

Article

Echinohalimane A, a Bioactive Halimane-Type Diterpenoid from a Formosan Gorgonian *Echinomuricea* sp. (Plexauridae)

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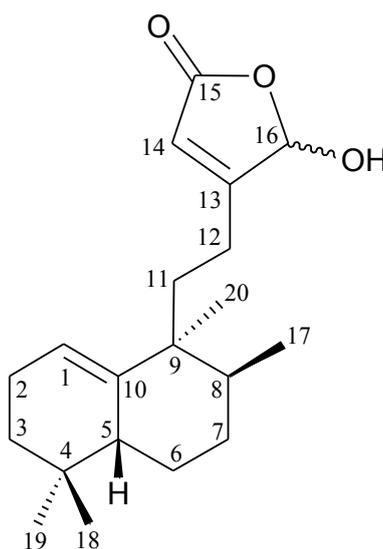
Abstract: A new halimane-type diterpenoid, echinohalimane A (**1**), was isolated from a gorgonian, identified as *Echinomuricea* sp. The structure of **1** was determined by spectroscopic methods and this compound was found to exhibit cytotoxicity toward various tumor cells and display an inhibitory effect on the release of elastase by human neutrophils. Echinohalimane A (**1**) is the first halimane analogue from the marine organisms belonging to phylum Cnidaria.

Keywords: halimane; echinohalimane; *Echinomuricea*; cytotoxicity; elastase

1. Introduction

The search for bioactive natural products from marine organisms has been remarkably successful [1] and octocorals have proven to be rich sources of interesting natural products [2,3]. In continuation of our search for new natural products from the marine invertebrates collected off the waters of Taiwan at the intersection of the Kuroshio current and the South China Sea surface current, a new bioactive substance, echinohalimane A (**1**) (Figure 1), was isolated from the gorgonian *Echinomuricea* sp. In this paper, we describe the isolation, structure determination and biological activities of echinohalimane A (**1**).

Figure 1. The structure of echinohalimane A (**1**).



2. Results and Discussion

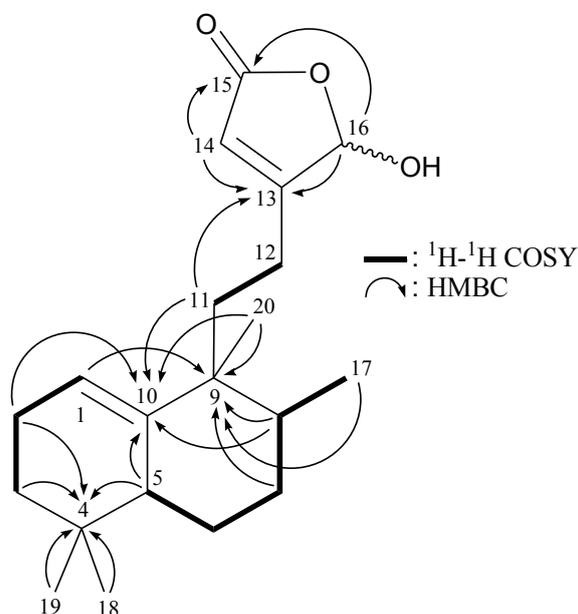
In previous studies, two bisabolane-type sesquiterpenoids, (7*S*,10*R*)-(+)-10,11-epoxycurcuphenol and (+)-curcuphenol [4]; a labdane-type diterpenoid, echinolabdane A [5]; a clerodane-type diterpenoid, echinoclerodane A [6]; and a steroid analogue, 6-*epi*-yonarasterol B [5], had been isolated from a Formosan gorgonian coral identified as *Echinomuricea* sp. (Plexauridae). We have further isolated a new halimane-type diterpenoid, echinohalimane A (**1**), from *Echinomuricea* sp.

