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Antiplasmodial halogenated monoterpenes from the marine red alga *Plocamium cornutum*

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ABSTRACT

In our continuing search for antimalarial leads from South African marine organisms we have examined the antiplasmodial organic extracts of the endemic marine red alga *Plocamium cornutum* (Turner) Harvey. Two new and three known halogenated monoterpenes were isolated and their structures determined by standard spectroscopic techniques. The 3,7-dimethyl-3,4-dichloro-octa-1,5,7-triene skeleton is common to all five compounds. Interestingly, compounds bearing the 7-dichloromethyl substituent showed significantly higher antiplasmodial activity toward a chloroquine sensitive strain of *Plasmodium falciparum*.

1. Introduction

Malaria is still a worldwide health problem and impacts most devastatingly on developing nations, especially in Africa (Greenwood et al., 2005). Widespread resistance to commonly used antimalarial drugs such as chloroquine and quinine has necessitated the search for new lead compounds to ensure a supply of new drugs (Hyde, 2005). While terrestrial plants have been a productive source of drugs and lead compounds for the treatment of malaria, the marine environment has received relatively little attention in this context. In recent years however, a number of important antimalarial leads such as manzamine A, the phloeodictynes, homofascaplysin A and the plakortides have been reported from marine invertebrates (for a recent review of antiplasmodial marine natural products see Laurent and Pietra (2006)). Therefore, in our continuing search for new antimalarial lead compounds from South African marine organisms we have screened a number of algae for activity against a chloroquine sensitive strain of *Plasmodium falciparum*. Organic extracts of *Plocamium cornutum* (Turner) Harvey showed promising activity (IC_{50} < 10 μg/mL) in this assay and were selected for further study.

The genus *Plocamium* contains more than 40 species that are widely distributed throughout the world's oceans (Saunders and Lehmkuhl, 2005). These algae typically produce acyclic and cyclic halogenated monoterpenes of which more than 100 have been reported. Biological activities associated with these compounds include antimicrobial, antifungal, ichthyotoxic, cytotoxic and insecticidal activities (Kladi et al., 2004). We have previously isolated a series of new and known halogenated monoterpenes from the endemic Southern African marine alga *P. corallorhiza* (Knott et al., 2005; Mann et al., 2007), however, no previous phytochemical investigation of *P. cornutum* had been reported. Here, we report the isolation, structures and antiplasmodial activity of three known (**1**–**3**) and two new (**4** and **5**) halogenated monoterpenes from *P. cornutum*. For comparison, we also report the antiplasmodial activities of halogenated monoterpenes previously isolated from *P. corallorhiza* (Mann et al., 2007).

2. Experimental

2.1. General experimental

UV spectra were obtained using a GBC UV/vis 916 spectrophotometer while optical rotations were measured on a Perkin–Elmer 141 polarimeter. Infrared spectra were obtained using a Perkin–Elmer Spectrum 2000 FT-IR spectrometer as films on KBr disks. HRFABMS were obtained by Dr Louis Fourie at the University of the North West, Potchefstroom on a VG-7070E mass spectrometer. All NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer using standard pulse sequences. NMR spectra were referenced to residual $CHCl_3$ solvent signals (δ_H 7.25 and δ_C 77.0).

