Fucoxanthin, Tetraprenylated Toluquinone and Toluhydroquinone Metabolites from *Sargassum heterophyllum* Inhibit the *in vitro* Growth of the Malaria Parasite *Plasmodium falciparum*

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In the course of our search for antimalarial leads from marine algae, four metabolites, sargaquinoic acid, sargahydroquinoic acid, sargaquinal and fucoxanthin, were isolated from the South African alga *Sargassum heterophyllum*. Fucoxanthin and sargaquinal showed good antiplasmodial activity toward a chloroquine-sensitive strain (D10) of *Plasmodium falciparum* (IC_{50} 1.5 and 2.0 μM, respectively), while sargaquinoic acid and sargahydroquinoic acid were only moderately active (IC_{50} 12.0 and 15.2 μM, respectively).

Key words: Antiplasmodial Activity, Fucoxanthin, Sargaquinal

Introduction

Malaria remains one of the major diseases affecting sub-Saharan Africa, with 300–500 million clinical cases and 1–3 million mortality cases reported *per annum* (Fidock *et al*., 2004). Most of the fatalities are caused by *Plasmodium falciparum*, a one-celled apicomplexan parasite. Although natural products, such as quinine and artemisinin, and their synthetic derivatives have been the mainstay of antimalarial chemotherapy over the past 100 years, the development of resistance to these drugs has necessitated the search for new drugs for the treatment of the disease.

In our search for antimalarial leads from marine organisms, we have screened a number of crude organic extracts, obtained from marine algae collected from the South African coast, for their activity against a chloroquine-sensitive strain (D10) of *P. falciparum*. Organic extracts obtained from *Sargassum heterophyllum* exhibited promising antiplasmodial activity and were selected for further investigation. In the present report we describe the isolation, identification and antiplasmodial activity of three tetraprenylated toluquinols (**1**–**3**) and a carotenoid pigment (**4**) from *S. heterophyllum*.

Experimental

General experimental procedures

NMR spectra were recorded in $CDCl_3$ on a Bruker Avance 400 NMR spectrometer using standard pulse sequences. Spectra were referenced to residual solvent resonances at δ_H = 7.25 and δ_C = 77.0. Column chromatography was performed on silica gel 60 (0.04–0.063 mm; Merck, Darmstadt, Germany) and Diaion HP-20 resin (Supelco, Bellefonte, PA, USA). Semi-preparative HPLC was carried out on an HPLC system equipped with a Spectra-Physics IsoChrom pump, a Whatman Partisil 10 semi-preparative column (1 × 50 cm) and a SpectraSeries UV100 detector at a wavelength of 250 nm.

Plant material

Sargassum heterophyllum (Turner) C. Agardh (Sargassaceae) was collected at low tide from Noordhoek near Port Elizabeth, South Africa and identified by J. J. B. A voucher specimen (number NDK06–5) is kept at the Faculty of Pharmacy, Rhodes University, Grahamstown, South Africa.

Extraction and isolation

The frozen alga was submerged in MeOH (500 mL) at 4 °C for 1 h, the solvent decanted and the alga re-extracted with CH_2Cl_2/MeOH (2:1, 3 × 500 mL) at 38 °C for 30 min. The MeOH and CH_2Cl_2/MeOH extracts were separately extracted with CH_2Cl_2 (with the addition of sufficient water for phase separation) and the two organic fractions combined and concentrated. The CH_2Cl_2 extract was prefractionated by successive solvent partitioning between hexane/MeOH/H_2O (5:4:1 v/v/v), CH_2Cl_2/MeOH/H_2O (5:3:2) and EtOAc/H_2O (1:1) to give hexane (Fr. A, 2.0 g), CH_2Cl_2 (Fr. B, 2.0 g), EtOAc (Fr. C, 0.1 g) and aqueous fractions. The aqueous fraction was passed through an HP-20 column (2 × 10 cm) and eluted successively with MeOH (20 mL) and CH_2Cl_2 (20 mL) to give two additional crude organic fractions (Frs. D and E, 0.8 and 0.09 g, respectively). Crude fractions B, C and E were active in the antiplasmodial assay and were thus further fractionated. A portion (500 mg) of the CH_2Cl_2 fraction (Fr. B) was applied to a silica gel column (10 g, 2.5 × 6 cm) and successively eluted with 50 mL portions of an EtOAc/hexane mixture in the ratios of 0:1 (Fr. B1), 1:9 (Fr. B2), 2:8 (Fr. B3), 3:7 (Fr. B4), 4:6 (Fr. B5), 6:4 (Fr. B6), 8:2 (Fr. B7) and 10:0 (Fr. B8) followed by a 1:1 EtOAc/MeOH mixture (Fr. B9). Fractions B4 and B5 gave pure **2** (283 mg), while fractions B2 (3 mg) and B3 (11 mg) were combined and further purified by normal phase HPLC (EtOAc/hexane, 1:9) to give **3** (4.1 mg). Normal phase HPLC of fraction B6 using a mobile phase of EtOAc/hexane (1:1) gave pure **4** (20.9 mg). Silica gel column chromatography (hexane) of the EtOAc fraction (Fr. C) and the HP-20 CH_2Cl_2 fraction (Fr. E) gave pure **1** (99.5 mg).