Echinohalimane A (**1**) was isolated as a yellowish oil that gave a pseudomolecular ion (M + Na)⁺ at *m/z* 341.2089 in the HRESIMS, indicating the molecular formula C₂₀H₃₀O₃ (calcd for C₂₀H₃₀O₃ + Na, 341.2093) and implying six degrees of unsaturation. IR absorptions were observed at 3375 and 1755 cm⁻¹, suggesting the presence of hydroxy and ester groups in **1**. The ¹³C NMR for **1** confirmed the presence of 20 carbon signals (Table 1), which were characterized by DEPT as four methyls, six sp³ methylenes, three sp³ methines, two sp² methines, two sp³ quaternary carbons and three sp² quaternary carbons. A suite of resonances at δ_C 172.2 (C-15), 171.6 (C-13), 116.7 (CH-14) and 99.4 (CH-16), could be assigned to an α,β-unsaturated-γ-lactone moiety [6]. A trisubstituted olefin was deduced from the ¹³C NMR data at δ_C 145.0 (C-10) and 118.1 (CH-1). Thus, from the reported data, the proposed skeleton of **1** was suggested to be a diterpenoid with three rings.

Table 1. ^1H (400 MHz, CDCl_3) and ^{13}C (100 MHz, CDCl_3) NMR data, ^1H - ^1H COSY and HMBC correlations for diterpenoid **1**.

Position	δ_{H} (J in Hz)	δ_{C} , Multiple	^1H - ^1H COSY	HMBC
1	5.40 br s	118.1, CH	H ₂ -2	C-2, -9
2	2.02 m	23.2, CH ₂	H-1, H ₂ -3	C-1, -3, -4, -10
3	1.11 m, 1.28 m	31.2, CH ₂	H ₂ -2	C-2, -4, -18, -19
4		31.2, C		
5	1.49 br d (14.8)	43.7, CH	H ₂ -6	C-3, -4, -7, -10
6	1.09 m, 1.80 m	30.0, CH ₂	H-5, H ₂ -7	n.o.
7	1.42 m, 1.55 m	31.1, CH ₂	H ₂ -6, H-8	C-8, -9
8	1.32 m	44.3, CH	H ₂ -7, H ₃ -17	C-7, -9, -10, -17
9		42.5, C		
10		145.0, C		
11	1.32 m, 1.90 m	28.2, CH ₂	H ₂ -12	C-10, -13, -20
12	2.10 m	22.3, CH ₂	H ₂ -11	
13		171.6, C		
14	5.79 s	116.7, CH		C-13, -15, -16
15		172.2, C		
16	5.98 s	99.4, CH		C-13, -14, -15
17	0.86 d (6.4)	16.3, CH ₃	H-8	C-7, -8, -9
18	0.85 s	27.5, CH ₃		C-3, -4, -5, -19
19	0.84 s	27.7, CH ₃		C-3, -4, -5, -18
20	1.04 s	22.9, CH ₃		C-8, -9, -10, -11

n.o. = not observed.

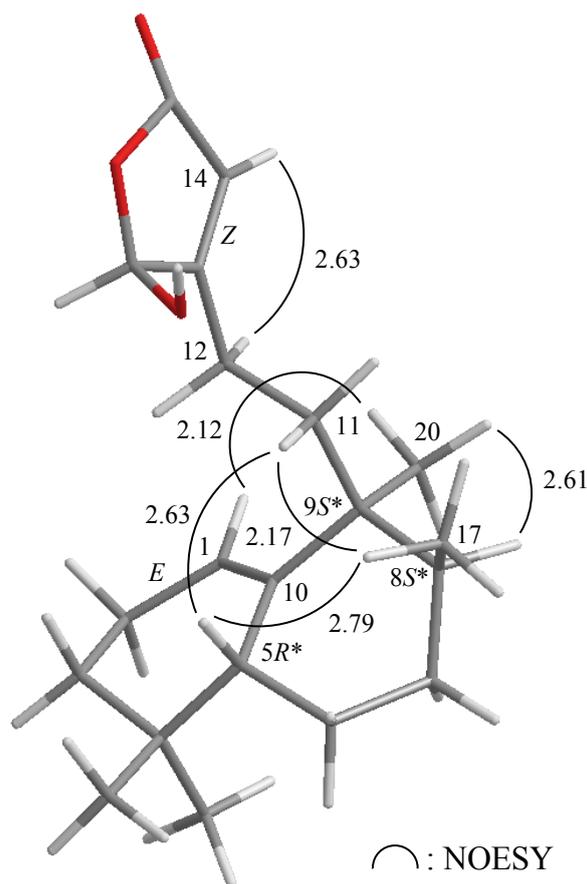
Figure 2. The ^1H - ^1H COSY and selective HMBC correlations (protons \rightarrow quaternary carbons) for diterpenoid **1**.

