PERGAMON

Phytochemistry 52 (1999) 1047–1053

PHYTOCHEMISTRY

Plocamium hamatum and its monoterpenes: chemical and biological investigations of the tropical marine red alga[☆]

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Received 25 January 1999; received in revised form 1 April 1999; accepted 18 May 1999

Abstract

The polyhalogenated monoterpene content of six samples of the tropical marine red alga *Plocamium hamatum*, collected from the southern, central and northern regions of The Great Barrier Reef, Australia, was assessed. In all but two of the samples, the polyhalogenated monoterpene content was shown to differ markedly. In total, eleven previously reported compounds were isolated and characterised (**1**–**11**). Compound **2** was obtained for the first time as a pure natural product. For compound **4** a single crystal X-ray crystallographic analysis was undertaken which established its absolute configuration as (1*S*,2*S*,4*R*,5*R*,1′*E*)-2-bromo-1-bromomethyl-1,4-dichloro-5-(2′-chloroethenyl)-5-methylcyclohexane. Complete and unambiguous ^{1}H and ^{13}C NMR data are reported for **2** and **4**. For **6**–**8**, some prior ^{13}C NMR assignments are revised. The biological activities of compounds **2**–**8** and **11** were assessed and indicated **4** to have potent antialgal activity towards *Chlorella fusca* in an agar diffusion bioassay, as well as being moderately antitubercular and cytotoxic. Compound **6** demonstrated moderate cytotoxicity. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Plocamium hamatum; Halogenated monoterpenes; X-ray; Antialgal; Biological activity; *Chlorella fusca*; Great Barrier Reef

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1. Introduction

Plocamium hamatum J. Agardh (Gigartinales, Plocamiaceae) is a red algal species commonly found on The Great Barrier Reef (GBR), Australia. It grows on inshore, midshelf and outer reefs in relatively well shaded habitats, on stony substrates at variable depths, usually 2–12 m. The surfaces of these plants are typically not fouled by other algal species or other common fouling organisms. It also seems that *P. hamatum* does not form a main dietary component for any of the many reef herbivores.

A number of studies of *Plocamium* species have shown that the secondary metabolite chemistry of the collected materials seems to be dependent upon when and where a particular sample is collected (Capon et al., 1984; Coll, Skelton, White & Wright, 1988; Crews, Kho-Wiseman & Montana, 1978; Higgs, Vanderah & Faulkner, 1977; Naylor, Hanke, Manes & Crews, 1983). In the current study six samples of *P. hamatum* from the northern, central and southern regions of the GBR were investigated for their secondary metabolite content, affording eleven halogenated monoterpenes. Thorough NMR investigations allowed the spectroscopic data for compounds **2**, **4** and **6**–**8** to be unambiguously assigned. The absolute configuration of the strongly antialgal metabolite **4** was established by X-ray crystallography.

2. Results

Samples of *P. hamatum* were collected from six different locations on the GBR; Milne and Telford

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PII: S0031-9422(99)00284-8

Table 1
Plocamium hamatum: Collection site and identified natural products

Location	Compounds (Reference)[a]
Milne Reef	**1** (3–6, 8), **2** and **3** (6), **4** (4, 8), **5** (8), **6** (6)
Telford Reef	**1** (3–6, 8), **2** and **3** (6), **4** (4, 8), **5** (8), **6** (6)
Orpheus Island	**7** (6)
Rib Reef	**2** and **3**, **7–9** (6)
Potter Reef	**2**, **3**, **6** and **10** (6)
Heron Island	**2** and **3** (6)

[a] (3–6, 8) = Crews et al. (1978), Higgs et al. (1977), Capon et al. (1984), Coll et al. (1988), Stierle & Sims (1979). (6) = Coll et al. (1988). (4, 8) = Higgs et al. (1977), Stierle & Sims (1979). (8) = Stierle & Sims (1979).

Reefs (Cairns Section), Orpheus Island, Rib and Potter Reefs (Central Section) and Heron Island (Mackay/Capricorn Section).

All *P. hamatum* samples, except the one from Orpheus Island, contain the acyclic and highly halogenated monoterpenes **2** and **3**. The Orpheus Island sample is also the only sample to have a predominance ($>95\%$) of a single halogenated monoterpene (**7**), all other samples containing two to five related cyclic and acyclic terpenoid compounds. The results of the phytochemical investigations are summarised in Table 1.