Sargaquinoic acid (**1**): Yellow oil. – ^{1}H (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$): data for **1** are consistent with literature values (Kusumi *et al*., 1979).

Sargahydroquinoic acid (**2**): Yellow oil. – ^{1}H (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$): data for **2** are consistent with literature values (Segawa and Shirahama, 1987).

Sargaquinal (**3**): Yellow oil. – ^{1}H (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$): see Table II. – EIMS (70 eV): *m/z* (int, %) = 137 (27), 175 (basepeak), 201 (23), 215 (27), 253 (27), 271 (18), 408 (18).

Fucoxanthin (**4**): Red-orange oil. – ^{1}H (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$): data for **4** are consistent with literature values (Mori *et al*., 2004).

Antiplasmodial assay

All samples were tested in duplicate on one occasion against a chloroquine-sensitive strain (D10) of *Plasmodium falciparum*. Sample stock solutions were prepared to a concentration of 2 mg/mL in 10% MeOH or 10% DMSO and 2 μg/mL for the reference drug, chloroquine. All samples were stored at –20 °C until use and were further diluted to the required concentrations in complete medium on the day of the experiment.

The *in vitro* antiplasmodial assays were performed as previously described (Clarkson *et al*., 2004). Continuous *in vitro* cultures of asexual erythrocyte stages of *P. falciparum* were maintained using a modified method of Trager and Jensen (1976). Quantitative assessment of the *in vitro* antiplasmodial activity was determined by the parasite lactate dehydrogenase assay using a modified method described by Makler *et al.* (1993). Percentage parasite viability for test samples was initially tested at three concentrations (50, 25 and 12.5 μg/mL), while the positive control chloroquine diphosphate (Sigma) was tested at concentrations of 30, 15 and 7.5 ng/mL. The 50% inhibitory concentration (IC_{50}) values were obtained using a nonlinear dose-response curve fitting analysis *via* Microsoft Excel and GraphPad Prism v. 4.0 software. The starting concentration of a full dose-response was 100 μg/mL, which was serially diluted 2-fold in complete medium to give 10 concentrations with a lowest concentration of 0.195 μg/mL.

Cytotoxicity assay

Crude fractions and pure compounds showing antiplasmodial activity were also tested for *in vitro* cytotoxicity toward a Chinese hamster ovarian (CHO) cell line using emetine (Sigma) as positive control. All samples were tested in triplicate on one occasion using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay. The MTT assay is a colorimetric assay for investigating the cellular growth and survival, and compares well with other available assays (Mosmann, 1983; Rubinstein *et al*., 1990). The highest concentration of solvent to which the cells were exposed to had no measurable effect on the cell viability. Full dose-responses were done for the selected samples

with a starting concentration of 100 μg/mL, which was serially diluted in complete medium with 10-fold dilutions to give five concentrations, the lowest being 0.01 μg/mL. The starting concentration of emetine was 100 μg/mL, which was serially diluted in complete medium with 10-fold dilutions to give 6 concentrations, the lowest being 0.001 μg/mL. The 50% inhibitory concentration (IC_{50}) values were obtained using a nonlinear dose-response curve fitting analysis *via* Microsoft Excel and GraphPad Prism v. 4.0 software.

Results and Discussion

The MeOH and CH_2Cl_2/MeOH (2:1) extracts of *S. heterophyllum* were prefractionated by solvent partitioning and HP-20 column chromatography to give five fractions (Frs. A–E) that were tested for their antiplasmodial activity against a chloroquine-sensitive strain (D10) of *Plasmodium falciparum*. The most active fractions were the CH_2Cl_2 (Fr. B, IC_{50} = 2.8 μg/mL) and EtOAc (Fr. C, IC_{50} = 5.9 μg/mL) fractions and the CH_2Cl_2 HP-20 column fraction (Fr. E, IC_{50} = of 4.1 μg/mL) (Table I).

