A New Quinoline Epoxide from the Australian Plant,

Drummondita calida

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Abstract

A drug discovery program aimed at identifying new antimalarial leads from a prefractionated natural product library has resulted in the purification of a new quinoline alkaloid (2'*R*)-2',3'-epoxy-*N*-methylatanine (**1**), along with eight known natural products, skimmianin (**2**), γ-fagarine (**3**), maculosidine (**4**), evolitrine (**5**), dictamnine (**6**), pteleine (**7**), *N*-methylatanine (**8**) and werneria chromene (**9**). Compound **1** displayed 74% inhibition at 80 μM against a chloroquine-resistant *Plasmodium falciparum* strain (Dd2).

Key words

Drummondita calida, Rutaceae, antimalaria, quinoline alkaloids, epoxide

Introduction

Malaria is an infectious disease caused by parasites belonging to the genus *Plasmodium*. The latest statistics show that two billion people live in areas at risk from the disease, and annually 1.7-2.5 million people die from malaria infection [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 [2]. Historically, medicinal plants have played a very important role in combating this disease. The stem bark of *Cinchona succiruba*, known as "fever tree" from South America and the young leaves of *Artemisia annua*, a traditional Chinese herb, commonly known as "Qinghao" have been used to treat malaria for centuries [3]. Subsequent chemical investigations of *Cinchona succiruba* and *Artemisia annua*, identified the major active metabolites to be quinine and artemisinin, respectively [4]. Further research on these two important compounds has led to the development of numerous antimalarial drugs [4].

As part of our continuing research into the discovery of new antimalarial leads [5-8], we undertook high-throughput screening (HTS) of a prefractionated natural product extract library. From the HTS data, we identified one fraction derived from the stems of the Australian plant *Drummondita calida* (F. Muell.) (Rutaceae) that showed activity in an antimalarial imaging assay, with no cytotoxicity towards the human embryonic cell line, HEK293. (+)-LRESIMS analysis of the active fraction identified an ion at *m/z* 274 that was predicted to correspond to the bioactive natural product. Mass-directed fractionation on the large-scale organic extract of *D. calida* resulted in the purification of the new quinoline alkaloid, (2'*R*)-2',3'-epoxy-*N*-methylatanine (**1**). Further chemical investigations of the organic extract resulted in the isolation of eight known natural products. Herein we report the isolation, structure elucidation and antimalarial activity

for (2'*R*)-2',3'-epoxy-*N*-methylatanine (**1**).

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Materials and Methods

General experimental procedures

NMR spectra were recorded at 30 ºC on Varian INOVA 500 and 600 MHz NMR spectrometers (Varian). 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 $CDCl_3$ at δ_H 7.27 and δ_C 77.2. Standard parameters were used for the 2D experiments, which included gradient gCOSY, gHSQC ($^{1}J_{CH}$ = 140 Hz) and gHMBC ($^{n}J_{CH}$ = 8.3 Hz). LRESIMS were recorded on a Waters ZQ mass spectrometer (Waters). HRESIMS were measured on a Bruker Daltonics Apex III 4.7e Fourier Transform Mass Spectrometer (Bruker), fitted with an Apollo API source. IR and UV spectra were recorded on a Bruker Tensor 27 spectrometer (Bruker), and a Jasco V-650 UV/Vis spectrophotometer (Jasco), respectively. Optical rotations were recorded on a Jasco P-1020 polarimeter (Jasco). A BIOLINE orbital shaker (Edwards Instrument Company) was used for the large-scale extraction of plant material. A ThermoElectron C_{18} Betasil 5 µm 143 Å column (21.2 × 150 mm) (Thermo Scientific) and a YMC-Pack diol 5 µm, (20 × 150 mm) were used for semi-preparative HPLC. A Phenomenex C_{18} Onyx monolithic column (4.6 × 100 mm) (Phenomenex) was used for compound purity analysis. A Waters 600 pump fitted with a 996 Photodiode Array Detector and 717 plus Autosampler (Waters) was used for the semi-preparative and analytical HPLC separations. End-capped Sepra C_{18} bonded silica (Phenomenex) was used for pre-adsorption work. Machery Nagel Polyamide CC6 (0.05-0.016 mm) was used for tannin/polyphenolic removal (Machery Nagel). Water was Millipore Milli-Q PF

