Halioxepine, a New Meroditerpene from an Indonesian Sponge *Haliclona* sp.

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Chemical investigations on a sponge *Haliclona* sp. found a meroditerpene 1 having a new carbon skeleton. By analyzing spectroscopic data, the structure was elucidated to comprise a substituted hydroquinone, a tetrahydrooxepine, and a cyclohexene, and these components were united with C1 and C2 units. Compound 1 showed moderate cytotoxicity against NBT-T2 cells with IC_{50} 4.8 μg/ml and also antioxidant activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) with IC_{50} 3.2 μg/ml.

Key words marine natural product; sponge; antioxidant activity; cytotoxicity; 1,1-diphenyl-2-picrylhydrazyl; NBT-T2

Extensive investigations on marine natural products have shown that marine sponges are nearly inexhaustible sources of new secondary metabolites.[1)] From a structural viewpoint, various classes of molecules including macrolides,[2)] polypeptides,[3)] terpenes,[4)] steroids,[5)] alkaloids,[6)] and others[7)] have been isolated, and a number of different modes of biological activity have been reported. The sponges belonging to the genus *Haliclona* are also prolific sources of unique molecules such as manzamines,[8)] haliclonamines,[9)] haliclonamides,[10)] halipeptins,[11)] and others.

In our continuing search for new cytotoxic metabolites from Indonesian sponges,[12,13)] we collected a small sponge belonging to *Haliclona* at Baubau, Indonesia. After the ^{1}H-NMR spectrum of a methanolic extract from the sponge indicated the presence of a unique molecule, we examined the chemical contents. In this note we describe the isolation, structure characterization, and bioactivity of the new compound, which we named halioxepine (**1**).

Results and Discussion

The sponge (45 g, wet) was soaked in MeOH to provide a crude extract, whose ^{1}H-NMR spectrum showed the presence of a unique metabolite as one of principal components. The extract was triturated with CH_2Cl_2 to provide a lipophilic fraction (136.5 mg) followed by purification on silica HPLC to give compound **1** (25.3 mg).

Compound **1** was obtained as a slightly purple glass, $[\alpha]_D^{25}$ +49. The electrospray ionization (ESI)-MS showed a quasimolecular ion at *m/z* 437.26620 $[M+Na]^+$ compatible with the molecular formula $C_{26}H_{38}O_4$ indicating eight degrees of unsaturation. Its Fourier transform (FT)-IR spectrum exhibited a strong absorption at 3356 cm^{-1} due to the presence of hydroxyl groups.

Analysis of the ^{1}H- and ^{13}C-NMR spectra of **1** allowed us to identify the presence of a 1,2,4-trisubstituted aromatic ring: δ_H 6.72 d (J=8.7 Hz), 6.64 dd (J=3.0, 8.7 Hz), 6.57 d (J=3.0 Hz); δ_C 115.0 d, 115.5 d, 118.0 d, 127.6 s, 148.6 s, and 149.4 s. With 2D-NMR analysis and relatively higher chemical shifts for the aromatic protons, this structural unit I was assigned as 2-substituted hydroquinone (Fig. 1). Further analysis on correlation spectroscopy (COSY) and heteronuclear single quantum coherence (HSQC) revealed the presence of unit II with two oxymethines: δ_H 4.98 d (J=2.6 Hz), 4.46 br s; δ_C 75.5 d, 76.8 d and unit III –$C(CH_3)$=CH–CH_2–CH_2–: δ_H 1.77 d (3H, J=1.3 Hz), 5.66 qd (J=1.3, 6.7 Hz), 2.43 m, 1.88 m, and 1.66 m (2H); δ_C 21.4 q, 135.6 s, 130.0 d, 23.3 t, and 37.5 t. Another structural unit IV is made of two successive methylenes: δ_H 1.27 dt (J=2.5, 12.6 Hz), 1.49 dt (J=3.6, 12.6 Hz), 1.24 dt (J=3.6, 12.6 Hz), and 1.40 dt (J=2.5, 12.6 Hz); δ_C 34.7 t and 30.6 t, while the last structural unit V is composed of –$CH(CH_3)$–CH_2–CH_2–CH=C< corresponding to NMR signals at δ_H 1.69 m; 0.81 d (3H, J=7.0 Hz), 1.43 m (2H), 1.96 m (2H), and 5.41 br q (J=1.6 Hz); δ_C 33.2 d, 15.6 q, 27.0 t, 25.5 t, and 124.2 d.