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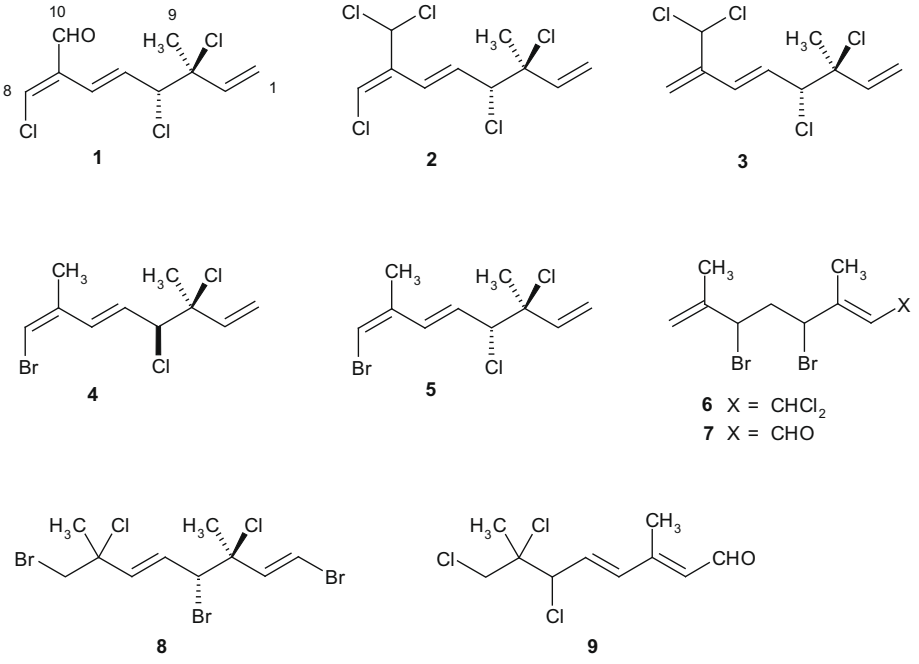

2.2. Plant material

P. cornutum is endemic to temperate coasts of southern Africa, from Northern Namibia (Rocky Point) to the Eastern Cape of South Africa (Morgan Bay). It is a very distinctive *Plocamium* with unusually fleshy, overlapping branchlets, main axes up to 1.5 mm in diameter, and with a reddish-brown colour (see Stegenga et al. (1997) for detailed description and illustrations). It occurs in dense stands in the mid to lower intertidal of rocky shores with heavy wave action (Stegenga et al., 1997).

Specimens of *P. cornutum* was collected at low tide from Kalk Bay on the west coast and Noordhoek on the south east coast of South Africa and identified by JJB. Voucher specimens (KB06-03 and NDK06-36) is kept at the Division of Pharmaceutical Chemistry, Rhodes University, Grahamstown, South Africa.

2.3. Extraction and isolation

P. cornutum (KB06-3, 26.02 g dry weight) was stored frozen until it was extracted. The frozen sample was sequentially extracted with MeOH and MeOH–CH_2Cl_2 (1:2) to give two organic extracts that were combined and concentrated *in vacuo*. The crude extract was partitioned between hexane and MeOH–H_2O (9:1) and the hexane partition layer collected. The water content of the aqueous MeOH fraction was increased to 20% and was extracted with CH_2Cl_2. Finally, the MeOH was removed under vacuum and the remaining aqueous fraction extracted with EtOAc. The hexane, CH_2Cl_2 and EtOAc fractions (A–C) were dried over anhydrous sodium sulphate and evaporated under reduced pressure to give a hexane fraction (Fr A, 784 mg), a CH_2Cl_2 fraction (Fr B, 706 mg) and an EtOAc fraction (Fr C, 135 mg). Silica gel column chromatography of the hexane fraction (A) using a step gradient of *n*-hexane–EtOAc of increasing polarity afforded nine fractions (A1–A9). Fraction A1, which eluted with 100% hexane, was fractionated by semi-preparative normal phase HPLC using hexane as mobile phase to give a 1:1 mixture of compounds **4** and **5** (fraction A1a, 33.0 mg) and pure **2** (46.3 mg). Repeated semi-preparative normal phase HPLC of fraction A1a using 100% hexane as mobile phase gave pure **4** (13.3 mg) and **5** (18.6 mg). Fraction A2, which eluted with hexane–EtOAc (9:1), was also fractionated by semi-preparative normal phase HPLC using 100% hexane as mobile phase to give pure **3** (3.8 mg) and more **2** (7.5 mg). Silica gel column chromatography of the CH_2Cl_2 fraction (B) using a step gradient of *n*-hexane–EtOAc of increasing polarity afforded nine fractions (B1–B9) of which B1 (100% hexane) was pure **2** (131.7 mg) and B2 (hexane–EtOAc, 9:1) was pure **1** (65.4 mg). The percentage isolated yield (based on extracted and dried alga) for each compound was as follows: **1** (64.4 mg, 0.25%), **2** (185.5 mg, 0.71%), **3** (3.8 mg, 0.015%), **4** (13.3 mg, 0.05%) and **5** (18.6 mg, 0.071%).