(Millipore) filtered, while all other solvents used were Lab-Scan HPLC grade (RCI Lab-Scan). Parasite strain 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). 4′,6-Diamidino-2-phenylindole (DAPI) stain and Alamar Blue were from Invitrogen (Invitrogen). Triton-X, saponin, puromycin (>98%), chloroquine (>98%) and artemisinin (98%) were all from Sigma Aldrich (Sigma-Aldrich). HEK293 cells were purchased from the American Tissue Culture Collection (ATCC). The 384-well Falcon sterile tissue culture treated plates were from Becton Dickinson (BD).

Plant Material

The stems of *Drummondita calida* (F. Muell.) (Rutaceae) were collected on April 23, 1998 from Bulleringa National Park, 80 km NW of Mt Surprise, Red River area, Queensland, Australia. The plant sample was identified by G. P. Guymer and P. I. Forster from the Queensland Herbarium. A voucher specimen (AQ605109) has been lodged at the Queensland Herbarium.

Extraction and isolation

The dried and ground plant material (10 g) was sequentially extracted with *n*-hexane (250 mL), CH_2Cl_2 (250 mL) and CH_3OH (250 mL × 2). All CH_2Cl_2/CH_3OH extractions were combined and dried under reduced pressure to yield a crude extract (2.4 g). 1.2 g of the extract was resuspended in CH_3OH (150 mL) and loaded onto a column of polyamide gel (30 g) then flushed with CH_3OH (300 mL). This was dried to give a fraction (1.07 g) that was subsequently pre-adsorbed to C_{18}-bonded silica (1.0 g), then packed into a stainless steel cartridge (10 × 30 mm) and attached to a C_{18} semi-preparative HPLC column. Isocratic HPLC conditions of 90% H_2O (0.1%

TFA)/10% CH_3OH (0.1% TFA) were initially employed for the first 10 min, then a linear gradient to CH_3OH (0.1% TFA) was run over 40 min, followed by isocratic conditions of CH_3OH (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 analysed by (+)-LRESIMS. Fractions 23-27 contained the ion of interest (*m/z* 274). These fractions were combined and further purified by C_{18} HPLC using a linear gradient from 95% H_2O (0.1% TFA)/5% CH_3OH (0.1% TFA) to CH_3OH (0.1% TFA) to yield (2'*R*)-2',3'-epoxy-*N*-methylatanine (**1**, 3.2 mg, 0.064% dry wt). Fractions 40-60 were further purified by C_{18} HPLC with a linear gradient from 90% H_2O (0.1% TFA)/10% CH_3OH (0.1% TFA) to CH_3OH (0.1% TFA) to yield **2** (0.76 mg, 0.015% dry wt), **3** (2.1 mg, 0.042% dry wt), **4** (1.3 mg, 0.026% dry wt), **5** (0.3 mg, 0.0060% dry wt,), **6** (1.2 mg, 0.024% dry wt,), **7** (2.3 mg, 0.046% dry wt), **8** (0.9 mg, 0.019% dry wt), and **9** (0.60 mg, 0.0120% dry wt).

In order to obtain large quantities of **1** for stereochemistry investigations, the remaining CH_2Cl_2/CH_3OH extract (1.2 g) was purified by diol semi-preparative HPLC using a linear gradient from 90% CH_2Cl_2/10% CH_3OH to CH_3OH which afforded compound **1** (5.4 mg, 0.11% dry wt). Compounds **1-9** were analysed for purity by analytical C_{18} HPLC (254 nm) and shown to all be >95%.

