Antimalarial Benzylisoquinoline Alkaloid from the Rainforest Tree *Doryphora sassafras*

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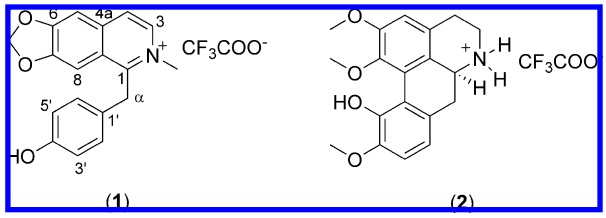
Mass-directed isolation of the CH_2Cl_2/MeOH extract of *Doryphora sassafras* resulted in the purification of a new benzylisoquinoline alkaloid, 1-(4-hydroxybenzyl)-6,7-methylenedioxy-2-methylisoquinolinium trifluoroacetate (**1**), and the known aporphine alkaloid (*S*)-isocorydine (**2**). The structures 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 IC_{50} values of 3.0 and 4.4 μM, respectively. Compound **1** was tested for cytotoxicity toward a human embryonic 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 our prefractionated natural product library has recently been undertaken in order to discover new antimalarial compounds. Two fractions derived from a leaf extract of *Doryphora sassafras* (Monimiaceae) showed inhibitory activity in a malaria HTS assay, with no cytotoxicity identified toward a human embryonic kidney cell line (HEK293). MS analysis of the two active fractions identified ions in the (+)-LRESIMS at *m/z* 294 and 328, which were predicted to correspond to the bioactive natural products. Mass-directed isolation of the large-scale CH_2Cl_2/MeOH extract of *D. sassafras* afforded the new benzylisoquinoline alkaloid 1-(4-hydroxybenzyl)-6,7-methylene-dioxy-2-methylisoquinolinium trifluoroacetate (**1**) and the known aporphine alkaloid (*S*)-isocorydine (**2**).[2,3] The genus *Doryphora* has previously yielded aporphines, isoquinolines, benzylisoquinolines, and bisbenzylisoquinolines.[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 CH_2Cl_2/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 benzylisoquinoline 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 benzylisoquinoline 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 supported the presence of a methylenedioxy group. This methylenedioxy singlet showed gHMBC correlations to two downfield oxygenated aromatic carbons at δ_C 151.8 and 155.2. HMBC 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*-methylisoquinolinium 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 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

Table 1. ^1H (600 MHz) and ^{13}C (150 MHz) NMR Data for Compound **1** in 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_2O	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.

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

	IC_{50} (μM)			
compound	Dd2	3D7	HEK293	selectivity index
1	4.4	3.0	0% @ 120 μM	>27 (Dd2), >40 (3D7)
2	19.0	17.0	0% @ 120 μM	>6 (Dd2/3D7)
artemisinin[a]	0.021	0.021	0% @ 2 μM	>100 (Dd2/3D7)
chloroquine[a]	0.130	0.025	0% @ 2 μM	>16 (Dd2), >80 (3D7)

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

and 125.3, revealing the *p*-oxygenated benzyl group was attached to the isoquinoline moiety at C-1. A hydroxy group was attached to C-4′ of the benzyl moiety based on gHMBC correlations from the exchangeable proton at δ_H 9.44 to δ_C 156.4 (C-4′) and δ_C 115.5 (C-3′/C-5′). Therefore, compound **1** was assigned as 1-(4-hydroxybenzyl)-6,7-methylenedioxy-2-methylisoquinolinium trifluoroacetate. The related natural product 1-(4-methoxybenzyl)-6,7-methylenedioxy-2-methylisoquinolinium chloride has been previously isolated from the leaves of another sample of *D. sassafras* that was also collected in Queensland, Australia.[6] This 4′-*O*-methyl analogue of **1** had been previously reported to display weak activity in a κ-opioid receptor binding assay.[6] Comparison of the NMR data of both these benzylisoquinoline alkaloids showed only the expected differences due to hydroxy/methoxy replacement.[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 toward human cells was also investigated for **1** and **2** using a human embryonic kidney cell line (HEK293). Neither compound **1** or **2** showed any cytotoxicity toward the HEK293 cells up to and including 120 μM.

