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HIERRIDIN B AND 2,4-DIMETHOXY-6-HEPTADECYL-PHENOL, SECONDARY METABOLITES FROM THE CYANOBACTERIUM *PHORMIDIUM ECTOCARPI* WITH ANTIPLASMODIAL ACTIVITY

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Key Word Index—Cyanobacteria; biological activity; antimalaria; *Phormidium*; aromatic lipids.

Abstract—Bioassay guided fractionation of the lipophilic extract of the marine cyanobacterium *Phormidium ectocarpi* yielded a new natural product hierridin B and the previously described compound 2,4-dimethoxy-6-heptadecyl-phenol. Both structures were secured by extensive spectroscopic analysis (1D and 2D NMR, MS, GC-MS, IR). The isolate (mixture) showed antiplasmodial activity towards *Plasmodium falciparum*.

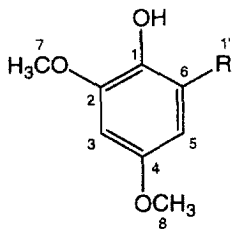
INTRODUCTION

Cyanobacteria of the genus *Phormidium* are hardly investigated regarding their secondary metabolite production. One of the few natural products described, is the secondary amine alkaloid, homoanatoxin-A, derived from *P. formosum* (*Oscillatoria formosa*) [1]. This compound is similar to anatoxin-A [2], and showed potent neuromuscular blocking properties in the mouse bioassay [3]. Glycolipids isolated from a freshwater *P. tenue* strain showed anti-tumor-promoting activity in an Epstein Barr virus activation test system [4]. This family of compounds also exhibited autolytic properties when tested against *P. tenue* itself [5, 6].

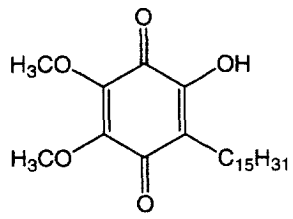
In the course of the current studies, cyanobacteria from freshwater and marine environments were screened primarily for their antiplasmodial activity. The results of these assays indicated the methylene dichloride extract of *P. ectocarpi* to inhibit the growth of *Plasmodium falciparum*. Guided by the antiplasmodial bioassay, fractionation of the extract led to the isolation of the active components. In this report, we detail the extraction, isolation and structure elucidation of the compounds **1** and **2**, the first natural products from a marine cyanobacterium with antiplasmodial activity.

RESULTS AND DISCUSSION

For the current investigation, 18 strains of cyanobacteria (*Anabaena* sp., *Aphanizomenon flos-aquae*, *Chroococcus turgidus*, *Lyngbya* sp., *Microcystis aeruginosa* (5 strains), *Nostoc sphaericum*, *Oscillatoria* sp., *Phormidium ectocarpi*, *P. persicum*, *Synechocystis* sp., *Staniera* sp., *Tolypothrix byssoi-*



1 R = $C_{15}H_{31}$ (1'-15')
2 R = $C_{17}H_{35}$ (1'-17')
3 R = $C_{21}H_{34}$ (1'-21')



2-hydroxy-5,6-dimethoxy-3-pentadecyl-1,4-benzoquinone (**4**)

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dea, T. distorta, Xenococcus sp.) from marine and terrestrial habitats were chosen.

Strains were cultured in Erlenmeyer flasks with 100 ml of BG-11- (for freshwater strains) or HW-medium (for marine strains). Cultures were constantly shaken, and illuminated with cool white fluorescent light. Harvested cells were frozen, lyophylized and extracted with methylene dichloride followed by methanol. The two extracts were tested for their antiplasmodial (*Plasmodium falciparum*), antibacterial (*Escherichia coli, Bacillus megaterium*), antifungal (*Ustilago violacea, Eurotium repens, Fusarium oxysporum, Mycotypha microspora*), and antialgal (*Chlorella fusca*) properties, as well as for their cytotoxicity. Furthermore, ELISA based tests for HIV-1-reverse-transcriptase and tyrosine–kinase-inhibition were performed. The results of this screening showed the methylene dichloride extract of *P. ectocarpi* to be active in the antiplasmodial test system ($IC_{50} = 2.1\ \mu g\ ml^{-1}$). This cyanobacterium was thus chosen for large scale cultivation in a 30 l photobioreactor.