(2'R)-2',3'-epoxy-N-methylatanine (**1**): isolated as a light yellow amorphous solid; $[\alpha]_D^{25}$ + 11.0 (0.1, CH_3OH); UV (CH_3OH) λ_{max} (log ε) 317 sh (3.91), 305 sh (4.03), 294 (4.09), 237 (4.51) nm; IR ν_{max} (film) 3743, 3392, 1685, 1640, 1501, 1371, 1202, 983 cm^{-1}; ^1H NMR ($CDCl_3$ with 2 drops of MeOH-d_4, 600 MHz): δ_H 8.19 (1H , d, *J* = 8.4 Hz, H-5), 7.84 (1H, dd, *J* = 8.6, 7.6 Hz, H-7), 7.76 (1H, d, *J* = 8.6 Hz, H-8), 7.56 (1H, dd, *J* = 8.4, 7.6 Hz, H-6), 5.11 (1H, dd, *J* = 9.2, 6.7 Hz, H-2'), 4.42 (3H, s, 4-OCH_3),

4.00 (3H, s, *N*-CH_3), 3.94 (1H, dd, *J* = 15.5, 6.7 Hz, H-1'), 3.84 (1H, dd, *J* = 15.5, 9.2 Hz, H-1'), 1.37 (3H, s, H-4'), 1.21 (3H, s, H-5'); ^{13}C NMR ($CDCl_3$ with 2 drops of MeOH-d_4, 125 MHz): 167.1 (C-2), 163.8 (C-4), 137.1 (C-10), 134.5 (C-7), 127.5 (C-6), 125.3 (C-5), 120.1 (C-9), 116.7 (C-8), 103.7 (C-3), 94.9 (C-2'), 71.1 (C-3'), 60.1 (4-OCH_3), 34.5 (*N*-CH_3), 28.4 (C-1'), 29.9 (C-4'), 25.0 (C-5'); (+)-LRESIMS *m/z* (rel. int.) 274 (100) $[M+H]^+$; (+)-HRESIMS *m/z* 274.1430 $[C_{16}H_{20}NO_3]^+$ (calcd 274.1437, Δ 2.7 ppm).

Preparation of (-)-*S*-edulinine (10) from compound 1

1 (3 mg) in methanolic $NH_3.H_2O$ (5% w/w, 3 mL) was stirred at 45 ºC for 3 h. The reaction mixture was evaporated under reduced pressure. The residue was subjected to a Sephadex LH-20 column (30 × 1 cm) with CH_3OH to yield 2.9 mg of (-)-*S*-edulinine (**10**) [10].

Antimalarial Assay

Compounds **1**-**4** and **6**-**9** were incubated in the presence of 2 or 3% parasitemia (Dd2) and 0.3% hematocrit in a total assay volume of 50 µL, for 72 h at 37 ºC and 5 % CO_2, 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 OPERA™ HTS confocal imaging system (PerkinElmer). The digital images obtained were analysed using the PerkinElmer Acapella spot detection software where fluorescent spots which fulfilled the criteria established for a stained parasite are counted. The % inhibition of parasite replication was calculated using DMSO and 2 µM artemisinin control data. Artemisinin [IC_{50} = 0.021 µM (Dd2)] and chloroquine [IC_{50} = 0.130 µM (Dd2)] were used as positive controls.

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 ºC and 5 % CO_2. After incubation, the supernatant was aspirated out of 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 emissionusing a VICTOR II (PerkinElmer). The % inhibition of cell growth was calculated using DMSO and 10 μM puromycin control data. IC_{50} values were obtained by plotting % inhibition against log dose using Prizm4 graphing package and non-linear regression with variable slope plot. Puromycin was used as a positive control and displayed an IC_{50} of 0.372 μM.

Results and Discussion

The air-dried and ground stems of *D. calida* were sequentially extracted with *n*-hexane, CH_2Cl_2, and CH_3OH. The CH_2Cl_2/CH_3OH extracts were combined, suspended in CH_3OH and passed through a polyamide gel column to remove tannins. The resulting CH_3OH eluent was then purified by several steps of reversed-phase C_{18} HPLC to yield compounds **1**-**9**. In order to obtain larger quantities of **1** for stereochemistry investigations, we used normal phase diol-bonded silica chromatography in an attempt to improve yields. Compound **1** was isolated in an improved yield using diol chromatography [0.064% dry wt (C_{18} HPLC) vs. 0.11% dry wt (diol HPLC)].