Experimental Section

General Experimental Procedures. UV and IR spectra were recorded on an Agilent 8453 UV/vis spectrophotometer (Agilent, Santa Clara, CA) 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). 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. Standard parameters were used for the 2D experiments, which included gCOSY, gHSQC ($^1J_{CH}$ = 140 Hz), and gHMBC ($^nJ_{CH}$ = 8.3 Hz). LRESIMS were recorded on a Waters ZQ mass spectrometer (Waters, Milford, MA). 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 C_{18} Betasil 5 μm 143 Å column (21.2 mm × 150 mm) (Thermo Scientific, Los Angeles, CA) was used for semipreparative HPLC. A Waters 600 pump fitted with a 996 photodiode array detector and 717 Plus autosampler (Waters, Milford, MA) was used for the semipreparative HPLC separations. End-capped Sepra C_{18} bonded silica (Phenomenex, Torrance, CA) was used for preadsorption 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) filtered, while all other solvents 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). 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 leaves of *D. sassafras* Endlicher (Monimiaceae) were collected during August 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 air-dried and ground leaves of *D. sassafras* (10 g) were poured into 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. 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 CH_2Cl_2/MeOH extractions were combined and dried under reduced pressure to yield a crude extract (1.32 g). This material was resuspended 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 preadsorbed on C_{18}-bonded silica and packed into a stainless steel cartridge (10 × 30 mm) that was subsequently attached to a C_{18} semipreparative HPLC column. Isocratic HPLC conditions of 90% H_2O (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 analyzed 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 C_{18} HPLC. Fractions 24−29 (98.5 mg): A linear gradient from 100% H_2O (0.1% TFA) to 60% H_2O (0.1% TFA)/40% MeOH (0.1% TFA) was employed for 45 min, followed by a linear gradient to 100% MeOH (0.1% TFA) run over 15 min, at a flow rate of 10 mL/min. Sixty fractions (60 × 1 min) were collected from time = 0 min and analyzed by (+)-LRESIMS. Fraction 29 contained the ion of interest (*m/z* 294) and required final purification by C_{18} HPLC (3.6 mg). A linear gradient from 90% H_2O (0.1% TFA)/10% MeOH (0.1% TFA) to 85% H_2O (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% H_2O (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 analyzed by (+)-LRESIMS. Fractions 30 and 31 contained the ion of interest (*m/z* 294) and following lyophilization 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 C_{18} fractionation step was undertaken using the following HPLC conditions: A linear gradient from 100% H_2O (0.1% TFA) to 85% H_2O (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% H_2O (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 analyzed by (+)-LRESIMS. Fraction 36 contained the ion of interest (*m/z* 328) and following lyophilization yielded the TFA salt of (*S*)-isocorydine (**2**, 1.6 mg, 0.016% dry wt).

1-(4-Hydroxybenzyl)-6,7-methylenedioxy-2-methylisoquinolinium trifluoroacetate (1): brown gum; UV (MeOH) λ_{max} (log ε) 346 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}; ^{1}H and ^{13}C NMR, see Table 1; (+)-LRESIMS (rel int) *m/z* 294 (100); (+)-HRESIMS *m/z* 294.1111 ($C_{18}H_{16}NO_3$ [M − CF_3COO^-]$^+$ 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 μL, for 72 h at 37 °C and 5% CO_2, in poly-D-lysine-coated Cell Carrier 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). The digital images obtained were analyzed using the Perkin-Elmer

Acapella spot detection software, where fluorescent spots that fulfilled the criteria established for a stained parasite were counted. The % inhibition of parasite replication was calculated using DMSO and 2 μM artemisinin control data.

Cytotoxicity Assay. 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 % inhibition of cell proliferation was calculated using DMSO and 10 μM puromycin control data. IC_{50} values were obtained by plotting % inhibition against log dose using the Prizm4 graphing package and nonlinear regression with variable slope plot.

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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.

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