P. cornutum, collected from Noordhoek (NDK06-36, 16.93 g dry weight), was extracted following the same procedure as KB06-3. The ^{1}H NMR spectrum of the combined MeOH and MeOH–CH_2Cl_2 (1:2) extract showed only the presence of compound **2** (1.14 g, 6.7% dry weight).

2.4. (1*Z*,3*E*,5*S**,6*S**)-1-bromo-5,6-dichloro-2,6-dimethyl-octa-1,3,7-triene (**4**)

Colourless oil: $[\alpha]_D$ +5.0° (*c* 0.09, hexane); λ_{max} (hexane) 252 nm; IR ν_{max} (KBr): 1460, 2852 and 2921 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) (see Table 1); HMBC correlations, H-1/C-3; H-4/C-5, C-6, C-9; H-5/C-3, C-8; H-6/C-4, C-8, C-10; H-10/C-6, C-7, C-8; H-9/C-2, C-3, C-4; GC-EIMS (70 eV) *m*/*z* (rel. int.): 131 $[M–Br–Cl_2]^+$ (32), 167/169 $[M–Br–Cl]^+$ (52/19), 193/195/197 $[M–C_4H_6Cl]^+$ (29/43/12), 203/205 $[M–Br]^+$ (19/12); HRFABMS obsd. *m*/*z*. 281.9572; calc. *m*/*z* 281.9578.

2.5. (1*Z*,3*E*,5*R**,6*S**)-1-bromo-5,6-dichloro-2,6-dimethyl-octa-1,3,7-triene (**5**)

Colourless oil: $[\alpha]_D$ −38.6° (*c* 0.15, hexane); λ_{max} (hexane) 252 nm; IR ν_{max} (KBr): 1460, 2852 and 2921 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) (see Table 1); HMBC correlations, H-1/C-3; H-4/C-2, C-3, C-6; H-5/C-3, C-4, C-7; H-6/C-5, C-7, C-8; H-8/C-7; H-10/C-6, C-7; H-9/C-2, C-3, C-4; GC-EIMS (70 eV) *m*/*z* (rel. int.): 131 $[M–Br–Cl_2]^+$ (32), 167/169 $[M–Br–Cl]^+$ (52/19), 193/195/197 $[M–C_4H_6Cl]^+$ (29/43/12), 203/205 $[M–Br]^+$ (19/12); HRFABMS obsd. *m*/*z*. 281.9579; calc. *m*/*z* 281.9578.

Table 1
^{1}H (400 MHz, $CDCl_3$) and ^{13}C (100 MHz, $CDCl_3$) NMR data for compounds **4** and **5**.

Carbon[a]	**4**		**5**	
1a	117.0	5.30, d, 10.6	116.2	5.27, d, 10.6
1b		5.45, d, 16.9		5.39, d, 17.0
2	138.3	6.11, dd, 10.7, 16.9	139.7	6.06, dd, 10.6, 17.0
3	72.2	–	71.9	–
4	69.7	4.60, d, 9.1	69.5	4.59, d, 9.0
5	128.9	5.95, dd, 9.1, 15.5	128.7	5.96, dd, 9.1, 15.5
6	132.3	6.83, d, 15.5	132.3	6.83, d, 15.5
7	135.6	–	135.6	–
8	107.5	6.17, s	107.6	6.17, br s
9	27.9	1.78, s	25.3	1.77, br s
10	19.5	1.89, s	19.5	1.89, br s

[a] For ease of comparison with literature data, the atom numbering used for compounds **1** – **5** is consistent with that reported by Mynderse and Faulkner (1975).