From the ^1H - ^1H COSY experiment of **1** (Table 1 and Figure 2), it was possible to establish the spin systems that map out the proton sequences from H-1/H₂-2/H₂-3, H-5/H₂-6/H₂-7/H-8/H₃-17 and

H₂-11/H₂-12, which was accomplished with the assistance of an HMBC experiment (Table 1 and Figure 2). The key HMBC correlations between protons and quaternary carbons of **1**, including H₂-2, H₂-3, H-5, H₃-18, H₃-19/C-4; H-1, H₂-7, H-8, H₃-17, H₃-20/C-9; H₂-2, H-5, H-8, H₂-11, H₃-20/C-10; H₂-11, H-14, H-16/C-13; and H-14, H-16/C-15, permitted the elucidation of the carbon skeleton of **1**. The tertiary methyls at C-4, C-8 and C-9 were confirmed by the HMBC correlations between H₃-18/C-3, -4, -5, -19; H₃-19/C-3, -4, -5, -18; H₃-17/C-7, -8, -9; and H₃-20/C-8, -9, -10, -11. The methine unit at δ_C 99.4 (CH-16) was more shielded than that expected for an oxygenated C-atom and correlated with the methine proton at δ_H 5.98 (H-16) in the HMQC spectrum, and this proton showed ³J-correlations with C-14 and C-15 in the HMBC spectrum and concluded to be a part of a hemiketal constellation.

The relative configuration of **1** was elucidated mainly from a NOESY spectrum as being compatible with that of **1** ascertained using molecular mechanics calculations (MM2), which suggested the most stable conformations, as shown in Figure 3 [7], in which the close contacts of atoms in space calculated were consistent the NOESY correlations. In the NOESY experiment of **1**, H-5 exhibited correlations with H₃-17 and a proton of C-11 methylene (δ_H 1.90), but not with H-8 and H₃-20, indicated that H-5 and Me-17 were situated on the same face in **1**, and these were assigned as β protons, since the C-20 methyl is an α -substituent at C-9. The *E*-configuration of C-1/10 double bond was elucidated from a correlation between H-1 and H₃-20. Based on the above findings, the main structure of **1** was elucidated unambiguously, and the chiral carbons for **1** were assigned as 5*R**, 8*S**, and 9*S** although the configuration for 16-hydroxy group was not determined at this stage by this method.

Figure 3. The stereoview of **1** (generated from computer modeling) and the calculated distances (Å) between selected protons having key NOESY correlations.



The cytotoxicity of diterpenoid **1** toward K562 (human erythromyeloblastoid leukemia), MOLT-4 (human acute lymphoblastic leukemia), HL-60 (human acute promyelocytic leukemia), DLD-1 (human colorectal adenocarcinoma), LoVo (human colorectal adenocarcinoma) and DU-145 (human prostate carcinoma) cells was studied, and the results were shown in Table 2. These data showed that echinohalimane A (**1**) exhibited cytotoxicity toward MOLT-4, HL-60, DLD-1 and LoVo cells. Furthermore, the *in vitro* anti-inflammatory effects of diterpenoid **1** were tested and echinohalimane A (**1**) displayed a significant inhibitory effect on the release of elastase by human neutrophils (Table 3).

Table 2. Cytotoxic data of diterpenoid **1**.

Compounds	Cell lines IC ₅₀ (µg/mL)					
	K562	MOLT-4	HL-60	DLD-1	LoVo	DU-145
1	6.292	2.111	2.117	0.967	0.563	NA ^b
Doxorubicin ^a	0.171	0.001	0.048	2.322	0.959	0.005

^a Doxorubicin was used as positive control; ^b NA = not active at 20 µg/mL.

Table 3. Inhibitory effects of diterpenoid **1** on the generation of superoxide anion and the release of elastase by human neutrophils in response to FMLP/CB.

Compounds	Superoxide anion		Elastase release
	IC ₅₀ (µg/mL)	Inh% ^a	IC ₅₀ (µg/mL)
1	>10	20.55 ± 5.18	0.38 ± 0.14
DPI ^b	0.80 ± 0.21		
Elastatinal ^b			31.95 ± 5.92

^a Percentage of inhibition (Inh%) at a concentration of 10 µg/mL; ^b DPI (diphenylene indonium) and elastatinal were used as positive control.

