectrophotometer

(Bruker, Karlsruhe, Germany), respectively. NMR spectra were recorded at 30°C on Varian INOVA 500 and 600 MHz NMR spectrometers (Varian, Walnut Creek, CA, USA). The latter spectrometer was equipped with a triple resonance cold probe. The ^1H 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. Standard parameters were used for the 2D experiments, which included gradient gCOSY, gHSQC ($^1J_{\text{CH}} = 140$ Hz) and gHMBC ($^nJ_{\text{CH}} = 8.3$ Hz). LRESIMS were recorded on a Waters ZQ mass spectrometer (Waters, Milford, MA, USA). HRESIMS were measured on a Bruker Daltonics Apex III 4.7e Fourier Transform Mass Spectrometer (Bruker, Karlsruhe, Germany), fitted with an Apollo API source. A BIOLINE orbital shaker (Edwards Instrument Company, Narellan, NSW, Australia) was used for the large-scale extraction of plant material. A ThermoElectron C₁₈ Betasil 5 μm 143 Å column (21.2 mm \times 150 mm) (Thermo Scientific, Los Angeles, CA, USA) was used for semi-preparative HPLC. A Waters 600 pump fitted with a 996 Photodiode Array Detector and 717 plus Autosampler (Waters, Milford, MA, USA) was used for the semi-preparative HPLC separations. End-capped Septra C₁₈ bonded silica (Phenomenex, Torrance, CA, USA) was used for pre-adsorption work. Machery Nagel Polyamide CC6 (0.05-0.016 mm) was used for tannin/polyphenolic removal (Machery Nagel, Düren, Germany). Water was Millipore Milli-Q PF (Millipore, Billerica, MA, USA) filtered, while all other solvents used were Lab-Scan HPLC grade (RCI Lab-Scan, Bangkok, Thailand). 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, USA). 4',6-Diamidino-2-phenylindole (DAPI) stain and Alamar Blue were from Invitrogen (Invitrogen, Carlsbad, CA, USA). Triton-X, saponin, puromycin, and artemisinin were all from Sigma Aldrich (Sigma-Aldrich, St. Louis, MO, USA). HEK 293 cells were purchased from the American Tissue Culture Collection (ATCC, Manassa, VA, USA). The 384-well Falcon sterile tissue culture treated plates were from BD (BD, Franklin Lakes, NJ, USA).

Plant Material. The leaves of *D. sassafras* Endlicher (Monimiaceae) were collected during August of 1994 from The Head, 1.5 km NW of Wilsons Peak, Darling Downs, Queensland, Australia. A voucher specimen (No. 601204) has been lodged at the Queensland Herbarium.

Extraction and Isolation. The freeze-dried and ground plant material (10 g) was poured into a conical flask (1L), *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. CH₂Cl₂ (250 mL) was added to the de-fatted plant material in the conical flask and shaken at 200 rpm for 2 h. The resulting extract was filtered under gravity, and set aside. 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 MeOH (250 mL), while being shaken at 200 rpm for 16 h. All CH₂Cl₂/MeOH extractions were combined and dried under reduced pressure to yield a crude extract (1.32 g). This material was re-suspended in MeOH (150 mL), loaded onto a MeOH conditioned polyamide gel (30 g) column and washed with MeOH (300 mL). The resulting MeOH eluent (0.97 g) was pre-adsorbed on C₁₈-bonded silica, then packed into a stainless steel cartridge (10 × 30 mm) that was subsequently attached to a C₁₈ semi-preparative HPLC column. Isocratic HPLC conditions of 90% H₂O (0.1% TFA)/10% MeOH (0.1% TFA) were 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 and analysed by (+)-LRESIMS. Fractions 24-29 (*m/z* 294) and 30-33 (*m/z* 328) contained the ions of interest and were combined (98.5 and 21 mg, respectively) before undergoing further C₁₈ HPLC. Fractions 24-29 (98.5 mg): A linear gradient from 100% H₂O (0.1% TFA) to 60% H₂O (0.1% TFA)/40% MeOH (0.1% TFA) was employed for 45 min, followed by a linear gradient to 100% MeOH (0.1% TFA) was run over 15 min, at a flow rate of 10 mL/min. Sixty fractions (60 × 1 min) were collected from time = 0 min and analysed by (+)-LRESIMS. Fraction 29 contained the ion of interest (*m/z* 294) and required final purification by C₁₈ HPLC (3.6 mg). A linear gradient from 90% H₂O (0.1% TFA)/10% MeOH (0.1% TFA) to 85% H₂O (0.1% TFA)/15% MeOH (0.1% TFA) was employed for 1 min, followed by isocratic

conditions for 19 min, then a linear gradient to 65% H₂O (0.1% TFA)/35% MeOH (0.1% TFA) in 30 min and finally a linear gradient to 100% MeOH (0.1% TFA) in 10 min, at a flow rate of 10 mL/min. Sixty fractions (60 × 1 min) were collected from time = 0 min and analysed by (+)-LRESIMS. Fractions 30-31 contained the compound of interest (*m/z* 294), and following lyophilisation yielded 1-(4-hydroxybenzyl)-6,7-methylenedioxy-2-methylisoquinolinium trifluoroacetate (**1**, 0.8 mg, 0.008% dry wt). Further purification of fractions 30-33 (21 mg) from the first C₁₈ fractionation step, was undertaken using the following HPLC conditions: A linear gradient from 100% H₂O (0.1% TFA) to 85% H₂O (0.1% TFA)/15% MeOH (0.1% TFA) was employed for 2 min, followed by isocratic conditions for 23 min, then a linear gradient to 70% H₂O (0.1% TFA)/30% MeOH (0.1% TFA) was run over 20 min, and finally a linear gradient to 100% MeOH (0.1% TFA) in 10 min, at a flow rate of 10 mL/min. Sixty fractions (60 × 1 min) were collected from time = 0 min and analysed by (+)-LRESIMS. Fraction 36 contained the compound of interest (*m/z* 328), and following lyophilisation yielded the trifluoroacetate salt of (*S*)-isocorydine (**2**, 1.6 mg, 0.016% dry wt).

