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ORIGINAL ARTICLE

Hymerhabdrin A, a novel diterpenoid with antifouling activity from the intertidal sponge *Hymerhabdia* sp.

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Hymerhabdrin A (1), a diterpenoid possessing a novel 6/6/5 fused-ring skeleton, together with four known sterols were isolated from an intertidal marine sponge *Hymerhabdia* sp. Their structures were elucidated by extensive spectroscopic methods, and the relative and absolute configurations of 1 were determined by NOESY analysis and electronic circular dichrosim calculations, respectively. Hymerhabdrin A (1) exhibited significant antifouling activity against *Balanus amphitrite* larval with LC_{50} (lethal concentration 50) value of 3.6 μg ml^{-1}.
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INTRODUCTION

Biofouling is an extensive phenomenon in the marine environment and cause significant economic and environment losses worldwide every year.[1] Because the metal-containing (like organotin and copper) and biocide-based (like Irgarol 1051 and diuron) antifoulants have toxicity to nontarget marine organisms, the nontoxic and environment-friendly antifoulants are urgently needed.[2] Sessile marine organisms such as sponges, corals and algae are widely believed to possess chemical defense systems against predators, larvae of other sessile organisms and pathogenic microorganisms.[2] Therefore, their secondary metabolites might be the promising sources of novel antifoulants. In fact, in the past few decades, many compounds with potent antifouling activity have been isolated from various marine organisms.[2–4] In particular, marine sponges have proven to be the richest source of structurally diverse antifouling compounds.[5] As part of our continuing search for the bioactive compounds from marine organisms, a novel antifouling diterpenoid, namely hymerhabdrin A (**1**) (Figure 1), together with four known sterols cholestanol (**2**),[6] ergosta-5,24(28)-dien-3β-ol (**3**)[7] and 24*S*- and 24*R*-saringosterol (**4** and **5**)[8] have been isolated from the MeOH extract of an orange sponge *Hymerhabdia* sp. collected from the intertidal zone of Yantai, Shandong Province of China. Herein, we report the isolation, structural elucidation, plausible biogenetic pathway as well as antifouling activity of **1**.

RESULTS AND DISCUSSION

Hymerhabdrin A (**1**) was obtained as a white amorphous solid and had a molecular formula of $C_{20}H_{34}O_2$ by positive HR electrospray ionization MS measurements (*m/z* 329.2449 $[M+Na]^+$, calcd for 329.2457), requiring 4 degrees of unsaturation. The IR absorption at 3406 cm^{-1} indicated the presence of hydroxyl groups. In the ^{1}H NMR spectrum of **1** (Table 1), the signals of a terminal double bond at δ_H 4.90 (1H, s) and 4.69 (1H, s), two oxymethine groups at δ_H 4.13 (1H, m) and 3.23 (1H, d, $J = 4.0$ Hz) and five methyl groups at δ_H 1.74 (3H, s), 1.20 (3H, s), 1.04 (3H, s), 1.03 (3H, s) and 0.70 (3H, s) were clearly apparent. The ^{13}C NMR and DEPT spectra revealed the presence of 20 carbon signals, including five methyls, six methylenes, five methines and four quaternary carbons. Among them, the terminal double bond (δ_C 145.6 and 111.2), two oxymethines (δ_C 78.8 and 71.1) and five methyl groups (δ_C 29.7, 24.8, 16.9, 16.2 and 14.9) were also easily observed (Table 1). From the above information, together with the 4 degrees of unsaturation, indicated that hymerhabdrin A (**1**) should be a diterpenoid possessing a tricyclic aliphatic ring skeleton.

The proton and carbon resonances in the NMR spectra of **1** were unambiguously assigned by interpretation of HSQC (heteronuclear single quantum correlation), COSY and HMBC (heteronuclear multiple bond correlation) spectroscopic data (Figure 2). The HMBC correlations of H-1/C-2, C-3, C-5, C-10 and C-20; H-7/C-5, C-6, C-8 and C-9; H-18 and H-19/C-3, C-4 and C-5; and H-20/C-1, C-5, C-9 and C-10, together with the COSY correlations of H-1/H-2/H-3 and H-5/H-6/H-7, indicated the presence of the six-membered rings A and B, respectively. Similarly, the HMBC correlations of H-17/C-7, C-8, C-9 and C-13, along with the spin system of H-9/H-11/H-12/H-13 in the COSY spectrum, suggested the presence of a five-membered ring C. Additional HMBC correlations of the two protons at H-15 with C-13 and C-16, of H-16 with C-13, C-14 and C-15 provided proof of a isopropenyl moiety attachment at C-13. Thus, the planar structure of hymerhabdrin A (**1**) is a 6/6/5 fused tricyclic diterpenoid as shown in Figure 1.