The above five structural units were connected by observing the following key heteronuclear multiple bond connectivity (HMBC) correlations: δ_H 4.98/δ_C 127.6, 149.4; δ_H 6.57/δ_C 75.5 for units I and II, δ_H 1.77/δ_C 76.8, 135.6, 130.0; δ_H 2.43, 1.88/δ_C 135.6, 130.0, 37.5 for II and III, and δ_H

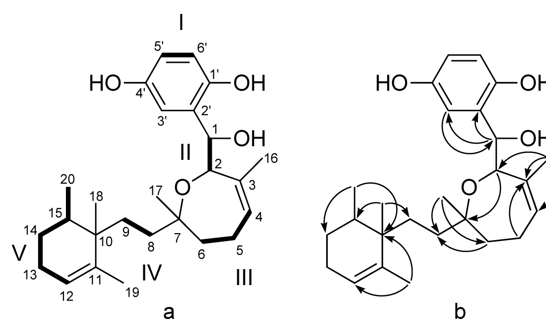

Fig. 1. Selected COSY (a) and HMBC (b) Correlations in **1**

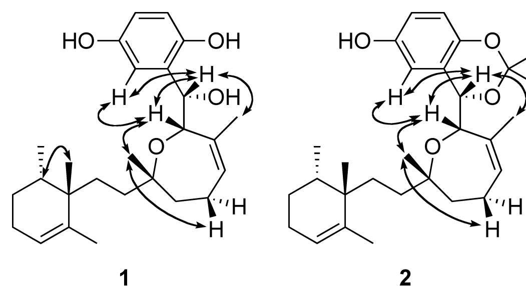

Fig. 2. Selected NOEs for **1** and Acetonide **2**

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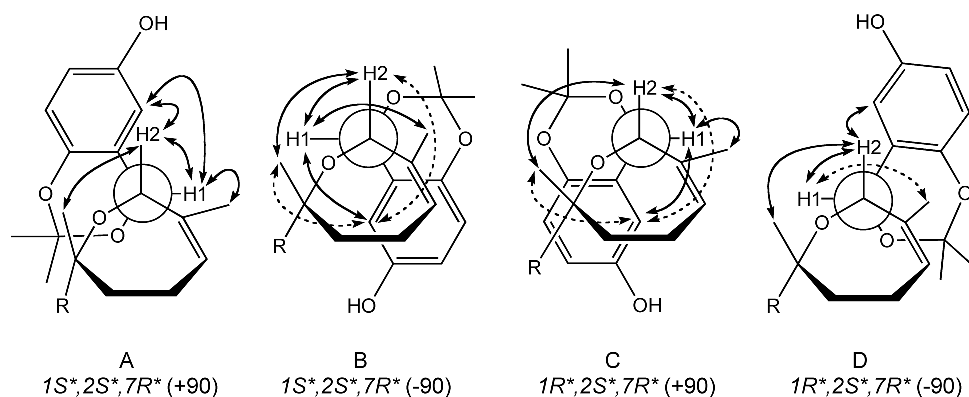

Fig. 3. Four Possible Structures A—D

$0.82/\delta_C$ 37.5, 78.4, 34.7 for III and IV. HMBC correlations: δ_H $0.86/\delta_C$ 30.6, 40.1, 33.2, 139.5; δ_H $0.81/\delta_C$ 40.1; δ_H $1.61/\delta_C$ 40.1, 124.2, 139.5, to connect the units IV and V also supported the presence of a cyclohexene ring. Of the total eight degrees of unsaturation, it can be explained as, four to a hydroquinone, two to a cyclohexene, and one to a double bond. The remaining one can be assigned to a tetrahydrooxepine ring as an HMBC correlation was observed between δ_H 4.46 (H-2)/δ_C 78.4 (C-7).

For the relative stereochemistry of the cyclohexene and the tetrahydrooxepine rings, it was elucidated to have 10*S**,15*S** and 2*S**,7*R** with nuclear Overhauser effects (NOEs) between H-15 and Me-18 and also between H-2 (δ 4.46 br s) and Me-17 (δ 0.82 s). As the coupling constant between H-1 and H-2 was quite small, the dihedral angle of H-1/C-1/C-2/H-2 is close to $+90°$ or $-90°$. It is caused by restricted rotation of the bonds C-1/C-2 and C-1/C-2′ with steric hindrance between the hydroquinone and the tetrahydrooxepine ring. This relationship was also confirmed in acetonide **2** prepared from **1** (Fig. 2). To elucidate the configuration at C-1, we examined four possible cases A—D having either 1*R** or 1*S** configuration and $+90°$ or $-90°$ of the dihedral angle for H-1/C-1/C-2/H-2 as in Fig. 3. Among them, only the structure A (1*S**,2*S**,7*R**, $+90°$ for the dihedral angle) is consistent with observed NOEs: H-2/H-3′, H-1/H-3′, and H-1/Me-16. Therefore, the relative stereochemistry of halioxepine (**1**) can be summarized either 1*S**,2*S**,7*R**,10*S**,15*S** or 1*S**,2*S**,7*R**,10*R**,15*R**.