2.1. ^{1}H and ^{13}C NMR assignments and reassignments

For compounds **2** and **4** complete and unambiguous ^{1}H and ^{13}C NMR data are reported in Table 2. From the INADEQUATE spectrum of **7** it was evident that the original ^{13}C NMR assignments made for C-3 and C-6 (Coll et al., 1988) had been reversed and have to be 39.8 (t), 52.7 (t), respectively, ^{1}H NMR data were, however, correctly assigned. This result also indicates that the corresponding assignments for C-3 and C-6 in mertensene (**8**) (Coll et al., 1988) have to be reversed as well. From the ^{1}H-^{13}C shift-correlated 2D NMR (proton non-decoupled HMQC) spectrum of **6** it was clear that the original assignments made for C-5 and C-7 (Coll et al., 1988) were also reversed and should be 59.8 (d) and 62.8 (d), respectively.

2.2. X-ray Crystallographic analysis of 4

As **4** had significant biological activity and its absolute configuration was unknown, a single crystal X-ray crystallographic analysis was undertaken. The crystal data are given in Table 3. The crystal structure of **4** revealed no unusual features. As the compound crystallises in a polar space group, it is enantiomerically pure and the absolute structure was confidently confirmed by the diffraction experiment (see Experimental). There are two symmetry-independent molecules in the asymmetric unit. Both molecules are of the same enantiomer and there are no significant differences in the conformations of the two molecules. The maximum difference in the torsion angles is a twist of approximately 7° about the C(5)–C(8) bond. As depicted in Fig. 1, compound **4** has the 1*S*,2*S*,4*R*,5*R* configuration, which is opposite to that indicated by earlier workers (Higgs et al., 1977; Stierle & Sims, 1979). It should be noted that no attempts were made to determine the optical purity of **4**.

2.3. Biological activity assessments

The antifungal, antibacterial and antialgal activities of **2**–**8** and **11** were evaluated against the fungi *Ustilago violacea* (Pers.) Roussel (Ustomycetes),

Table 2
^{1}H ($CDCl_3$, 300 MHz) and ^{13}C NMR ($CDCl_3$, 75.5 MHz) data of **2** and **4**[a]

Carbon	δ_C (**2**)	δ_H (**2**)	δ_C (**4**)	δ_H (**4**)
1	116.3 (t)[b]	5.28 (d, *J* 10.7 Hz) 5.39 (d, *J* 17.3 Hz)	70.9 (s)	
2	139.8 (d)	6.07 (d, *J* 10.7, 17.3 Hz)	51.2 (d)	4.48 (d, *J* 4.1, 12.4 Hz)
3	71.4 (s)		39.1 (t)	2.58 (ddd, *J* 4.1, 4.1, 13.6 Hz) 2.78 (ddd, *J* 12.4, 12.4, 13.6 Hz)
4	60.7 (d)	4.64 (d, *J* 9.2 Hz)	64.6 (d)	3.69 (dd, *J* 4.1, 12.4 Hz)
5	128.7 (d)	6.08 (d, *J* 9.2, 15.3 Hz)	42.0 (s)	
6	136.7 (d)	5.93 (d, *J* 15.3 Hz)	48.5 (t)	2.27 (d, *J* 15.5 Hz) 2.40 (d, *J* 15.5 Hz)
7	67.1 (s)		40.4 (t)	3.57 (d, *J* 10.9 Hz) 3.95 (d, *J* 10.9 Hz)
8	41.6 (t)	3.68 (d, *J* 10.7 Hz) 3.72 (d, *J* 10.7 Hz)	135.4 (d)	6.56 (d, *J* 13.6 Hz)
9	27.8 (q)	1.83 (s)	119.0 (d)	6.07 (d, *J* 13.6 Hz)
10	25.7 (q)	1.81 (s)	27.4 (q)	1.28 (s)

[a] All assignments are based on extensive 1D and 2D-NMR experiments.
[b] Implied multiplicity by DEPT (s = C, d = CH, t = CH_2, q = CH_3).

