Bioactive Constituents from the Marine Crinoid *Himerometra magnipinna*

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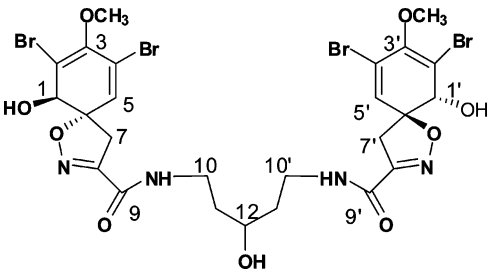
A new tetrabromospirocyclohexadienylisoxazole, (+)-12-hydroxyhomoaerothionin (**1**), together with the known compounds (+)-aerothionin (**2**) and crinemodin-rhodoptilometrin bianthrone (**3**), were isolated from the marine crinoid *Himerometra magnipinna*, which had been collected in the South China Sea, Malaysia. The structure of **1** was elucidated by interpretation of 1D ^{1}H and ^{13}C NMR spectra and 2D ^{1}H−^{1}H COSY, HMQC, and HMBC spectra. This is the first report of tetrabromospirocyclohexadienylisoxazole compounds from a crinoid of *Himerometra*. Compounds **1**−**3** were evaluated for their inhibitory activity with the hyphae formation inhibition assay in *Streptomyces* 85E.

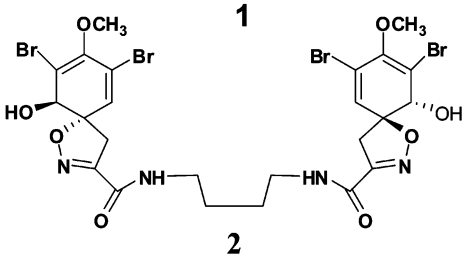
Protein kinases (PK) are a large family of homologous proteins comprising two major subfamilies: serine/threonine kinases and tyrosine kinases. The phosphorylation of proteins on these three residues by the ~520 protein kinases in the human genome is one of the major regulatory mechanisms for processes including apoptosis, cell proliferation, and metabolism. Abnormal phosphorylation of regulatory molecules associated with these pathways is often the cause of diseases. In this regard, protein kinases have emerged as extremely promising targets in the chemotherapeutic treatment of cancer.[1,2]

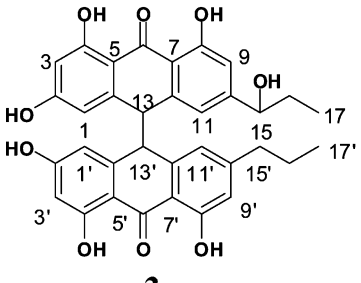
Marine organisms offer a rich source of as yet unexamined bioactive molecules, and many diverse secondary metabolites remain to be discovered for the development of new pharmaceutical agents.[3] To this end, we have been screening marine organisms for protein kinase inhibitors using a prokaryotic whole cell *in vivo* assay, hyphae formation inhibition (HFI) in *Streptomyces* 85E.[4] In the course of primary screening, an organic extract of the crinoid *Himerometra magnipinna*, collected in the South China Sea, Malaysia, exhibited inhibitory activity in the HFI assay, giving 14 mm bald and clear phenotypes at a concentration of 80 μg/disk. In addition, previous phytochemical or biological studies on this species are rare. To date, only two papers reported the isolation of sulfated steroids,[5] bianthrones, and related polyketides[6] from *H. magnipinna*. In this investigation, the structure elucidation of a new compound (**1**) and the biological evaluation of **1**−**3** are reported.

The crinoid *H. magnipinna* was extracted with CH_2Cl_2−MeOH (1:1) followed by MeOH. The organic extracts were combined, concentrated, and subjected to Si gel MPLC (medium-pressure liquid chromatography). Final separation of the active fractions by Sephadex LH-20 and RP-HPLC afforded a new compound (**1**) and two known compounds (**2** and **3**). Compound **2** was identified to be aerothionin[7] by comparing its spectroscopic data with those reported in the literature.

