Two new compounds from an Indonesian sponge Dysidea sp.

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On our joint bioprospecting research on Indonesian marine invertebrates, we found moderate cytotoxicity on an extract of the sponge *Dysidea* sp. collected at Biak, West Papua. Separation of the extract provided two new compounds, biaketide (**1**) and debromoantazirine (**2**), along with four known molecules **3**–**6**. The new structures were elucidated by spectroscopic analyses and by comparison with those reported. Compounds **1** and **2** showed moderate cytotoxicity against NBT-T2 cells with IC_{50} values of 8.3 and 4.7 $\mu g\,ml^{-1}$, respectively.

Keywords: marine natural products; polyketide; azirine; cytotoxicity; sponge

1. Introduction

Eastern Indonesian water is located at the center of Coral Reef Triangle region, where it is recognized as the epicenter of marine biodiversity [1]. On our bioprospecting research from this region to discover new bioactive molecules, we have reported several molecules from Alor [2], Kupang [3], and South-East Sulawesi [4]. From more than 100 specimens collected at Biak, an extract prepared from a *Dysidea* sponge showed moderate cytotoxicity. *Dysidea* sponges have been known to contain various unique molecules such as macrolides [5], polybrominated diphenyl ethers [6], hydroquinone-containing terpenes [7], amino acid derivatives [8], fatty acids [9], and sterols [10]. From our material, we found two new metabolites, for which the structures and bioactivity are the subjects of this report (Figure 1).

2. Results and discussion

A frozen specimen of the sponge *Dysidea* sp. was extracted with MeOH to provide a crude extract showing cytotoxicity at 10 $\mu g\,ml^{-1}$ against NBT-T2 cells. As its EtOAc soluble portion also retained cytotoxicity at 10 $\mu g\,ml^{-1}$ against the cell, it was separated on a silica gel column followed by repetitive silica HPLC, affording two new compounds **1** and **2**, with four known compounds, antazirine (**3**), dysidazirine (**4**), neoavarol (**5**), and frondosin (**6**). The known compounds were identified by comparing the spectroscopic data with those published in the literature [11–14].

Compound **1** was isolated as a white solid with optical activity, $[\alpha]_D^{25} + 20$. HR-ESI-MS of **1** established the molecular formula as $C_{17}H_{27}ClO_2$ by observing pseudomolecular ions at *m/z* 321.15730 and 323.15678 $[M + Na]^+$ in a 3:1 intensity ratio.

^{1}H NMR spectrum showed the presence of two olefinic (δ_H 5.74 s, 5.22 d), one oxymethine (δ_H 4.29 d), one vinyl, two doublet, and one triplet methyls (δ_H 1.60 d, 1.07 d, 0.93 d, and 0.90 t), along with 12 aliphatic protons resonated between δ_H 1.4 and δ_H 2.7 (Table 1). ^{13}C NMR signals were

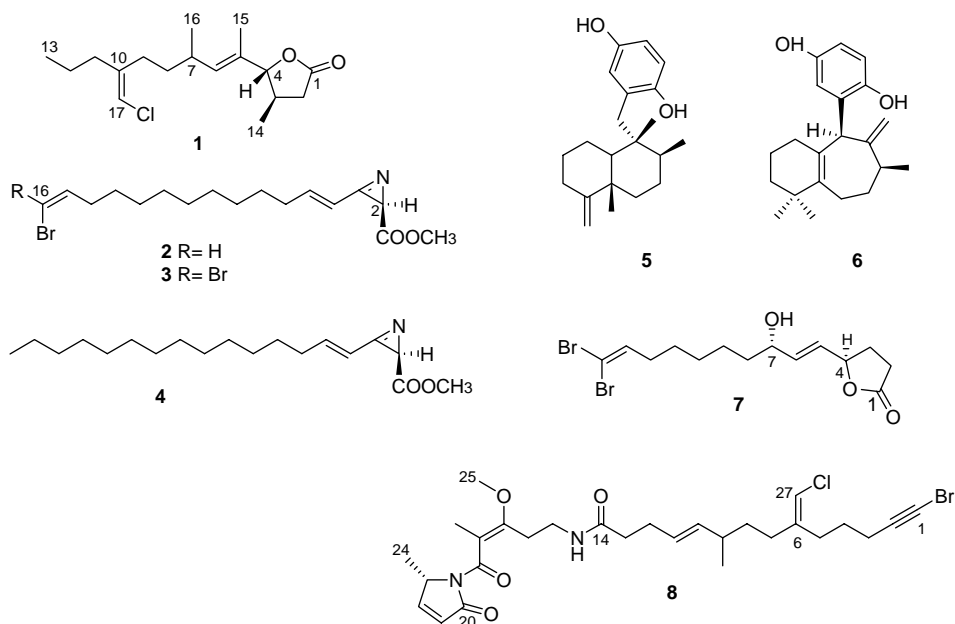*Corresponding author. trianto_telawur@yahoo.co.id