Compound **1** was isolated as an optically active amorphous solid with a optical rotation

value of $[\alpha]_D^{25}$ + 11.0 (c 0.1, CH_3OH). The molecular formula of **1** was determined to be $C_{16}H_{19}NO_3$ by (+)-HRESIMS of the $[M+H]^+$ ion at *m/z* 274.1430 (calcd 274.1437), indicating eight degrees of unsaturation. The characteristic absorption at 1630 cm^{-1} in the IR spectrum and the λ_{max} at 237, 294, 305, and 317 nm in the UV spectrum indicated that compound **1** was a 2-quinolone alkaloid [9]. The ^1H NMR and ^1H-^1H COSY spectra of **1** displayed four aromatic protons at δ_H 8.19 (1H, d, *J* = 8.4 Hz, H-5), 7.84 (1H, dd, *J* = 8.6, 7.6 Hz, H-7), 7.76 (1H, d, *J* = 8.6 Hz, H-8) and 7.56 (1H, dd, *J* = 8.4, 7.6 Hz, H-6), attributed to a 1,2-disubstituted aromatic moiety, as well as two *C*-methyl singlets at δ_H 1.21 (3H) and 1.37 (3H), and two heteroatom substituted methyl singlets at δ_H 4.00 (3H) and 4.42 (3H). Furthermore, the ^1H NMR and ^1H-^1H COSY spectra of **1** showed coupled signals at δ_H 5.11 (1H, dd, *J* = 9.2, 6.7 Hz), 3.94 (1H, dd, *J* = 15.5, 6.7 Hz), and 3.84 (1H, dd, *J* = 15.5, 9.2 Hz), suggesting the presence of a -CH_2-CH-O- moiety in **1**. All direct proton-carbon connectivities were assigned following gHSQC data analysis, and allowed for further substructures of **1** to be assigned. HSQC cross-peaks of the downfield methyl singlets at δ_H 4.04 and 4.42 to carbons at δ_C 34.5 and 60.1, respectively, identified the presence of one *N*-methyl and one methoxy group in **1**. HMBC data analysis allowed the construction and confirmation of the quinoline skeleton of **1**. Key HMBC correlations are shown in Figure 2. With the quinoline nucleus assigned, seven of the eight degrees of unsaturation for **1** had been satisfied. With all the atoms accounted for **1**, the remaining one degree of unsaturation had to be assigned to an additional ring, which was determined to be an epoxide. Long range HMBC correlations of the two *C*-methyls at δ_H 1.21 and 1.37 to the oxygenated carbons at δ_C 94.9 and 71.1 and the methylene moiety carbon at δ_C 28.4, supported the presence of an epoxide system and furthermore established an 2,3-epoxy-3-methylbutyl moiety. This epoxy side chain was attached to C-3 of the quinoline nucleus on the basis of HMBC and ROESY correlations. The methylene protons at δ_H 3.84 and 3.94 both

showed HMBC correlations to the quinoline carbons at δ_C 103.7 (C-3) and 167.1 (C-2), and the ROESY cross-peak between the methoxy at δ_H 4.42 and methylene protons confirmed this assignment (Fig. 2). Therefore, the planar structure of **1** was determined as 2',3'-epoxy-*N*-methylatanine (**1**) (Fig. 1).

With more sample now available, the stereochemistry of **1** was determined by converting **1** to the known natural product (-)-*S*-edulinine. Cleavage of the epoxide ring was undertaken using mild alkaline conditions ($NH_3.H_2O$, MeOH) to yield (-)-*S*-edulinine (90% yield, $[\alpha]_D^{25}$ -31.0, *c* 0.1, CH_3OH) (Fig. 3) [10]. So, the absolute stereochemistry of C-2' was assigned as *R* due to inversion of the chiral centre during epoxide opening [11]. Thus, compound **1** was assigned as 2'*R*-2',3'-epoxy-*N*-methylatanine.