Further fractionation and purification of the CH_2Cl_2 fraction (Fr. B) gave sargahydroquinoic acid (**2**), sargaquinal (**3**) and fucoxanthin (**4**) as the main constituents while sargaquinoic acid (**1**) was obtained from the EtOAc fraction (Fr. C) and the CH_2Cl_2 fraction of the HP-20 column (Fr. E).

The ^{13}C NMR spectrum of **1** showed 27 signals of which those at δ 188.0, 188.1 and 172.2 were characteristic of benzoquinone and carboxyl carbonyl carbon atoms, respectively. All spectroscopic data of **1** were in agreement with literature data reported for sargaquinoic acid (Fig. 1) (Kusumi *et al*., 1979).

The noticeable difference between the ^{13}C NMR spectra of **1** and **2** was the absence of the carbonyl signals of the benzoquinone ring in **2**. These were replaced with two phenolic carbon signals at δ 146.4 and 148.7. Thus, compound **2** was considered to be the hydroquinone derivative of sargaquinoic acid. Further analysis of the ^1H and ^{13}C NMR data and comparison with literature values confirmed that **2** was sargahydroquinoic acid (Fig. 1) (Segawa and Shirahama, 1987).

The ^1H and ^{13}C NMR spectra of **3** were similar to those of **1** except for the disappearance of the signal at δ 172.2 in the ^{13}C NMR spectrum and the appearance of a new signal at δ 195.1. This, together with a proton shift at δ 9.33, is indicative of an aldehyde functionality in **3**. Although the ^1H NMR data for **3** were in close agreement with the literature data for sargaquinal (Fig. 1) (Kusumi *et al*., 1979), no additional NMR data for this compound have been reported. We, therefore include here for the first time the complete NMR assignment of this compound (Table II).

Compound **4** was isolated as an red-orange pigment which exhibited 42 resonances in its ^{13}C NMR spectrum which were identical to those reported for fucoxanthin (Fig. 1) (Mori *et al*., 2004).

Compounds **1**–**4** displayed moderate antiplasmodial activity with fucoxanthin (**4**) being the most active one (IC_{50} = 1.5 μM) (Table I). Although the antiplasmodial activities of compounds **1**–**4** may be related to their antioxidant properties, further studies are required in order to clarify their mode of action.

In order to assess the selectivity of fucoxanthin (**4**) and sargaquinal (**3**) for *P. falciparum*, they were evaluated for toxicity against a Chinese hamster ovarian (CHO) cell line (Table I). The relatively low cytotoxicity of fucoxanthin (**4**, IC_{50} = 83.7 μM)

Sample	D10: IC_{50} [μg/mL]	CHO: IC_{50} [μg/mL]	SI
Fr. A (hexane)	ND(A)	ND	ND
Fr. B (CH_2Cl_2)	2.8	3.7	1.3
Fr. C (EtOAc)	5.9	12.3	2.1
Fr. D (HP-20 CH_2Cl_2)	ND(D)	ND	ND
Fr. E (HP-20 MeOH)	4.1	9.8	2.4
Sargaquinoic acid (**1**)	5.1 (12.0 μM)	ND	ND
Sargahydroquinoic acid (**2**)	6.5 (15.2 μM)	ND	ND
Sargaquinal (**3**)	0.8 (2.0 μM)	8.8 (20.7 μM)	11
Fucoxanthin (**4**)	1.3 (1.5 μM)	70.5 (83.7 μM)	54
Chloroquine	9.3 ng/mL		
Emetine		0.07	

Table I. *In vitro* antiplasmodial (D10) and cytotoxic (CHO) activities of crude fractions A–E and compounds **1**–**4**.

ND, not determined.
ND(A), IC_{50} not determined; 40% of parasites survived at a concentration of 12.5 μg/mL.
ND(D), IC_{50} not determined; 55% of parasites survived at a concentration of 12.5 μg/mL.
SI (selectivity index) = antiplasmodial IC_{50}/cytotoxicity IC_{50}.