Figure 1. Structures of new compounds **1** and **2**, (*E*)-antazirine (**3**), dysidazirine (**4**), neoavarol (**5**), frondosin (**6**), and the model compounds **7** and **8**.

observed for an ester carbonyl (δ_C 175.2 s), four olefinic (δ_C 142.2 s, 136.4 s, 129.7 d, and 112.2 d), one oxymethine (δ_C 92.3), two methine (δ_C 34.3 and 31.8), five methylene (δ_C 37.0, 35.1, 32.6, 32.0, and 20.4), and four methyl carbons (δ_C 20.9, 16.8, 14.0, and

Table 1. ^1H and ^{13}C NMR spectral data for **1** in CDCl$_3$.

No.	^{13}C	^1H (*J* in Hz)	COSY	HMBC
1	175.2 s	–		
2	37.0 t	2.66 (1H, dd, *J* = 17.1, 6.6)	H-2β, H-3	C-1, C-3, C-4
		2.20 (1H, dd, *J* = 17.1, 10.6)	H-2α, H-3	C-1, C-3, C-4
3	34.3 d	2.34 (1H, m)	H-2α/β, H-4, H-14	C-1, C-4, C-14
4	92.3 d	4.29 (1H, d, *J* = 8.6)	H-3	C-3, C-5, C-14
5	129.7 s	–		
6	136.4 d	5.22 (1H, d, *J* = 9.1)	H-7	C-4, C-5, C-7
7	31.8 d	2.36 (1H, m)	H-6, H-8α/β	C-8
8	35.1 t	1.33 (1H, m)	H-7, H-8β, H-9α/β	C-7, C-9
		1.45 (1H, m)	H-7, H-8β, H-9α/β	C-7, C-9
9	32.0 t	2.13 (1H, m)	H-8αβ, H-9β	C-8, C-10, C-11, C-17
		2.16 (1H, m)	H-8αβ, H-9 α	C-8, C-10, C-17
10	142.2 s	–		
11	32.6 t	1.99 (2H, m)	H-12	C-10, C-17
12	20.4 t	1.40 (1H, m)	H-12β, H-13	C-11, C-13
		1.45 (1H, m)	H-12α, H-13	C-11, C-13
13	14.0 q	0.90 (3H t, *J* = 7.3)	H-12αβ	C-11, C-12
14	16.8 q	1.07 (3H, d, *J* = 6.6)	H-3	C-2, C-3, C-4
15	11.3 q	1.60 (1H d, *J* = 1.2)	–	C-4, C-5, C-6
16	20.9 q	0.93 (1H, d, *J* = 7.1)	H-7	C-6, C-7, C-8
17	112.2 q	5.74 (1H, s)	–	C-9, C-10, C-11

11.3). Therefore, totally four degrees of unsaturation were accounted for two double bonds and the lactone moiety (1786 cm^{-1}).

Correlation spectroscopy (COSY) analysis revealed three substructures in **1**. The first partial structure I was consisted of —CH—CH(CH$_3$)—CH$_2$— with proton signals at δ_H 4.29 d, 2.34 m, 1.07 d, 2.66 dd, and 2.20 dd (Figure 2). The second partial structure II linked aliphatic groups as —CH—CH(CH$_3$)—CH$_2$—CH$_2$— observed at δ_H 5.22 d, 2.36 m, 0.93 d, 1.33 m, 1.45 m, 2.13 m, and 2.16 m. The last structural unit III appeared as an *n*-propyl group at δ_H 1.99 m (2H), 1.40 m, 1.45 m, and 0.90 t. The remaining isolated proton was at δ_H 5.74 s.

The presence of γ-lactone including unit I was supported by heteronuclear multiple-bond correlation spectroscopy (HMBC) correlations between methylene protons (δ_H 2.66 dd, 2.20 dd, $^2J = 17.1$ Hz) and the lactone carbonyl and by the presence of the sole oxymethine proton (δ_H 4.29 d). Substructures I and II were linked by HMBC correlations from vinyl methyl (δ_H 1.60) to lactone oxymethine (δ_C 92.3) and also to olefinic carbons (δ_C 129.7 and 136.4).