Compound **1**, obtained as a yellow solid, showed in the low-resolution ESIMS spectrum a 1:4:6:4:1 quintet at *m*/*z* 867, 869, 871, 873, and 875 for the pseudomolecular ion peak, indicating the presence of four bromine atoms. A high-resolution measurement on the peak at *m*/*z* 871 suggested the molecular formula $C_{25}H_{28}Br_4N_4O_9$ (*m*/*z* 870.8446 $[M + Na]^+$, calcd for 870.8458), indicating 12 degrees of unsaturation. IR absorption showed the presence of hydroxyl and amino groups (ν_{max} 3369 cm^{-1}) and α-iminoamide (ν_{max} 1655, 1599, 1543 cm^{-1}) functionalities. The ^{1}H NMR spectrum of **1** (Table 1) showed signals for two olefinic protons at δ_H 6.58 (s, 2H), three oxymethine protons at δ_H 4.18 (s, 2H) and 3.76 (m, 1H), two *O*-methyl groups at δ_H 3.72 (s, 6H), methylene protons at δ_H 3.18 (d, J = 18.0 Hz, 2H), 3.84 (d, J = 18 Hz, 2H), 3.36 (m, 2H), and 3.52 (m, 2H), and two sets of aliphatic protons at δ_H 1.66 (m, 2H) and 1.74 (m, 2H). Thirteen signals were observed in the ^{13}C NMR spectrum; a DEPT spectrum revealed the presence of three methylenes (δ_C 39.5, 36.8, 36.6), one methoxyl (δ_C 59.6), two sp^3 (δ_C 74.5 and 67.2) and one sp^2 (δ_C 132.7) methines, five quaternary carbons (δ_C 154.6, 148.1, 122.1, 113.2, 91.0), and one amide carbonyl (δ_C 160.0). A molecular formula of $C_{25}H_{28}Br_4N_4O_9$ was determined by HRESIMS, indicating that the molecule contains 25 carbons. However, only 13 peaks were observed in the carbon spectrum. This disparity suggested that there may be molecular symmetry to account for the rest of the carbons, with two identical substructures connected to a single carbon atom.







Comparison of ^{1}H NMR and ^{13}C NMR data of **1** with values for

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Table 1. ^{1}H and ^{13}C NMR Data of Compounds **1** and **2**

	1			**2**	
position	δ_C	δ_H[a]	HMBC (H → C#)	δ_C	δ_H[a]
1, 1′	74.5 CH	4.18 s	2/2′, 3/3′, 5/5′, 6/6′	75.4 CH	4.18 s
2, 2′	113.2 C			113.9 C	
3, 3′	148.1 C			148.9 C	
4, 4′	122.1 C			122.1 C	
5, 5′	132.7 CH	6.58 s	1/1′, 2/2′, 3/3′, 4/4′, 6/6′, 7/7′	132.5 CH	6.52 s
6, 6′	91.0 C			91.6 C	
7, 7′	39.5 CH_2	3.18 d (18.0); 3.84 d (18.0)	1/1′, 5/5′, 6/6′, 8/8′	40.3 CH_2	3.18 d (18.0); 3.83 d (18.0)
8, 8′	154.6 C			155.4 C	
9, 9′	160.0 C			160.1 C	
10, 10′	36.8 CH_2	3.36 m; 3.52 m	9/9′, 11/11′, 12	39.6 CH_2	3.33 m
11, 11′	36.6 CH_2	1.66 m; 1.74 m	10/10′, 12	27.6 CH_2	1.62 m
12	67.2 CH	3.76 m	10/10′, 11/11′		
3, 3′-OMe	59.6 CH_3	3.72 s	3/3′	60.3 CH_3	3.73 s

[a] δ_H values are referenced to the internal signal for acetone-d_6 at 2.05 ppm. *J* values (Hz) are shown in parentheses. Assignments are based on ^{1}H−^{1}H COSY, HMQC, and HMBC spectra.