The relative configuration of **1** was assigned by the NOESY spectrum (Figure 3). The NOE correlations of H-17/H-20 and H-18/H-20 indicated that they were β-oriented and located in an axial orientation, whereas the NOE correlations of H-3/H-5, H-5/H-9 and H-9/H-13 showed that they were oriented in the other direction.

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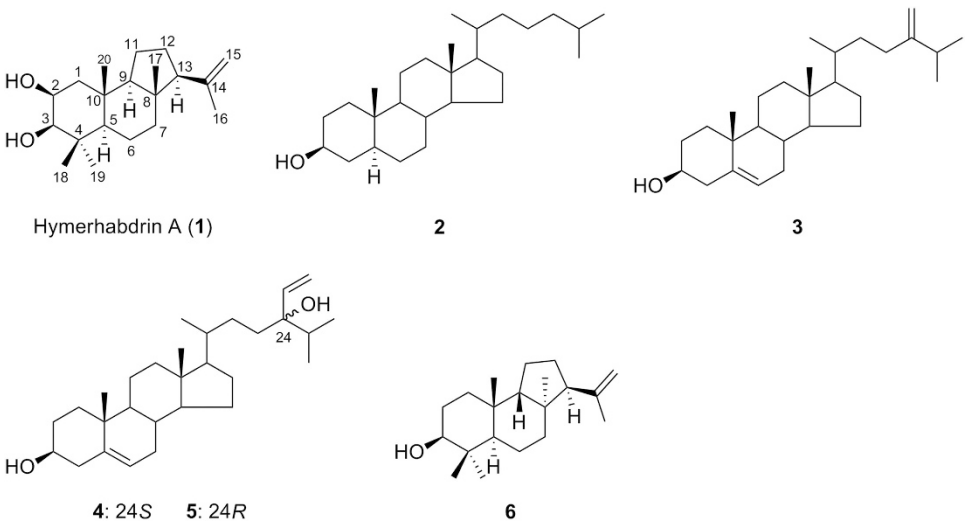

Figure 1 The structures of the compounds **1**–**6**.

Table 1 ^{13}C and ^{1}H NMR (125/500 MHz) data of 1 recorded in $CDCl_3$

Position	δ_C	δ_H *(mult, J in Hz)*	*HMBC*
1	43.9, CH_2	2.01 (1H, dd, 14.5, 2.5)	C-2, 3, 5, 10, 20
		1.21 (1H, m)	
2	71.1, CH	4.13 (1H, m)	C-1, 3, 10
3	78.8, CH	3.23 (1H, d, 4.0)	C-4, 18, 19
4	38.1, C		
5	56.3, CH	0.91 (1H, dd, 11.7, 3.0)	
6	19.0, CH_2	1.62 (2H, m)	
7	41.0, CH_2	1.89 (1H, dt, 12.5, 3.3)	C-5, 6, 8, 9, 17
		1.24 (1H, m)	
8	43.5, C		
9	63.5, CH	1.18 (1H, m)	
10	36.5, C		
11	19.6, CH_2	1.50 (2H, m)	
12	24.9, CH_2	1.79 (1H, m)	
		1.68 (1H, m)	
13	58.4, CH	1.97 (1H, brd, 9.6)	C-14, 15
14	145.6, C		
15	111.2, CH_2	4.90 (1H, s)	C-13, 16
		4.69 (1H, s)	
16	24.8, CH_3	1.74 (3H, s)	C-13, 14, 15
17	14.9, CH_3	0.70 (3H, s)	C-7, 8, 9, 13
18	16.9, CH_3	1.04 (3H, s)	C-3, 4, 5, 19
19	29.7, CH_3	1.03 (3H, s)	C-3, 4, 5, 18
20	16.2, CH_3	1.20 (3H, s)	C-1, 5, 9, 10
2,3-OH		2.21 (1H, brs)	
		2.15 (1H, brs)	

Abbreviation: HMBC, heteronuclear multiple bond correlation.

Moreover, the correlations of H-2/H-3 suggested the α-orientation of H-2 and H-3, and the corresponding β-orientation of the 2-OH and 3-OH. To determine the absolute configuration, we tried to get suitable crystals of **1** for X-ray diffraction but were unsuccessful at various conditions. Alternatively, electronic circular dichrosim (ECD) analysis was used as an indirect determination of absolute configuration of **1**. By comparing its CD spectrum and the ECD spectra of two possible enantiomers, which were calcd using time-dependent density-functional theory at the B3LYP/6-311+G(2d, p) level in methanol solvent, as shown in Figure 4, the ECD spectra of (2*S*, 3*R*, 5*S*, 8*R*, 9*S*, 10*S*, 13*R*)-**1** was in accordance with the experimental CD spectra of **1**. Thus, the absolute configuration of **1** was established as 2*S*, 3*R*, 5*S*, 8*R*, 9*S*, 10*S* and 13*R*.