Table 3
Crystallographic data for **4**

Crystallised from	Hexane
Empirical formula	$C_{10}H_{13}Br_2Cl_3$
Formula weight (g mol^{-1})	399.38
Crystal colour, habit	colourless, prism
Crystal dimensions (mm)	0.30 × 0.35 × 0.35
Temperature (K)	173 (1)
Crystal system	orthorhombic
Space group	$P2_12_12_1$
Z	8
Reflections for cell determination	25
2θ range for cell determination (°)	33–40
Unit cell parameters	
a (Å)	13.067(5)
b (Å)	17.295 (4)
c (Å)	12.606 (4)
V (Å^3)	2849 (2)
D_x (g cm^{-3})	1.862
μ (MoK_α) (mm^{-1})	6.238
$2\theta_{(\max)}$ (°)	55
Transmission factors (min; max)	0.698; 1.000
Total reflections measured	8462
Symmetry independent reflections	6520
Reflections used ($I > 2\sigma(I)$)	4054
Parameters refined	271
R	0.0446
wR	0.0286
Goodness of fit	1.254
Final $\Delta_{\max}/\sigma$	0.0007
$\Delta\rho$ (max; min) (e Å^{-3})	0.70; −0.77

Mycotypha microspora Fenner (Zygomycetes), *Eurotium repens* Corda (Ascomycetes) and *Fusarium oxysporum* Schltdl. (Deuteromycetes), the bacteria *Bacillus megaterium* de Bary (gram +ve) and *Escherichia coli* (Migula) Castellani and Chambers (gram −ve) and the alga *Chlorella fusca* Shih Krauss (Chlorophyceae) as described by Schulz et al., (1995). The results presented in Table 4 indicate that, with the exception of compounds **4** and **11**, all metabolites are essentially inactive. Compound **11**, an artefact derived from **1**, has weak antimicrobial properties. Compound **4** proved to be potently active against the alga *Chlorella fusca*. This activity was further assessed at the 22 µg level and compared with that of irgarol 1071 (**12**), a commercial antialgal agent. At the 22 µg level **4** gave an inhibition zone of 0.9 cm radius in the agar diffusion bioassay. In contrast, irgarol 1071 was inactive in the same assay at the 25 µg level. Further testing with **4** revealed that it still has antialgal activity at the 11 µg level (total inhibition on the filter disc but no inhibition on the agar around the disc) and partial activity at the 2.8 µg level, but not at 1.4 µg. It was possible to show that the antialgal activity of **4** was not due to the inhibition of photosynthesis (König, Wright, Draeger, Aust & Schultz). Compounds **3**, **4** and **6** were also tested for their antimalarial (Angerhofer et al., 1992), antitubercular (TB) (Collins & Franzblau, 1997) and cytotoxic properties (Likhitwitayawuid, Angerhofer, Ruangrungsi, Cordell & Pezzuto, 1993). All three compounds showed no antimalarial activities, **3** and **6** were TB inactive and **3** had no cytotoxic properties. Compound **4**, aside from being antialgal, was also moderately cytotoxic (IC$_{50}$:

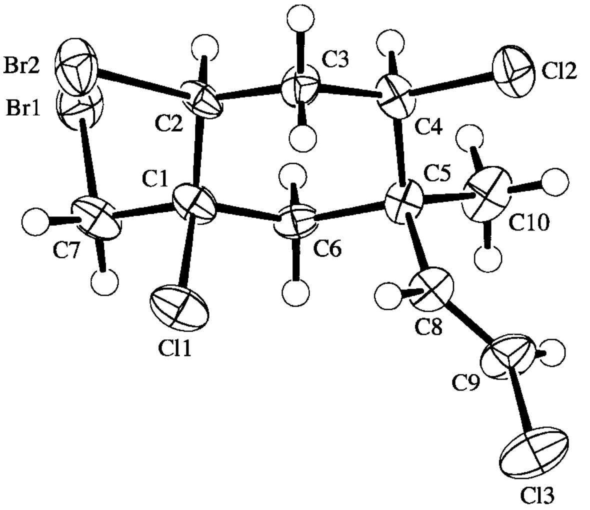


Fig. 1. *ORTEP* (Johnson, 1976) representation of one of the two symmetry-independent molecules in the crystal structure of **4** (50% probability ellipsoids; H-atoms given arbitrary thermal parameters for clarity).