aerothionin (**2**)[7] (Table 1) indicated that **1** was a bromotyrosine derivative. Compounds **1** and **2** are very closely related bromotyrosine alkaloids, with the major differences being the presence of a sp^3 oxygenated methine (δ_C 74.5; δ_H 3.76, m, 1H) appearing in **1**. In addition, the signal for C-11/11′ (δ_C 36.6) in **1** was shifted to a lower field than that in **2** (δ_C 27.6), and the signal for C-10/10′ (δ_C 36.8) in **1** was shifted to a higher field than that in **2** (δ_C 39.6). The location of the OH at C-12 of **1** was confirmed by an HMBC experiment, in which correlations were observed for the resonances at H-11/ H-11′ (δ_H 1.66, m, 1H; 1.74, m, 1H) and H-10/ H-10′ (δ_H 3.36, m, 1H; 3.52, m, 1H) with the sp^3 oxygenated methine signal (δ_C 74.5, C-12). Additional correlations were observed for the resonances at H-10/H-10′ (δ_H 3.36, m, 1H; 3.52, m, 1H) with signals at δ_C 36.6 (C-11), 67.2 (C-12), and 160.0 (C-9). The cross-peaks of H-11/ H-11′ (δ_H 1.66, m,1H; 1.74, m, 1H) to H-12 (δ_H 3.76, m, 1H) appearing in the ^{1}H−^{1}H COSY spectrum further supported the above assignment. Detailed analysis of the ^{1}H−^{1}H COSY, HMQC, and HMBC experiments allowed the complete assignment for structure **1**.

Comparison of the spectra of compound **1** with literature values indicated **1** was a 12-hydroxy derivative of homoaerothionin,[8] a known tetrabromospirocyclohexadienylisoxazole, which was isolated from the marine sponge *Aplysina aerophoba* and other sponges of the order *Verongida*.[9] The absolute stereochemistry of (+)-aerothionin (**2**) has been determined by X-ray crystallographic analysis and circular dichroism (CD) spectrum ($[\theta]_{284}$ +70 500, $[\theta]_{245}$ +78 200).[10] These positive optical rotation values and the positive Cotton effect with absolute configuration 1*R*, 6*S* for (+)-aerothionin[10] were consistent with the data obtained for compound **2** (see Experimental Section). Recent work that reported the first synthesis of optically pure (+)-aerothionin ($[\alpha]_D^{20}$ +203.8) and (−)-aerothionin ($[\alpha]_D^{20}$ −205.2) further established the absolute configuration of (+)-aerothionin.[11] On the basis of these studies, the specific rotation ($[\alpha]_D^{20}$ +106), the positive Cotton effect observed in the CD spectrum for **1**, and biosynthetic considerations, the absolute configuration of **1** was deduced to be 1*R*, 1′*R*, 6*S*, and 6′*S*. This is the first report of tetrabromospirocyclohexadienylisoxazole compounds from the marine crinoid *Himerometra* sp. Previous studies have reported the occurrence of tetrabromospirocyclohexadienylisoxazoles from a sponge in the genus *Aplysina* (formerly known as *Verongia*).[7,10,12,13]

Compound **3** was determined to be crinemodin-rhodoptilometrin bianthrone[6] by a combination of ^{1}H−^{1}H COSY, HMQC, HMBC, and NOESY experiments. Although it had been isolated from the same marine crinoid species in 1985, there were no literature reports on its ^{13}C NMR or ^{1}H NMR data assignments. This is the first report of the full NMR assignment of crinemodin-rhodoptilometrin bianthrone (**3**).

The initial crude organic extract of *H. magnipinna* gave 14 mm bald and clear phenotypes at a concentration of 80 μg/disk in the HFI assay in *Streptomyces* 85E, according to an established protocol.[4] Compounds **1**−**3** obtained in this investigation were evaluated for their inhibitory activity in the HFI assay. Compounds **1** and **3** exhibited inhibitory activity to *Streptomyces* 85E as a "bald" zone (11 mm) at the concentration of 80 μg/disk, whereas compound **2** was inactive. Compounds **1** and **3** were inactive when tested at the lower concentration of 40 μg/disk. Aeroplysinin, previously reported from *Aplysina aerophoba*, was recently shown to inhibit tyrosine kinase activity in the sponge *Suberites domuncula*.[14] As a tyrosine kinase inhibitor, it blocked the binding of (1→3)-β-D-glucan of fungi to the receptor, which was the (1→3)-β-D-glucan binding protein in *S. domuncula*. This modulates the recognition system of sponges for fungi and their ability to protect themselves from potential parasitic microorganisms.[14] Taken together, previous studies and our findings support the rationale that the HFI assay is a potentially powerful tool with which to rapidly screen and identify chemotherapeutic candidate compounds that target general serine/threonine and/or tyrosine activation and potentially cancer cell kinase activity.