Bioassay-guided fractionation of the methylene dichloride extract of *P. ectocarpi* led to the isolation of an active sample, which was an inseparable (by HPLC) mixture of two compounds, as indicated by GC-MS. The GC-MS measurement revealed the sample to be a 1:1 mixture of **1** and **2**. HRMS measurement of the ions at *m/z* 364 for **1** [M^+], and *m/z* 392 for **2** [M^+], showed them to have the molecular formulae $C_{23}H_{40}O_3$ and $C_{25}H_{44}O_3$, respectively. In the ^1H NMR spectrum of **1** and **2** signals for two *meta*-coupled aromatic protons (δ 6.35 (*d*), $J = 2.8$ Hz; δ 6.29 (*d*), $J = 2.8$ Hz), indicated the presence of a 1,2,4,6-substituted aromatic ring, and thus accounted for all of the elements of unsaturation indicated by the molecular formulae of **1** and **2**. Further, from the IR, UV, ^1H NMR and ^{13}C NMR data it was possible to discern the presence of a hydroxyl (3416 cm^{-1}, δ 5.25 *s*), two methoxyl groups (1030 cm^{-1}, δ 3.85 *s*; 3.75 *s*, 55.9 *q*; 56.1 *q*), and two alkyl chains (**1**: $C_{15}H_{31}$ and **2**: $C_{17}H_{35}$), as the aromatic substituents. The characteristic fragment ion for the benzylic moiety in the GC-MS study of **1** and **2** was at *m/z* 168. The alkyl chain for **1** thus had to be $-C_{15}H_{31}$ and for **2** $-C_{17}H_{35}$. The positions of the four substituents on the aryl ring were deduced from NOE difference measurements. Thus, irradiation at the resonance frequency of the aromatic proton, H-3 (δ 6.35 *d*) caused enhancement of the resonances associated with H_3-7 (δ 3.85 *s*), and H_3-8 (δ 3.75 *s*). Irradiation of the resonance for H_3-7 (δ 3.85 *s*) caused enhancement of the resonance for H-3 (δ 6.35 *d*), and 3-O*H* (δ 5.25 *s*). Irradiation of the resonances associated with H_3-8 (δ 3.75 *s*) enhanced the H-5 (δ 6.29 *d*), and H-3 (δ 6.35 *s*) resonances. Finally, irradiation of the resonance for H-5 (δ 6.29 *d*) caused enhancement of the resonances for H_3-8 (δ 3.75 *s*) and H_2-1′ (δ 2.60 *t*, $J = 7.7$ H). From these results, it is evident that compound **1** is best described as 2,4-dimethoxy-6-pentadecyl-phenol (hierridin B) and **2** as 2,4-dimethoxy-6-heptadecyl-phenol. For **1** and **2** we propose the trivial names of hierridin B and A, respectively.

The ^{13}C and ^1H NMR data of **1** and **2** were in good agreement with those reported for hierridin (**3**), first isolated by González *et al.* [7]. Compound **2** was formerly reported from the acetone extract of *Phaffia rhodozyma*, a yeast, and patented as potential antioxidant [8].

BIOLOGICAL ACTIVITY

The mixture of **1** and **2** was tested for antimicrobial (50 μg/filter disk) [9], antialgal (50 μg/filter disk) [9], HIV-1-reverse-transcriptase-inhibitory (66 $\mu g\ ml^{-1}$) [10] and tyrosine–kinase-inhibitory (200 $\mu g\ ml^{-1}$) [11], activity. The test results indicated the mixture of **1** and **2** to have no significant activities in the applied test systems. The mixture did, however, show antiplasmodial activity towards strains of *P. falciparum* [12]. The IC_{50}s against clones D6 (chloroquine sensitive) and W2 (chloroquine resistent) were determined to be 5.2 μg ml and 3.7 $\mu g\ ml^{-1}$, respectively.