2.6. Antiplasmodial assay

Compounds **1**–**9** were tested in duplicate against a chloroquine sensitive (CQS) *P. falciparum* D10 strain. Stock solutions (2 mg/mL) of compounds **1**–**9** in 10% MeOH or DMSO were stored at −20 °C until use and further diluted to the required concentration in complete medium on the day of the experiment.

The *in vitro* antiplasmodial assays were performed as previously described (Clarkson et al., 2004). The continuous *in vitro* culture of the asexual erythrocytic stage used in the experiment was maintained using a modified method of Trager and Jensen (1976). Quantitatively assessment of the *in vitro* antiplasmodial activity was determined by the lactate dehydrogenase assay by using a modified method described by Makler et al. (1993). The full dose–response experiment of the compounds was started at a concentration of 100 μg/mL and was serially diluted 2-folds to give 10 concentrations with a minimum of 0.195 μg/mL. Chloroquine was tested at a starting concentration of 100 ng/ml. The highest concentration of solvent used did not have any measurable effect on the parasites. The 50% inhibitory concentration (IC_{50}) values were obtained using a non-linear dose–response curve fitting analysis via GraphPad Prism v.4.0 software.

3. Results and discussion

Specimens of *P. cornutum* were successively extracted with MeOH and MeOH–CH_2Cl_2 (1:2) and the organic extracts prefractionated by solvent–solvent partitioning. The antiplasmodial hexane and CH_2Cl_2 fractions were further fractionated and purified by silica gel column chromatography and semi-preparative normal phase HPLC to give pure compounds **1**–**5**. In an attempt to isolate more of these compounds for biological studies we extracted the second collection of *P. cornutum* which was obtained from Noordhoek, near Port Elizabeth. Interestingly, the ^{1}H NMR spectrum of the crude organic extract obtained from this collection indicated the presence of mainly compound **2** with only minute quantities of other metabolites. This variation is not due to a seasonal effect since the two collections were made within a two week period. Subsequent collections of this alga further confirmed this geographical variation of halogenated metabolites.

Compounds **1**–**3** were previously reported from *P. cartilagineum* and their structures were confirmed by comparison of their ^{1}H and ^{13}C NMR data with literature values (Abreu and Galindro, 1996; Crews and Kho, 1974; Mynderse and Faulkner, 1975). All five compounds contain the same C1–C6 unit which is discernible by two distinct AMX spin systems in their ^{1}H NMR spectra. For example, in compound **4**, the first AMX spin system appears at δ 5.30 (d, 10.6 Hz, H-1a), 5.45 (d, 16.9 Hz, H-1b) and 6.11 (dd, 10.7, 16.9 Hz, H-2) which is consistent with a monosubstituted double bond (Table 1). The second AMX system is evident at δ 6.83 (d, J = 15.5 Hz, H-6), 5.95 (dd, J = 15.5, 9.1 Hz, H-5) and δ 4.60 (d, J = 9.1 Hz, H-4) and is characteristic of a –CH=CH–CH(X)– moiety (Table 1). From this, it was clear that any structural differences between compounds **1**–**5** must be accounted for by the C7–C8 fragment or the stereochemistry of the compounds. The HRFABMS spectrum of **4** showed a molecular ion at m/z 281.9572 consistent with a molecular formula of $C_{10}H_{13}BrCl_2$ and three degrees of unsaturation. This, together with a signal at δ 107.5 in the ^{13}C NMR spectrum of **4** was evidence of a vinyl bromide moiety. In addition, the dichloromethyl group (δ_H 6.75 and δ_C 69.4) in **2** has been replaced by a vinyl methyl (δ_H 1.89 and δ_C 19.5) in **4**. The complete planar structure of **4** was confirmed by analysis of HMBC and ^{1}H–^{1}H COSY data. HMBC correlations from the vinyl methyl protons at δ 1.89 to the vinyl bromide carbon (δ 107.5) and two olefinic carbons at δ 135.6 and 132.3 is consistent with the substructure CH(X)=C(CH_3)–CH=. The second substructure, =CH–CH(X)–CX(CH_3)–CH=, was deduced from HMBC correlations from the methyl protons at δ 1.78 to δ 138.3, 72.2 and 69.7. An *E*-geometry was assigned to the $\Delta^{5,6}$ double bond from the coupling constants ($J_{5,6}$ = 15.5 Hz) for H-5 and H-6 while a NOESY correlation between H-8 (δ 6.17) and CH_3-10 (δ 1.89) was consistent with a *Z*-geometry for the $\Delta^{7,8}$ double bond. The assignment of *R* or *S* configurations to the chiral centers in halogenated monoterpenes is not trivial. Fortuitously, Mynderse and Faulkner (1975) observed, for compounds whose structures were confirmed by X-ray crystallography, that the ^{1}H NMR chemical shifts of CH_3-9 varied in a predictable manner depending on the relative configurations at C-3 and C-4. That is, a CH_3-9 chemical shift of δ 1.79 is characteristic of a 3R*,4R* relative configuration whereas δ 1.73 is characteristic of a 3S*,4R* configuration. Crews (1977) expanded these rules by making use of the more reliable and more discernable differences in ^{13}C chemical shifts for the CH_3-9 carbon atom. Chemical shifts of ca. δ 28 and 25 are characteristic of 3R*, 4R* (or 3S*, 4S*) and 3S*, 4R* (or 3R*, 4S*) relative configurations, respectively. Thus, the chemical shift of δ 27.9 is consistent with a 3R*, 4R* (or 3S*, 4S*) relative configuration and completed the structural assignment of compound **4**.