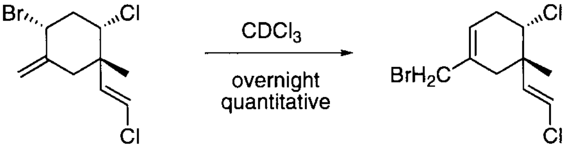
Table 4
Antifungal, antibacterial and antialgal activities of compounds **2**–**8**, and **11**

	Radius of inhibition zone (cm)						
Cpd (µg)	Fungi				Bacteria		Alga
	U.v.[a]	M.m.	E.r.	F.o.	B.m.	E.c.	C.f.
2 (30)	0	0.2	0	0	0.1	0	0
3 (28)	0	0.2	0	0	0.1	0	0
4 (44)	0.1	0.2	0	0	0.2	0	1.6
5 (40)	0	0	0	0	0.1	0	0.1
6 (45)	0	0	0	0	0	0	0
7 (25)	0	0	0	0	0.2	0	0
8 (40)	0	0	0	0	0.1	0	0.3
11 (37)	0.6	0.2	0.2	0.3	0	0.2	0.3
Controls							
Bp (85)[b]					2.0	0	
St (65)					0.5	0.3	
Mi (50)	Total[c]	0.7	1.8	0.1			
Cy (60)	Total	0.4	0.5	0.3			

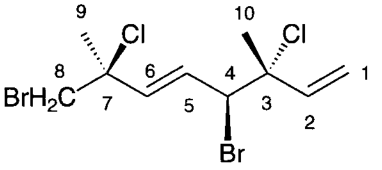
[a] Test Organisms: U.v. = *Ustilago violacea*, M.m. = *Mycotypha microspora*, E.r. = *Eurotium repens*, F.o. = *Fusarium oxysporum*, B.m. = *Bacillus megaterium*, E.c. = *Escherichia coli*, C.f. = *Chlorella fusca*.

[b] +ve Controls: Bp = Benzylpenicillin Potassium Salt, St = Streptomycin Sulphate, Mi = (±)-Miconazol Nitrate, Cy = Cycloheximide.

[c] Total indicates that the control substance completely inhibited fungal growth on the test plate.



1 **11**



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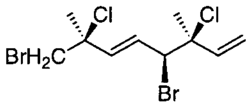

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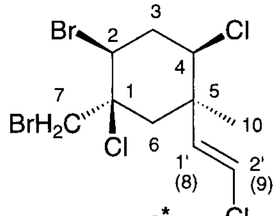
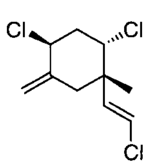


4*



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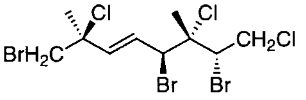

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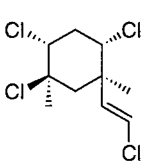

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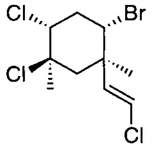

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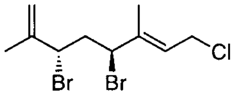

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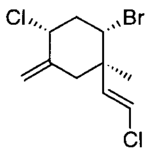

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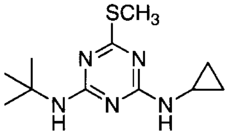

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*Numbers in brackets are those used in Table 2.

Lul 12.9 μg/ml, KB 13.3 μg/ml, ZR-75-1 7.8 μg/ml) and antitubercular (32 μg/ml minimum inhibition concentration (MIC) towards *Mycobacterium tuberculosis*, 64 μg/ml MIC towards *M. avium*). Compound **6** was moderately cytotoxic (IC$_{50}$: KB-V (-VBL) 5.3 μg/ml, KB 12.4 μg/ml, LNCaP 14.8 μg/ml). Compounds **2–8** and **11** were also tested for their HIV-1 reverse transcriptase inhibition activity (Eberle & Seibl, 1992) and all were found to be inactive.

3. Discussion

The secondary metabolite content of the six samples of *P. hamatum* varies considerably (Table 1). This could be due to prevailing environmental stresses at the respective collection sites. For example; the alga growing in the channel between Orpheus and Pelorus Islands was taken from an environment dominated by hard and soft corals. The single predominant metabolite from this sample was **7**, a compound which is able to cause tissue necrosis of soft corals (de Nys, Coll & Price, 1991). Other samples were collected from the ocean side fringing reefs of Heron Island, where the feeding habits and preferences of the local green turtle (*Chelonia midas*) population were being studied. Investigations made by G. Forbes, Department of Zoology, James Cook University, Townsville, Australia, during the turtle feeding study indicated that all of the algae in the region, with the exception of *P. hamatum*, had, at some time, been ingested by the turtles. This observation suggests that for whatever reason *P. hamatum* is not a preferred food source of the local turtles.