1-(4-Hydroxybenzyl)-6,7-methylenedioxy-2-methylisoquinolinium trifluoroacetate (1): isolated as a gum; UV (MeOH) λ_{max} (log ϵ) 326 sh (3.57), 332 sh (3.31), 313 (3.43), 283 (3.39), 253 (4.15), 228 (3.91) nm; IR ν_{max} (film) 3434, 1682, 1469, 1205, 1133 cm⁻¹; ¹H and ¹³C NMR, see Table 1; (+)-LRESIMS (rel. int.) *m/z* 294 (100); (+)-HRESIMS *m/z* 294.1111 (C₁₈H₁₆NO₃ [M-CF₃COO]⁺ requires 294.1125, Δ 4.8 ppm).

Antimalarial Assay. 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 μ L, for 72 h at 37 °C and 5 % CO₂, in poly-*D*-lysine coated CellCarrier Imaging plates. After incubation 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™ HTS confocal imaging system (PerkinElmer, Waltham, MA, USA). The digital images obtained were analysed using the Perkin Elmer Acapella spot detection software where

fluorescent spots which fulfill the criteria established for a stained parasite are counted. The % inhibition of parasite replication was calculated using DMSO and 2 μ M artemisinin control data.

Cytotoxicity Assay. Compounds were added to Falcon 384 well black/clear tissue treated assay plates containing 3000 adherent cells/well (HEK 293) in an assay volume of 45 μ L. The plates were incubated for 72 h at 37 °C and 5 % CO₂. After incubation the supernatant was aspirated out of the wells and 40 μ L 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, USA). The % inhibition of cell growth was calculated using DMSO and 10 μ M puromycin control data. IC₅₀ values were obtained by plotting % inhibition against log dose using Prism4 graphing package and non-linear regression with variable slope plot.

Acknowledgment. The authors would like to acknowledge Medicines for Malaria Venture for financial support. We thank H. The Vu from Griffith University for acquiring the HRESIMS measurements. The authors thank D. Camp, C. Lewis and K. Watts from the Molecular Libraries group (Eskitis Institute) for their assistance in the preparation of the screening library. We thank B. Aldred and R. Lang for technical assistance with the HTS. We also wish to thank P. I. Forster and G. P. Guymer for the collection and taxonomic identification of the plant material. 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 1-(4-hydroxybenzyl)-6,7-methylenedioxy-2-methylisoquinolinium trifluoroacetate (**1**). This material is available free of charge via the Internet at <http://pubs.acs.org>.

- (1) Medicines for Malaria Venture, <http://www.mmv.org/>.
- (2) Marsaioli, A. J.; Reis, F. De. A. M.; Magalhães, A. F.; Rúveda, E. A. *Phytochemistry* **1979**, *18*, 165-169.
- (3) *Dictionary of Natural Products on CD-Rom*, version 17.2, Chapman and Hall / CRC Press: London, UK, **2009**.
- (4) Bick, I. R. C.; Leow, H.-M.; Richards, M. J. *Aust. J. Chem.* **1980**, *33*, 225-228.
- (5) Chen, C. R.; Beal, J. L.; Doskotch, R. W.; Mitscher, L. A.; Svoboda, G. H. *Lloydia* **1974**, *37*, 493-500.
- (6) Carroll, A. R.; Davis, R. A.; Forster, P. I.; Guymer, G. P.; Quinn, R. J. *J. Nat. Prod.* **2001**, *64*, 1572-1573.
- (7) Hallock, Y. F.; Cardellina II, J. H.; Schäffer, M.; Stahl, M.; Bringmann, G.; François, G.; Boyd, M. R. *Tetrahedron* **1997**, *53*, 8121-8128.
- (8) Parai, M. K.; Panda, G.; Srivastava, K.; Puri, S. K. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 776-781.
- (9) Fernandez, L. S.; Buchanan, M. S.; Carroll, A. R.; Feng, Y. J.; Quinn, R. J.; Avery, V. M. *Org. Lett.* **2009**, *11*, 329-332.

Table 1. ^1H (600 MHz) and ^{13}C (150 MHz) NMR data for Compound **1** in $\text{DMSO-}d_6$

Position	δ_{C}^a	δ_{H} mult (J in Hz)
1	155.7 C	
2-CH ₃	45.4 CH ₃	4.25 s
3	136.3 CH	8.52 d (7.2)
4	122.3 CH	8.18 d (7.2)
4a	137.7 C	
5	103.2 CH	7.70 s
6	155.2 C	
6,7-OCH ₂ O	103.9 CH ₂	6.41 s
7	151.8 C	
8	103.3 CH	8.08 s
8a	125.3 C	
α	33.1 CH ₂	4.84 s
1'	124.3 C	
2'	128.8 CH	6.90 d (8.4)
3'	115.5 CH	6.70 d (8.4)
4'	156.4 C	
4'-OH		9.44 s
5'	115.5 CH	6.70 d (8.4)
6'	128.8 CH	6.90 d (8.4)

^a Chemical shifts obtained from 2D NMR experiments.

Table of Contents Graphic

An Antimalarial Benzyloisoquinoline Alkaloid from the Rainforest Tree, *Doryphora sassafras*

Malcolm S. Buchanan, Rohan A. Davis, Sandra Duffy, Vicky M. Avery and Ronald J. Quinn