Experimental Section

General Experimental Procedures. Optical rotations were obtained on a JASCO P-1010 automatic polarimeter. CD measurements were performed using a JASCO-600 CD spectropolarimeter. UV and IR spectra were recorded on a HP 8453 UV−visible spectrophotometer and a Perkin-Elmer BX FT-IR spectrometer. Mass spectra and high-resolution MS spectra were taken with a BioTOF II ESI mass spectrometer. 1D and 2D NMR spectra were recorded in acetone-d_6 and CD_3OD on Mercury Plus (300 MHz) and INOVA Unity (500 MHz) Varian spectrometers. Chemical shifts were referenced to the center of the solvent peaks of acetone-d_6 (δ_H = 2.05 and δ_C = 29.92) and methanol-d_4 (δ_H = 3.31 and δ_C = 49.15), respectively. Reversed-phase HPLC were performed on a Beckman Coulter Gold-168 system, equipped with a photodiode array detector, and an Alltech semipreparative Econosil C_{18} column (10 μm, 10 × 250 mm) run with a flow rate of 1.5 mL/min. Medium-pressure liquid chromatography (MPLC) was performed on a Büchi C-615 apparatus using Si gel 60 (200−400 mesh).

Animal Material. A crude organic extract of the marine crinoid *H. magnipinna* (C024691, 4.96 g) derived from 305 g wet weight of the initial collection was received from the Natural Product Branch, National Cancer Institute's Open Repository Program. The crinoid *H. magnipinna* was collected at a depth of 8 m from a coral reef off the North Coast of Sarawak in the South China Sea, Malaysia, in January 2003, and was identified by Charles Messing of Nova Southeastern University in Florida. A voucher specimen was deposited at the National Museum of Natural History, Smithsonian Institution Washington D.C. (0M915008).

Extraction and Isolation. The crinoid was frozen immediately upon collection and shipped to the National Cancer Institute (Frederick, MD). Following aqueous extraction of the frozen crinoid at 4 °C, frozen specimens were lyophilized prior to organic extraction. The crinoid

was extracted with a mixture of CH_2Cl_2−MeOH (1:1) and then with MeOH three times. The organic extracts were combined, evaporated, and stored at −30 °C in the Natural Products Open Repository at NCI. The extract (4.96 g) was subjected to medium-pressure liquid chromatography (MPLC) eluting with a gradient of $CHCl_3$−MeOH, affording a series of fractions. All of these fractions were tested for inhibitory activity of hyphae formation in *Streptomyces* 85E in the HFI assay. The bioactive fraction eluted with $CHCl_3$−acetone (5:1) was subjected to Sephadex LH-20 (MeOH) and then was further subjected to RP-C_{18} HPLC (MeOH−H_2O = 73:27) to yield pure compound **3** (20.5 mg). The bioactive fraction eluted with $CHCl_3$−MeOH (10:1) was chromatographed on a Si gel column ($CHCl_3$−acetone = 5:1) and then was subjected to Sephadex LH-20 (MeOH) to yield crude fractions containing compounds **1** and **2**, which were further purified by Si gel CC eluted with $CHCl_3$−acetone (5:1) to give compounds **1** (51.7 mg) and **2** (2.3 mg), respectively.

(+)-12-Hydroxyhomoaerothionin (1): yellow powder; $[\alpha]_D^{20}$ +106 (*c* 0.39, MeOH); UV (MeOH) λ_{max} (log ϵ) 231 (4.33) nm, 281 (3.99); CD (MeOH) $[\theta]_{250}$ + 60 000; IR (KBr) ν_{max} 3369, 2937, 1655, 1599, 1543, 1264 cm^{-1}; ^1H and ^{13}C NMR (acetone-d_6, 300 MHz), see Table 1; HRESIMS *m/z* 870.8446 for $[M + Na]^+$ (calcd for $C_{25}H_{28}Br_4N_4O_9Na$, 870.8458).