Halioxepine (**1**) showed moderate cytotoxicity against NBT-T2 cells with IC_{50} 4.8 μg/ml. Compound **1** also exhibited antioxidant activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) with IC_{50} 3.2 μg/ml as well as other hydroquinone-containing meroditerpenes.[14] A certain number of meroditerpenes have been reported from marine organisms, but so far there seems to be no molecule with exactly the same carbon skeleton as halioxepine (**1**).

Experimental

General Technical grade methanol (MeOH) was used for extraction. Reagent grade solvents were used for isolating compound **1**. Merck Si-60 (70—230 mesh) was used for silica gel column chromatography and Merck Si-60 F_{254} was used for analytical TLC. HPLC was performed either on a Waters 510 pump with a Waters 486 UV detector and a Shodex RI-101 or on a Hitachi L-6000 pump with a Hitachi L-4000 UV detector and a Shodex RI-101 using a Mightysil Si-60 (10×250 mm) column. Optical rotation was measured on a Jasco P-1010 polarimeter using a cell with 3.5 mm aperture. IR and UV spectra were recorded on a Jasco FT/IR-6100 instrument and on a Hitachi U-2001 spectrophotometer, respectively. High resolution (HR)-ESI-MS were measured on a Jeol JMS-T100LP spectrometer using reserpine as a standard. ^{1}H-, ^{13}C-, and 2D-NMR spectra were measured in $CDCl_3$ with tetramethylsilane (TMS) as an internal standard on a Bruker AVANCE III-500 in $CDCl_3$ or in CD_3OD. The ^{1}H- and ^{13}C-NMR chemical shifts were given in ppm, while coupling constants were in Hz. NOEs were measured as both nuclear Overhauser effect spectroscopy (NOESY) and difference spectra.

Sponge The sponge *Haliclona* sp. (family Chalinidae, order Haplosclerida) was collected off Baubau, Buton Island, South-East Sulawesi, Indonesia, at around 20 m depth by hand during SCUBA diving. The specimen was kept frozen until extraction. This sponge may be an endemic species in this region. Identification of the sponge was conducted by N. J. de Voogd, and the specimen is deposited at Naturalis with the code number RMNH POR 3930.

Extraction and Isolation After cutting into small pieces, the sponge (45 g, wet) was soaked in MeOH (100 ml) for 24 h, for three times. The solution was then concentrated under vacuum to obtain a crude extract. The methanolic extract (1.85 g) was triturated with CH_2Cl_2 to provide a lipophilic fraction (136.5 mg). After passing through a small amount of silica gel with EtOAc, the fraction was subjected to silica HPLC with a mixture of *n*-hexane and EtOAc in a ratio of 2 : 1 to provide halioxepine (**1**, 25.3 mg).

Halioxepine (**1**): Pale purple glass, $[\alpha]_D^{25}$ +49 (c=0.5, MeOH). ^{1}H- and ^{13}C-NMR see Table 1. IR (neat) 3356, 2962, 2922, 1505, 1456, 1377, 1238, 1078, 892, 742 cm^{-1}. UV λ_{max} (MeOH) nm (ε): 205 (7900), 293 (4500). HR-ESI-MS *m/z* 437.26620 (Calcd for $C_{26}H_{38}NaO_4^+$, 437.26623, Δ −0.03).

Conversion of 1 to Acetonide 2 To 0.5 mg of compound **1** was added 2,2-dimethoxypropane (0.5 ml) and 0.4 mg of 10-CSA (10-camphorsulfonic-acid). The mixture was stirred under N_2 atmosphere for 10 h at room temperature. After adding triethylamine (50 μl), the mixture was concentrated and the product was purified by preparative TLC (CH_2Cl_2–EtOAc, 10 : 1).