Quinones, such as **4**, were found to be very active in *in vitro* test systems for antiplasmodial activity [13]. These compounds, are reported to act as co-enzyme Q anti-metabolites, causing oxidative stress to the protozoans. After derivatisation of benzoquinones, it was noted [13], that a lipophilic side chain and the presence of methoxyl groups attached to quinones, such as in **4**, often led to an increased activity. The structural similarities between these molecules and **1** and **2**, after metabolic oxidation of the latter, probably explains the antiplasmodial activity observed in this study.

EXPERIMENTAL

General experimental procedures

GC-MS was performed using a Carlo Erba 5160 GC equipped with a 30 m × 0.32 mm fused silica capillary column, coated with a methyl silicone stationary phase DB-1 (J&W Scientific, California). Helium was used as carrier gas. Conditions: injector: 250°, split 1:20 temperature program: 150–320°; 6°C min^{-1}. The capillary column was directly coupled with a quadrupole mass spectrometer, Finnigan MAT 4515. Other details as previously published [14].

Plant material

A sample of *P. ectocarpi*, designated strain No. B60.90, was obtained from the Collection of Algal Cultures at the University of Göttingen, Göttingen.

This red pigmented strain was isolated as an epiphyte from *Udothea petiolata* collected at a depth of 15 m from the coast of Mallorca.

Anabaena sp., *C. turgidus*, *N. sphaericum*, *Oscillatoria* sp., *P. persicum*, *Synechocystis* sp., *Staniera* sp., *T. byssoidea*, *T. distorta*, *Xenococcus* sp. were also obtained from the Collection of Algal Cultures at the University of Göttingen.

A. flos-aquae was collected by Dr. Oberemm, Institute of Freshwater Ecology and Inland Fisheries Department IV, Berlin.

M. aeruginosa (5 strains) were obtained from Dr. Meyer, Max-Planck-Institute for Ecological Physiology, Plön. *Lyngbya* sp. was collected near Orpheus Island, Queensland.

Cultivation

Precultures were grown in 200 ml Erlenmeyer flasks with 100 ml of BG-11- (freshwater strains) Lit. [15] or HW-medium (seawater strains, modified inorganic medium HW-salt, with an additional 750 mg l^{-1} $NaNO_3$, 15.27 mg l^{-1} KH_2PO_4 and 1 mg ml^{-1} Vitamin B_{12}; Wiegandt, Krefeld). The cultures were stirred at 60 rpm at a temperature of 25° and illuminated with white fluorescent light. *P. ectocarpi* was mass cultured in a 30 l photobioreactor (Model Pluto, planktotec®, Regenstauf). The pH of the culture was adjusted to 8.80. The culture was continuously mixed with sterile air. During cultivation, pH-adjustment was achieved by a pH-electrode coupled magnetic vent, dosing additional CO_2 into the compressed air. Cultures were continuously illuminated with one white fluorescent lamp (OSRAM L 58W/11-860), and maintained at a constant temperature of 25°C.

Extraction and isolation

Freeze dried plant material (43.9 g/90 l culture medium) was extracted with CH_2Cl_2 (3 l) and then with MeOH (3 l). Vacuum liquid chromatography (VLC) of the CH_2Cl_2 extract (590 mg, 1.3%) over silica gel, employing a step gradient from hexane to EtOAc to MeOH, yielded 12 fractions, each of approximately 90 ml. Biological and chemical screening of these fractions indicated fraction 2 (3.8 mg, 0.09%) to be of further interest. HPLC purification (Lichrosorb Si-60, hexane–Me_2CO (99:1) as eluent) of this fraction yielded the homologues **1** and **2**.

Hierridin B (**1**) and 2,4-dimethoxy-6-heptadecylphenol (**2**): Amorphous solid (wax), (3.0 mg, 0.07%). IR ν_{max} film cm^{-1}: 3416, 3024, 2922, 1462, 1030. UV λ_{max} MeOH nm: 222, 254, 263, 274. ^1H and ^{13}C NMR see Table 1; HRDCIMS (*iso*-butane) (**1**) 364.2942 [M^+] (calcd for $C_{23}H_{40}O_3$ 364.2977), (**2**) 392.3267 [M^+] (calcd for $C_{25}H_{44}O_3$ 392.3290), GC-MS (Split 1:20, 150–320°C, 6°C/min) (**1**) 364 [M^+] (78), 168 (100), 167 (63); (**2**) 392 [M^+] (75), 168 (100), 167 (67).