(+)-Aerothionin (2): white powder; $[\alpha]_D^{20}$ +201.2 (*c* 0.40, MeOH) {lit.[9] $[\alpha]_D^{20}$ +210 (*c* 1.7, MeOH)}; UV (MeOH) λ_{max} (log ϵ) 231 (4.93) nm, 281 (4.69) nm; CD (MeOH) $[\theta]_{284}$ +70 879, $[\theta]_{245}$ +78 512 {lit.[9] $[\theta]_{284}$ +70 500, $[\theta]_{245}$ +78 200}; IR (KBr) ν_{max} 3347, 2941, 1658, 1600, 1551, 1263 cm^{-1}; ^1H and ^{13}C NMR (acetone-d_6, 500 MHz), see Table 1; HRESIMS *m/z* 840.8335 for $[M + Na]^+$ (calcd for $C_{24}H_{26}Br_4N_4O_8Na$, 840.8348).

(+)-Crinemodin-rhodoptilometrin bianthrone (3): yellow solid; ^1H NMR (CD_3OD, 500 MHz) δ_H 6.71 (s, 1H, H-9), 6.61 (s, 1H, H-9′), 6.39 (s, 2H, H-1, H-1′), 6.27 (d, 1H, *J* = 2.0 Hz, H-3), 6.26 (d, 1H, *J* = 2.0 Hz, H-3′), 6.11 (br s, 1H, H-11), 5.90 (br s, 1H, H-11′), 4.53 (br s, 2H, H-13), 4.46 (br s, 2H, H-13′), 4.36 (t, *J* = 7.0 Hz, 2H, H-15), 2.44 (t, *J* = 7.5 Hz, 2H, H-15′), 1.63 (m, 2H, H-16), 1.57 (m, 2H, H-16′), 0.95 (t, *J* = 7.5 Hz, 3H, H-17′), 0.93 (t, *J* = 7.5 Hz, 3H, H-17); ^{13}C NMR (CD_3OD, 125 MHz) δ_C 191.8 (C, C-6 and C-6′), 166.6 (C, C-4), 166.5 (C, C-4′), 165.8 (C, C-2 and C-2′), 163.0 (C, C-8), 162.9 (C, C-8′), 154.4 (C, C-10), 152.1 (C, C-10′), 146.9 (C, C-14), 146.6 (C, C-14′), 141.5 (C, C-12), 141.2 (C, C-12′), 121.8 (CH, C-11′), 118.6 (CH, C-11), 117.2 (CH, C-9′), 116.2 (CH, C-7), 115.5 (C, C-7′), 115.3 (CH, C-9), 112.0 (C, C-5), 111.8 (C, C-5′), 111.0 (CH, C-1 and C-1′), 103.1 (CH, C-3), 103.0 (CH, C-3′), 76.0 (CH_2, C-15), 57.7 (CH, C-13), 57.5 (CH, C-13′), 39.4 (CH_2, C-15′), 32.4 (CH_2, C-16′), 24.6 (CH_2, C-16), 14.4 (CH_3, C-17′), 10.9 (CH_3, C-17).

Hyphae Formation Inhibition Assay. An assay based on inhibition of aerial hyphae formation in *Streptomyces* 85E was performed by a method previously reported.[4] The mycelia fragments of *Streptomyces* 85E were spread on minimal medium ISP 4 agar plates to produce the bacteria lawn. Each sample was applied to a paper disk (6 mm) at a dose of 80 μg/disk, and the paper disk was then air-dried. The impregnated paper disks were applied directly on the surface of agar plates seeded with *Streptomyces* 85E. The growth inhibition zone was recorded after 30 h of incubation at 30 °C (during which the development of hyphae in *Streptomyces* species takes place). Two types of phenotypes were observed, a clear zone of inhibition and/or a bald phenotype around the disk. The sporulation inhibitor surfactin was used as a positive control, and MeOH was used as a negative control. An inhibition zone of greater than 9 mm is acceptable and is considered active. Subfractions and compounds **1**−**3** were tested at 80 μg/disk on 6 mm paper disks. Active compounds were tested at lower concentration, at 40, 20, 10, 5, and 2.5 μg/disk. The assays were performed in triplicate.

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