3. Experimental Section

3.1. General Experimental Procedures

Optical rotations were measured on a Jasco P-1010 digital polarimeter. Infrared spectra were recorded on a Varian Digilab FTS 1000 FT-IR spectrometer; peaks are reported in cm⁻¹. NMR spectra were recorded on a Varian Inova 500 or a Varian Mercury Plus 400 NMR spectrometers using the residual CHCl₃ signal (δ_H 7.26 ppm) as internal standard for ¹H NMR and CDCl₃ (δ_C 77.1 ppm) for ¹³C NMR. Coupling constants (*J*) are given in Hz. ESIMS and HRESIMS were recorded by a Bruker APEX II mass spectrometer. Column chromatography was performed on silica gel (230–400 mesh, Merck, Darmstadt, Germany). TLC was carried out on precoated Kieselgel 60 F₂₅₄ (0.25 mm, Merck); spots were visualized by spraying with 10% H₂SO₄ solution followed by heating. HPLC was performed using a system comprised of a Hitachi L-7100 pump, a Hitachi L-7455 photodiode array detector and a Rheodyne injection port. A normal phase column (Hibar 250 × 10 mm, Merck, silica gel 60, 5 µm) was used for HPLC.

3.2. Animal Material

Specimens of the gorgonian coral *Echinomuricea* sp. were collected by hand using scuba equipment off the coast of the southern Taiwan and stored in a freezer until extraction. This organism was identified by comparison with previous descriptions [8,9]. A voucher specimen (NMMBA-TW-GC-125) was deposited in the National Museum of Marine Biology and Aquarium, Taiwan.

3.3. Extraction and Isolation

The freeze-dried and minced material of *Echinomuricea* sp. (wet weight 1.68 kg, dry weight 428 g) was extracted with a mixture of methanol (MeOH) and dichloromethane (CH₂Cl₂) (1:1). The residue was partitioned with ethyl acetate (EtOAc) and H₂O. The EtOAc layer was partitioned between MeOH and *n*-hexane. The *n*-hexane layer was separated by silica gel and eluted using *n*-hexane/EtOAc/MeOH to yield 21 fractions A–U. Fraction M was separated on silica gel and eluted using a mixture of *n*-hexane/EtOAc to yield 10 fractions M1–M10. Fraction M3 was further purified by normal phase HPLC (*n*-hexane/acetone, 10:1) to yield compound **1** (33.7 mg).

Echinohalimane A (**1**): yellowish oil; $[\alpha]_D^{25}$ -102 (*c* 1.69, CHCl₃); IR (neat) ν_{\max} 3375, 1755 cm⁻¹; ¹H (CDCl₃, 400 MHz) and ¹³C (CDCl₃, 100 MHz) NMR data, see Table 1; ESIMS: *m/z* 341 (M + Na)⁺; HRESIMS: *m/z* 341.2089 (calcd for C₁₅H₁₈O₄ + Na, 341.2093).

3.4. Molecular Mechanics Calculations

Implementation of the MM2 force field [7] in CHEM3D PRO software from CambridgeSoft Corporation (Cambridge, MA, USA; ver. 9.0, 2005) was used to calculate molecular models.

3.5. Cytotoxic Assay

The cytotoxicity of diterpenoid **1** was assayed with a modification of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric method according to previously described procedures [10,11].

3.6. Superoxide Anion Generation and Elastase Release by Human Neutrophils

Human neutrophils were obtained by means of dextran sedimentation and Ficoll centrifugation. Measurements of superoxide anion generation and elastase release were carried out according to previously described procedures [12,13]. Briefly, superoxide anion production was assayed by monitoring the superoxide dismutase-inhibitable reduction of ferricytochrome *c*. Elastase release experiments were performed using MeO-Suc-Ala-Ala-Pro-Valp-nitroanilide as the elastase substrate.

4. Conclusions

In general, halimane-type diterpenoids exist in terrestrial plants, and are rarely found in marine organisms [14]. The compounds of this type were found to possess a carbon skeleton intermediate between that of labdanes and clerodanes [15,16]. It is worth noting that echinohalimane A (**1**) is the

first halimane-type derivative isolated from marine organisms belonging to phylum Cnidaria. The study material *Echinomuricea* sp. has begun to be transplanted in culturing tanks with a flow-through sea water system located in the National Museum of Marine Biology and Aquarium, Taiwan, for the extraction of additional natural products in order to establish a stable supply of bioactive material.

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Samples Availability: Not available.

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