Acetonide 2 ^{1}H-NMR ($CDCl_3$) δ: 6.88 (1H, br s, H-3′), 6.67 (1H, dd, J=1.3, 8.4 Hz, H-5′), 6.67 (1H, d, J=8.4 Hz, H-6′), 5.52 (1H, br d, J=5.1 Hz, H-4), 5.40 (1H, br s, H-12), 5.01 (1H, br s, H-1), 4.58 (1H, br s, H-2), 2.44 (1H, dd, J=2.5, 12.3 Hz, H-5b), 1.98 (1H, m, H-13b), 1.90 (1H, m, H-13a), 1.77 (1H, t, J=12.4 Hz, H-5a), 1.66 (3H, br s, Me-16), 1.65 (1H, m, H-15), 1.46 (3H, s, Me-19), 1.43 (3H, m, H-14), 1.42 (1H, m, H-9b), 1.28 (6H, s, Me-acetonide), 0.82 (3H, d, J=6.8 Hz, Me-20), 0.81 (3H, s, Me-18), 0.80 (3H, s, Me-17). HR-ESI-MS *m/z* 477.29629 (Calcd for $C_{26}H_{38}NaO_4^+$, 477.29808, Δ −1.79).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay The MTT assay was performed against the NBT-T2 cells (RIKEN) according to the method in the literature[15] with a slight modification to obtain an IC_{50} value. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotic-antimycotic (Sigma), fetal bovine serum (FBS) (Biowest), and minimum essential medium (MEM) nonessential amino acid (Gibco). Cultured cells were inoculated to a 96-well plate and treated with a series of concentration of **1**. After incubation, 20 μl of MTT solution (5 g/ml) in phosphate buffered saline (PBS) was added to each well, and diluted with 150 μl of dimethyl sulfoxide (DMSO) prior to measurement with a Tecan microplate reader at 590 nm with reference filter at 620 nm.

Scavenging Activity Test The DPPH radical scavenging activity was measured by the method of Yang *et al.* with a small modification.[16] DPPH was diluted in MeOH at a concentration of 60 μg/ml. Then, a series of

Table 1. NMR Data for Halioxepine (**1**) in $CDCl_3$

No	^{13}C	^{1}H (J in Hz)	COSY	HMBC
1	75.5 d	4.98 (1H, d, J=2.6)	H-2	C-3, C-1′, C-2′
2	76.8 d	4.46 (1H, br s)	H-1, H-4, H-5b, H-16	C-1, C-7
3	135.6 s	—	—	—
4	130.0 d	5.66 (1H, qd, J=1.3, 6.7)	H-2, H-5ab, H-16	C-2, C-5, C-6, C-16
5	23.3 t	1.88 (1H, m)	H-4, H-5b, H-6	C-3, C-4, C-6
		2.43 (1H, m)	H-2, H-4, H-5a, H-6	C-3, C-4, C-6
6	37.5 t	1.66 (2H, m)	H-5ab	C-4, C-5, C-7, C-8
7	78.4 s	—	—	—
8	34.7 t	1.27 (1H, dt, J=2.5, 12.6)	H-8b, H-9ab	C-7, C-9, C-17
		1.49 (1H, dt, J=3.6,12.6)	H-8a, H-9ab	C-6, C-7, C-9, C-17
9	30.6 t	1.24 (1H, dt, J=3.6, 12.6)	H-8ab, H-9b	C-8, C-7, C-15
		1.40 (1H, dt, J=2.5, 12.6)	H-8ab, H-9a	C-8, C-7, C-15
10	40.1 s	—	—	—
11	139.5 s	—	—	—
12	124.2 d	5.41 (1H, br q, J=1.6)	H-13, H-19	C-10, C-13, C-14
13	25.5 t	1.96 (2H, m)	H-12, H-14, H-15	C-12, C-14
14	27.0 t	1.43 (2H, m)	H-13, H-15	C-12, C-13, C-15
15	33.2 d	1.69 (1H, m)	H-13, H-14, H-20	C-10, C-11, C-14, C-20
16	21.4 q	1.77 (3H, d, J=1.3)	H-2, H-4	C-2, C-3, C-4
17	22.6 q	0.82 (3H, s)	—	C-6, C-7, C-8
18	21.2 q	0.86 (3H, s)	—	C-9, C-10, C-11, C-15
19	19.1 q	1.61 (3H, d, J=1.6)	H-12	C-10, C-11, C-12
20	15.6 q	0.81 (3H, d, J=7.0)	H-15	C-10, C-14, C-15
1′	149.4 s	—	—	—
2′	127.6 s	—	—	—
3′	115.0 d	6.57 (1H, d, J=3.0)	H-5′, H-6′	C-1, C-4′, C-5′
4′	148.6 s	—	—	—
5′	115.5 d	6.64 (1H, dd, J=3.0, 8.7)	H-3′, H-6′	C-1′, C-4′, C-6′
6′	118.0 d	6.72 (1H, d, J=8.7)	H-3′, H-5′	C-1, C-2′, C-4′, C-5′

methanolic solution of compound **1** was dispensed. Gallic acid and MeOH were used as positive and negative controls, respectively. After incubation for 20 min at room temperature, the absorbance was measured at 517 nm with a microplate reader. The scavenging activity of DPPH radicals was calculated according to the following equation: DPPH radical scavenging activity (%)=(1−absorbance of sample/absorbance of control)×100.[17)]

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