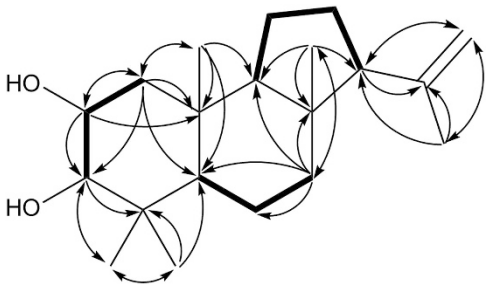

Figure 2 Key correlations of COSY (bold line) and heteronuclear multiple bond correlation (HMBC; arrow) experiments for **1**.

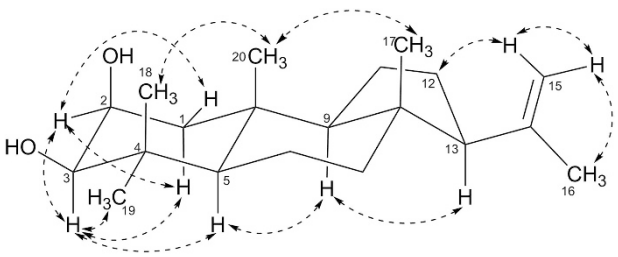

Figure 3 Key correlations of NOESY (arrow) experiment for **1**.

Hymerhabdrin A (**1**) represents a new tricyclic diterpenoid carbon skeleton with an unusual 6/6/5-fused ring system. According to the literatures, this type of diterpenoid has not been previously found in natural sources. Previously, several similar compounds including **6** (Figure 1) had been synthesized by Corey *et al.*,[9] but the six-membered ring B of them were in a boat conformation that is distinctly different from the chair conformation in ring B of hymerhabdrin A (**1**). Thus, compound **1** represented a new type of carbon skeleton in the family of diterpenoids from natural sources.

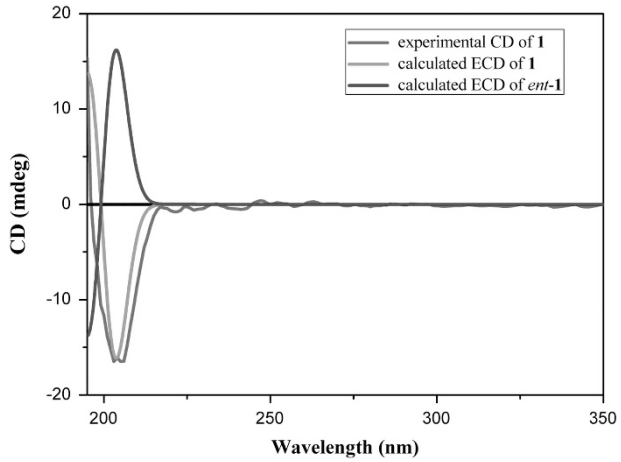

Figure 4 Experimental CD spectrum of **1**, and calcd electronic circular dichrosim (ECD) spectra of **1** and *ent*-**1**. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

The antifouling activity of hymerhabdrin A (**1**) was tested against the larval of barnacle *Balanus amphitrite* using an established method as described previously.[10] The compound displayed significant toxicity with LC_{50} (lethal concentration 50) value of 3.6 μg ml^{-1} for 24 h. This result indicated that hymerhabdrin A might play some role in the chemical defense of sponge *Hymerhabdia* sp. against predators or other fouling organisms, and this new type of diterpenoids could be used for further investigation as a potential antifouling agent.

METHODS

General experimental procedures

Optical rotations were determined on a Jasco P-1020 automatic digital polarimeter (Jasco, Tokyo, Japan). CD spectra were recorded on a Chirascan CD spectrometer (Applied Photophysics, Leatherhead, UK). IR spectra were measured on a Jasco FT/IR-4100 spectrometer (Jasco) with KBr pellets. The 1D and 2D NMR spectra were recorded on a Bruker Avance III 500 instruments (Bruker, Fallanden, Switzerland) with TMS as an internal standard. Chemical shifts were reported in units of δ (ppm) and coupling constants (J) were expressed in Hz. Atmospheric pressure chemical ionization MS was determined on a LCQ Fleet ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). HR electrospray ionization MS was measured on Agilent G6230 time-of-flight mass spectrometers (Agilent Technologies, Santa Clara, CA, USA). Column chromatography was performed over silica gel (200–300 mesh; Yantai Xinde Chemical Co., Ltd, Yantai, China), ODS gel (YMC, Kyoto, Japan) and Sephadex LH-20 (Amersham Pharmacia Biotech, Uppsala, Sweden). TLC was performed on the silica gel plates (Yantai Xinde Chemical Co., Ltd), and spots were visualized by spraying with 10% H_2SO_4 in EtOH, followed by heating.