Table 1. ^1H (300 MHz, $CDCl_3$) and ^{13}C NMR (75.5 MHz, $CDCl_3$) data* for **1** and **2**

Carbon	^1H	^{13}C†
1	5.25 (*s*, Ar–*OH*)	137.6 (*s*)
2	–	146.7 (*d*)
3	6.35 (*d*, J = 2.8 Hz)	96.9 (*d*)
4	–	152.8 (*s*)
5	6.29 (*d*, J = 2.8 Hz)	105.8 (*d*)
6	–	128.7 (*s*)
7	3.85 (*s*)	55.9 (*q*)
8	3.75 (*s*)	56.1 (*q*)
1′	2.60 (*t*, J = 7.7 Hz)	30.1 (*t*)
2′–13′/15′	1.25 (*t*)	29.4–30.1 (12 × *t* (**1**); 14 × *t* (**2**))
14′/16′	1.58 (*t*)	22.7 (*t*)
15′/17′	0.88 (*t*, J = 6.6 Hz)	14.3 (*q*)

*All assignments are based on extensive 1D and 2D NMR experiments, including COSY90, HMQC and HMBC.
†Multiplicity by DEPT, *s* = C, *d* = CH, *t* = CH_2, *q* = CH_3.

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REFERENCES

1. Skulberg, O. M., Carmichael, W. W., Andersen, R. A., Matsunaga, S., Moore, R. E. and Skulberg, R., *Environ. Toxicol. Chem.*, 1992, **11**, 321–329.
2. Devlin, J. P., Edwards, O. E., Gorham, P. R., Hunter, N. R., Pike, R. K. and Stavric, B., *Can. J. Chem.*, 1977, **55**, 1367–1371.
3. Lilleheil, G., Andersen, R. A., Skulberg, O. M. and Alexander, J., *Toxicon*, 1997, **35**, 1275–1289.
4. Shirahashi, H., Murakami, N., Watanabe, M., Nagatsu, K., Sakakibara, J., Tokuda, H., Nishiro, H. and Iwashima, A., *Chem. Pharm. Bull.*, 1993, **41**, 1664–1666.
5. Murakami, N., Imamura, H., Sakakibara, J. and Yamada, N., *Chem. Pharm. Bull.*, 1990, **38**, 3497–3499.
6. Murakami, N., Morimoto, T., Imamura, H., Ueda, T., Nagai, S., Sakakibara, J. and Yamada, N., *Chem. Pharm. Bull.*, 1991, **39**, 2277–2281.
7. González, A. G., Barrera, J. B., Rodriguez Pérez, E. M. and Hernández Padrón, C. E., *Planta Med.*, 1992, **58**, 214–218.

8. Hierridin, C., Patent of the Nippon Suisan Kaisha Ltd., Japan, for the use as antioxidant for food, Pat. Nr. JP 04145036 A2 920519 Heisei, JP 90-268683 901006.
9. Schulz, B., Sucker, J., Aust, H. J., Krohn. K., Ludewig, K., Jones, P. G. and Döring, D., *Mycol. Res.*, 1995, **8,** 1007–1015.
10. Eberle, J. and Seibl, R. J., *J. Virol. Methods*, 1992, **40,** 347–356.
11. Höller, U., Dräger, S., Schulz, B., Aust, H. J., Mattheé, G. F., Wright, A. D., König, G. M., *Mycol. Res.*, submitted.
12. Angerhofer, C. K., König, G. M., Wright, A. D, Sticher, O., Milhous, W. K. Cordell, G. A., Farnsworth, N. R., Pezzuto, J. M., in *Advances in Natural Product Chemistry*, ed., Atta-ur-Rahman. Harwood Academic Publishers, Chur, 1992, pp. 311–329.
13. Porter, T. H. and Folkers, K., *Angew. Chem.*, 1992, **86,** 635–645.
14. Greinwald, R., Canto, P., Bachmann, P., Witte, L. and Czygan, F. C., *Biochem. Syst. Ecol.*, 1991, **20,** 75–81.
15. Rippka, R., Herdman, M., *Pasteur Culture Collection, Catalogue of Strains*, 1992–93, 61.