A molecular formula of $C_{10}H_{13}BrCl_2$ for compound **5** was also deduced from HRFABMS data. The ^{1}H and ^{13}C NMR spectra of compounds **4** and **5** were almost identical with the main difference being a chemical shift of δ 25.3 for CH_3-9, in compound **5**. This is consistent with a 3S*,4R* (or 3R*, 4S*) relative configuration. All 2D NMR data are in agreement with the proposed structure for compound **5**.

The biosynthesis of all known halogenated monoterpenes, including **1**–**5**, can be rationalized by pathways involving Markovnikov addition of halogens (Br^+/Cl^+) into an ocimene or myrcene precursor (Naylor et al., 1983). These reactions are catalyzed by haloperoxidases which oxidize Br^- and Cl^- to "Br^+" and "Cl^+" in the presence of H_2O_2 (Butler and Carter-Franklin, 2004). It is likely that the aldehyde moiety in **2** is produced via hydrolysis of the *gem*-dichloride moiety. However, this needs to be verified by experiment.

Compounds **1**–**5** from *P. cornutum* together with compounds **6**–**9** which were available from a previous study of *P. corallorhiza* (Mann et al., 2007; Knott et al., 2005) were evaluated for antiplasmodial activity against the chloroquine sensitive strain of *P. falciparum* (Table 2). Although the compounds tested here were significantly less active than the standard (chloroquine IC_{50} = 0.036 μM) it is interesting to note that compounds **2** and **3** containing the 7-dichloromethyl moiety were the most active (IC_{50} values of 16 and 17 μM, respectively) followed by compound **1** which contains an aldehyde functional group at this position. Almost all the other compounds were essentially inactive, including

Table 2
In vitro antiplasmodial activity and cytotoxicity of compounds **1**–**9** against CQS D10 strain.

Compound	Antiplasmodial activity IC_{50} (μM)
1	27
2	16
3	17
4	230
5	210
6	95
7	142
8	>225
9	172
Chloroquine	0.036

plocoralide A (**6**) which contains a 1-dichloromethyl moiety. The importance of this functional group at position 7 is further emphasized when considering the lack of antiplasmodial activity of other related halogenated monoterpenes (König et al., 1999).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2009.02.010. Tables of ^{1}H and ^{13}C NMR data and spectra for compounds **1**–**5**.

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