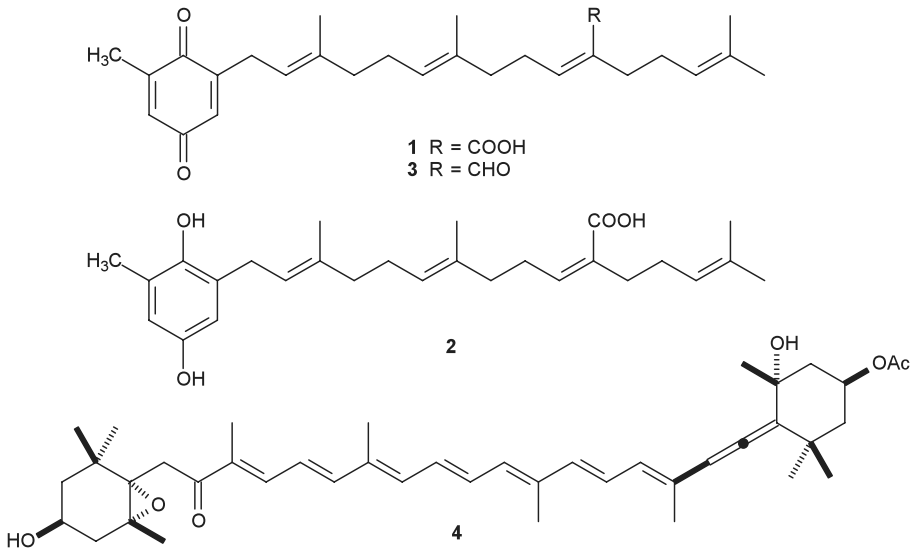

Fig. 1. Chemical structures of sargaquinoic acid (**1**), sargahydroquinoic acid (**2**), sargaquinal (**3**) and fucoxanthin (**4**).

Table II. NMR spectroscopic data for sargaquinal (**3**) in $CDCl_3$.

Carbon no.	δ_C	δ_C mult	δ_H mult, *J* [Hz]	COSY	HMBC
1	188.0	C			
2	146.0	C			
3	132.3	CH	6.54, br s		
4	187.9	C			
5	132.3	CH	6.43, m		
6	148.4	C			
7	16.0	CH_3	2.05, m		C-2, C-1
1′	27.5	CH_2	3.12, d, 7.1	H-2′	C-2′, C-3′, C-6, C-5, C-1
2′	118.2	CH	5.14, m	H-1′	
3′	139.7	C			
4′	39.5	CH_2	2.08, m	H-5′	C-3′, C-5′, C-20′
5′	26.4	CH_2	2.09, m	H-4′, H-6′	C-3′
6′	125.1	CH	5.11, m	H-5′	
7′	133.9	C			
8′	38.3	CH_2	2.14, m	H-9′	C-6′, C-7′
9′	27.0	CH_2	2.44, q, 7.45	H-8′, H-10′	C-8′, C-11′
10′	154.9	CH	6.41, m	H-9′	C-12′, C-18′
11′	143.2	C			
12′	27.5	CH_2	2.25, t, 7.62	H-13′	C-9′, C-11′
13′	25.7	CH_2	2.14, m	H-14′	C-12′
14′	123.6	CH	5.14, m	H-13′	C-12′
15′	133.2	C			
16′	24.3	CH_3	1.66, s		C-14′, C-15′, C-17′
17′	17.7	CH_3	1.56, br s		C-13, C14, C15
18′	195.1	CH	9.33, s		C-11′
19′	16.0	CH_3	1.63, s		C-6′, C-7′,
20′	16.1	CH_3	1.62, s		C-2′, C-3′, C-4′

in this assay translates into a promising selectivity index (SI = antiplasmodial IC_{50}/cytotoxicity IC_{50}) of 54. Fucoxanthin (**4**), which is commonly found in a number of edible marine brown algae such as *Hijikia fusiformis* (Yan *et al*., 1999), is also known for its antitumour (Kotake-Nara *et al*., 2005; Nishino, 1998), antiangiogenic (Sugawara *et al*., 2006) and antiobesity properties (Maeda *et al*., 2005).

Sargaquinoic acid (**1**) and sargahydroquinoic acid (**2**) are common constituents of a number of medicinal plants known for their antioxidant and anti-inflammatory activities (Pérez-Castorena *et al*., 2002). Additionally, sargaquinoic acid also shows neuroprotective effects (Tsang and Kamei, 2004) and is a potent cholinesterase inhibitor (Choi *et al*., 2007). No previous biological activity has been reported for sargaquinal (**3**).

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