During the purification process of 2'*R*-2',3'-epoxy-*N*-methylatanine, it was observed that several other HPLC peaks showed similar UV absorption profiles to **1**, suggesting that they most probably corresponded to other quinoline analogues. In order to explore preliminary structure-activity relationships for this class of compounds, further separation and purification of other quinoline analogues was performed. On the basis of 1D, 2D NMR and MS data, the known natural products, skimmianin (**2**) [12], γ-fagarine (**3**) [13], maculosidine (**4**) [14], evolitrine (**5**) [15], dictamnine (**6**) [16], pteleine (**7**) [17], N-methylatanine (**8**) [18] and werneria chromene (**9**) [19] were identified.

Compounds **1**-**4** and **6**-**9** were tested against a chloroquine-resistant (Dd2) *Plasmodium falciparum* strain. Preliminary toxicity towards human cells was investigated using a human embryonic kidney cell line, HEK293. 2'*R*-2',3'-epoxy-*N*-methylatanine (**1**) displayed 74% inhibition at 80 μM against Dd2, and was inactive against HEK293 at 80

μM. All other compounds were inactive against the Dd2 *Plasmodium falciparum* strain and the HEK293 cell line at 80 μM.

Supporting information

NMR data for (2'*R*)-2',3'-epoxy-*N*-methylatanine (**1**) and (-)-*S*-elulinine (**10**) are available as Supporting Information.

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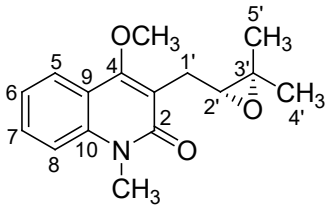
Figure Legends

Fig. 1: Chemical structures for the natural products **1-9**

Fig. 2: Key HMBC correlations (**A**) and ROESY correlations (**B**) for compound **1**

Fig. 3: Chemical conversion of **1** into *S*-edulinine (**10**)

Figure 1.



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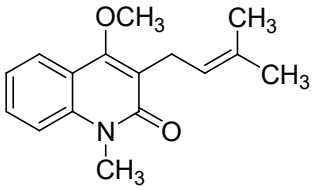

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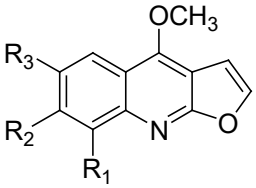

2 R_1,R_2=OCH_3, R_3=H
3 R_1=OCH_3, R_2,R_3=H
4 R_1,R_3=OCH_3, R_2=H
5 R_1,R_3=H, R_2=OCH_3
6 R_1,R_2,R_3=H
7 R_1,R_2=H, R_3=OCH_3

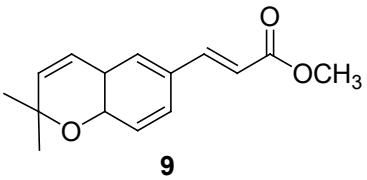

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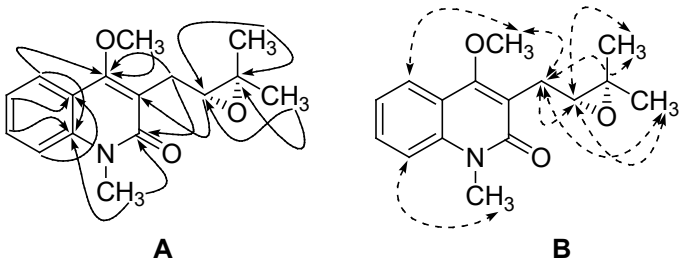
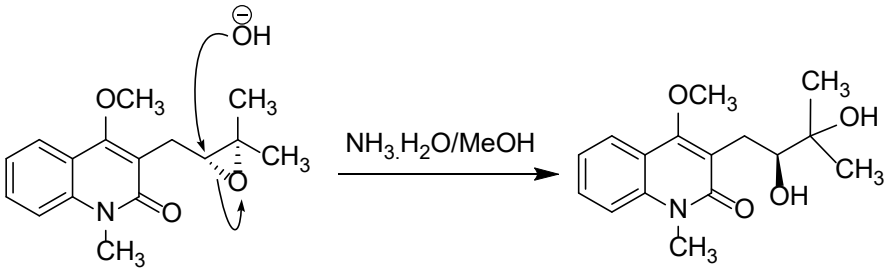
Figure 2.



A **B**

Figure 3.



1 *S*-edulinine (**10**)