It is evident that more studies aimed at investigating the ecological roles of these secondary metabolites need to be made to assess the role(s) of monoterpenes isolated in this and related studies, so as to clearly help in the understanding of why an apparent single species produces such an array of secondary metabolites in relatively high concentrations.

The biological activities of compound **4** are remarkable, above all its potent antialgal effect. This potent activity is only observed for compound **4** and not for the other closely related cyclic monoterpenes (**5**, **7**, **8**, **11**), suggesting a mode of action that is structure specific. A similar effect is seen when the cytotoxic activity of compounds **3** and **6** is compared. Despite close structural similarity compound **3** proved to be not cytotoxic, whereas **6** clearly demonstrates this type of activity.

4. Experimental

4.1. General experimental procedures

As previously reported (Coll et al., 1988; Wright et al., 1996).

4.2. Materials

As previously reported (Coll et al., 1988; Wright et al., 1996).

4.3. Natural materials

Orpheus Island see (Coll et al., 1988), Potter Reef see (Coll et al., 1988) and Rib Reef see (Coll et al., 1988). From Heron Island samples were collected during 1987 and 1988 from −2 to −8 m by divers and then frozen. At Milne Reef, alga were collected in July, 1991 by divers from crevices and caves in the 5–15 m depth range and then frozen. The Telford Reef samples of *P. hamatum* were also collected in July of 1991 by divers working at depths of 5–10 m.

4.4. Extraction of natural materials

Orpheus Island see (Coll et al., 1988), Potter Reef see (Coll et al., 1988) and Rib Reef see (Coll et al., 1988). A representative Heron Island sample was freeze dried and the dry tissue (72.8 g) extracted with dichloromethane (CH_2Cl_2, 3 l). The resultant extract (0.87 g, 1.2%) was chromatographed over silica (VLC), employing gradient elution from hexane to EtOAc, to yield 8 fractions each of 90 ml. Fractions 1–4 were predominantly **2**, **3** and lipid, which were separated from each other by HPLC, using normal phase silica and hexane as eluent. The Telford Reef samples of *P. hamatum* were freeze dried and the dry tissue (55.4 g) extracted with dichloromethane (CH_2Cl_2, 2 l). The resultant extract (0.87 g, 1.57%) was chromatographed over silica (VLC), employing gradient elution from hexane to EtOAc, to yield 8 × 90 ml fractions. By ^{1}H NMR and TLC analysis fractions 2–5 were identified as those containing halogenated monoterpenes. HPLC separations of these fractions using normal phase silica and hexane, or hexane–acetone mixtures (99.5:0.5 or 99:1), as eluents afforded compounds **1**–**6**. Freeze dried alga (44.9 g) collected from Milne Reef were extracted with dichloromethane (CH_2Cl_2, 2 l). The resultant extract (1.02 g, 2.27%) was chromatographed over silica (VLC), employing gradient elution from hexane to EtOAc, to yield 9 × 90 ml fractions. ^{1}H NMR and TLC analysis of these fractions indicated that they contained components essentially identical to those found in the Telford Reef sample.

4.5. Compound 1

As previously reported (Stierle & Sims, 1979).

4.6. Compound 2

An oil, (132 mg, 0.14%): $[\alpha]_D^{25}$ −1.9° ($CHCl_3$; *c* 0.48); IR ν_{max}^{film} cm^{-1}: 3250, 2920, 1450, 1010. ^{1}H NMR and ^{13}C NMR: Table 2. Mass spectral data as previously reported (Coll et al., 1988).

4.7. Compound 3

As previously reported (Coll et al., 1988).

4.8. (1S,2S,4R,5R,1′E)-2-bromo-1-bromomethyl-1,4-dichloro-5-(2′-chloroethenyl)-5-methylcyclohexane (4)

A white crystalline solid, (187 mg, 0.34%): MP 75–76°C cf 74–74.5°C (Higgs et al., 1977); $[\alpha]_D^{25}$ −61.4°, ($CHCl_3$; *c* 0.21) cf −43.8° (Higgs et al., 1977) and −67.8° Stierle et al., 1979); IR ν_{max}^{film} cm^{-1}: 3250, 2920, 1450, 1010. ^{1}H NMR and ^{13}C NMR: Table 2.

4.9. Compound 5

As previously reported (Stierle & Sims, 1979).

*4.10. Compounds **6–10***

As previously reported (Coll et al., 1988). For compound **6** the ^{13}C NMR assignments for C-5 and C-7 have been reversed.