Animal material

Specimens of an orange sponge *Hymerhabdia* sp. were collected from the intertidal zone of Yantai, Shandong Province of China, in October 2012. A voucher specimen (No. 20121023-2) was deposited at the Key Laboratory of Coastal Biology and Biological Resources Utilization, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, and identified by Professor Jin-He Li, Institute of Oceanology, Chinese of Academy of Sciences.

Extraction and isolation

The freshly collected sponges *Hymerhabdia* sp. were immediately frozen, and the lyophilized specimens were repeatedly extracted with MeOH (2L × 3) at room temperature. The combined extracts (97.84 g) was subjected to column chromatography on silica gel eluted with a gradient eluent of petroleum ether (PE)/acetone system (50:1 to 1:5, v/v) to provide six fractions (A–F). Fraction B (1.70 g) was chromatographed on Sephadex LH-20 (CH_2Cl_2/MeOH, 1:1, v/v), silica gel (PE/acetone, 5:1, v/v) and ODS (MeOH/H_2O, 80:20 to 98:2, v/v) columns to afford compounds **2** (55.8 mg) and **3** (35.9 mg). Fraction C (359 mg) was further purified by ODS column eluted with MeOH/H_2O gradient (70:30 to 95:5, v/v) to afford three subfractions (C1–C3). Subfraction C1 (175.2 mg) was finally submitted to Sephadex LH-20 (CH_2Cl_2/MeOH, 1:1, v/v) and repeated silica gel columns (PE/acetone, 5:1 and 8:1, v/v) to get compound **1** (6.5 mg). Subfraction C3 led to the isolation of a mixture of **4** and **5** (16.6 mg) by silica gel columns (PE/EtOAc 3:1 and CH_2Cl_2/EtOAc 20:1, v/v).

Hymerhabdrin A (**1**): white amorphous solid; $[\alpha]^{21.3}_{D}$ −7.1 (*c* 0.11, MeOH); CD (MeOH) λ_{max} ($\Delta\varepsilon$) 205 (−3.85) nm; IR (ν_{max}): 3406, 2935, 2873, 1639, 1446, 1373 and 1057 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; negative atmospheric pressure chemical ionization MS *m/z* 305 $[M-H]^-$; HR electrospray ionization MS *m/z* 329.2449 $[M+Na]^+$ (calcd for $C_{20}H_{34}O_2Na$, 329.2457).

Computational methods

Density functional theory calculations of compound **1** have been performed with the Gaussian09 series of programs using the B3LYP/6-311+G(2d, p) basis set to obtain the molecular structure and vibrational wavenumbers. Frequencies for the required structure were evaluated at the same level of theory to ascertain the nature of stationary points, and harmonic vibrational wave numbers were calcd using the analytic second derivatives to confirm the convergence to the minimum of the potential surface. ECD calculation was carried out in methanol solvent medium using time-dependent density-functional theory B3LYP/6-311+G(2d, p) level of theory on B3LYP/6-311+G(2d, p) optimized geometry through polarizable continuum model in methanol solution. The calcd ECD curve of **1** was generated using SpecDis with $\sigma = 0.15$ ev and UV shift 5 nm.

Antifouling activity assay

Adult barnacles, *B. amphitrite*, attached to discarded tires were collected from the intertidal zone in Yantai, China. When exposed to bright light, adults released the stage I and II nauplius upon immersion in filtered sea water after drying for 12 h. The antifouling activity assay (acute toxicity test) of metabolites was tested by using stage II nauplii of *B. amphitrite* within 2–4 h from nauplii collection. The test was performed by adding 15–25 nauplii II to 24-well polystyrene plates containing 2 ml of bioactive compound solution at different concentrations (0, 5, 10, 20, 40, 80 and 100 mg l^{-1}). Four replicates were prepared for each concentration of tested sample and the reported results are the mean values of the four replicates. The plates were stored for 24 h at 20 °C. After 24 h, the number of dead larvae was observed under a stereomicroscope. LC_{50} value was calcd as the concentration of tested sample causing 50% mortality to the exposed organisms after 24 h of contact.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)