*4.11. Compound **11***

As previously reported (Stierle & Sims, 1979).

*4.12. Crystal-structure determination of **4***

Crystals of **4**, obtained from hexane, were used for a low-temperature X-ray structure determination. All measurements were made on a Rigaku AFC5R diffractometer using graphite-monochromated Mo$K\alpha$ radiation ($\lambda = 0.71069$ Å) and a 12 kW rotating anode generator. The data collection was performed using $\omega/2\theta$ scans and included the measurement of the Friedel opposites of all unique reflections. The intensities of three standard reflections, which were measured after every 150 reflections, decreased by approximately 6% during the course of the data collection. A linear correction factor was applied to the intensities to account for this. The intensities were corrected for *Lorentz* and polarisation effects. An empirical absorption correction, based on azimuthal scans of several reflections (North, Phillips & Mathews, 1968), was applied. The space group was determined from the systematic absences and equivalent reflections, other than Friedel pairs, were merged. The structure was solved by Patterson methods using DIRDIF92 (Beurskens et al., 1992) which initially revealed the positions of the Br-atoms and expanded the Patterson solution by Fourier methods to yield the positions of all remaining non-hydrogen atoms. There are two symmetry-independent molecules in the asymmetric unit. Their conformations are very similar, but no additional symmetry could be found. The non-hydrogen atoms were refined anisotropically. All of the H-atoms were fixed in geometrically calculated positions with a [d(C–H) = 0.95 Å], the orientation on the methyl group H-atoms being determined from a difference electron density map. The H-atoms were assigned fixed isotropic displacement parameters with a value of 1.2U_{eq} of the parent C-atom. Refinement of the structure was carried out on F using full-matrix least-squares procedures which minimised the function $\sum w(|F_0|-|F_0|)^2$, where $w = [\sigma^2(F_0) + (0.005F_0)^2]^{-1}$. A correction for secondary extinction was not applied. The largest peaks of residual electron density were in the neighbourhood of the Br-atoms. Refinement (Carruthers & Watkin, 1986) of the absolute structure parameter (Bernardinelli & Flack, 1985; Flack, 1983) yielded a value of 0.00(2), which confidently confirms that the refined coordinates represent the true enantiomorph. A view of one of the independent molecules is shown in Fig. 1. Data collection and refinement parameters are given in Table 3. Neutral atom scattering factors for non-hydrogen atoms were taken from (Maslen, Fox & O'Keefe, 1992) and the scattering factors for H-atoms were taken from (Stewart, Davidson & Simpson, 1965). Anomalous dispersion effects were included in F_c (Ibers & Hamilton, 1964); the values for $\Delta f'$ and $\Delta f''$ were from (Creagh & McAuley, 1992). All calculations were performed using the TEXSAN crystallographic software package (Molecular Structure Corporation, 1989). Crystallographic data (excluding structure factors) for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication No. 113236. Copies of the data can be obtained, free of charge, on application to the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK. (fax: +44-1223-336033 or email: deposit@ccdc.cam.ac.uk).

4.13. Biological activity assessments

Fungicidal, antibacterial and algicidal activities were assessed using the methods described by Schulz et al., (1995), see Table 4. Inhibition of photosynthesis was investigated as described in König et al., (1999). The antimalarial, antitubercular (TB), cytotoxic properties and HIV-1 reverse transcriptase inhibition activities were assessed using the methods outlined in Angerhofer et al., (1992); Collins & Franzblau (1997); Likhitwitayawuid et al., (1993); Eberle & Seibl (1992), respectively.

Acknowledgements

We thank Dr. R. de Nys, School of Biological Sciences, University of NSW, Australia and Mr. G. Forbes, Department of Zoology, James Cook University, Townsville, Australia, for assistance with collections. All samples were identified by Dr. I. R. Price, Department of Botany, James Cook University, Townsville, Australia. For biological activity testing we thank, Prof. C. K. Angerhofer, Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago, Chicago, Illinois, USA, Prof. S. Franzblau, Pharmacology Research Department, G. W. L. Hansen's Disease Center and Louisiana State University, Baton Rouge, Louisiana, USA and Mr. U. Höller and Ms. G. Mattheé, Institute for Pharmaceutical Biology, Technical University of Braunschweig. Parts of the NMR work were performed by Dr. V. Wray and his team, GBF Braunschweig, Germany.

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