Characteristically, the isolated olefinic proton at δ_H 5.74 attaches on a double bond (δ_C 112.2, 142.2) which was indicative of a vinyl chloride as found in model compound **8** [chemical shifts of the model molecule, both ^{1}H (5.79 s) and ^{13}C (112.2 d, 142.2 s)] [15]. The latter carbon signal showed HMBC correlations with methylene protons at δ_H 2.13 and 2.16 of the unit II and also at δ_H 1.99 of the unit III indicating that the structural units II and III connect to this double bond. As the vinyl proton appeared as a sharp singlet and nuclear overhouser effect (NOE) was observed between the vinyl proton and one of methylene protons at C-11, the substitution pattern should be as depicted.

For the lactone portion, the relative stereochemistry at C-3 and C-4 was established as shown in Figure 1 by a positive NOE between C-3 methyl proton and H-4 and no NOE between H-3 and H-4. The double bond geometry at C-5 was confirmed to be *E* by an NOE between a proton at δ_H 5.22 (1H, d, $J = 9.1$, H-6) and a proton at δ_H 4.29 d ($J = 8.6$, H-4) and a typical chemical shift for C-15 [16]. The double-bond configuration at C-10,17 was also confirmed as *Z* by NOE data.

Compound **2** was obtained as a white solid with $[\alpha]_D^{25} - 20$. Its molecular formula C$_{17}$H$_{26}$BrNO$_2$ was based on mass spectra at *m/z* 378.10416 and 380.10224 [M + Na]$^+$, for which the intensities are in a ratio of 1:1 supporting the presence of one bromine atom. ^{1}H and ^{13}C NMR spectra of **2** resembled to those of 4*E*,15*Z*-(*S*)-antazirine (**3**) suggesting that **2** is an analog of **3**. Detailed comparison of the two molecules pointed out that the structural difference exists not on the azirine (δ_H 2.56 s, 3.71 s; δ_C 28.3 d, 156.6 s) or *E*-double bond portion (δ_H 6.53 dt, 6.68 dt; δ_C 112.9, 155.8 d) but on the terminal double bond (Table 2). Signals for the double bond in **2** were observed at δ_H 6.36 dt, 6.12 dt, δ_C 135.0 d, 107.6 d, while those in **3** were observed at δ_H 6.06 t, δ_C 133.9 d, 106.3 s indicating that compound **2** is a debromo analog of **3**. The geometry of this double bond was assigned as *Z* with their coupling constant
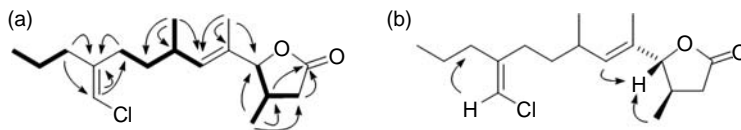

Figure 2. (a) COSY (bold) and key HMBC (arrows) correlations in compound **1**, (b) NOEs observed for compound **1**.

Table 2. ^{1}H and ^{13}C NMR spectral data for (4*E*)-(*S*)-antazirine and **2**.

| No. | (4*E*)-(*S*)-Antazirine (**3**) | | Compound **2** | |
	[13]C	[1]H	[13]C	[1]H
1	172.1 s	–	173.3 s[a]	
2	28.3 d	2.56 (1H, s)	28.3 d	2.56 (1H, s)
3	156.6 s	–	156.6 s	
4	112.9 d	6.53 (1H, d, $J = 16.0$ Hz)	112.9 d	6.53 (1H, dt, $J = 15.6$ and 1.4 Hz)
5	155.6 d	6.68 (1H, dt, $J = 16.0$ and 7.0 Hz)	155.8 d	6.68 (1H, dt, $J = 15.6$ and 6.8 Hz)
6	31.8 t	2,35 (2H, br q, $J = 7.0$ Hz)	31.8 t	2.35 (2H, dq, $J = 1.2$ and 6.8 Hz)
7	27.8 t	1.53–1.46 (2H, br m)	27.8 t	1.37 (2H, tt, $J = 6.9$ and 7.3 Hz)
8	29.3 t	1,23 (10 H, br s)	29.5 t	1.23 (10H, br s)
9	29.2 t		29.30 t	
10	29.1 t		29.27 t	
11	29.0 t		29.12 t	
12	29.1 t		29.05 t	
13	27.9 t	1.42–1.35 (2H, br m)	28.1 t	1.39 (2H, tt, $J = 6.8$ and 7.3 Hz)
14	33.2 t	2.10 (2H, q, $J = 7$ Hz)	32.9 t	2.07 (1H, dq, $J = 1.2$ and 7.0 Hz)
15	133.9 d	6.06 (1H, t, $J = 7.3$ Hz)	135.0 d	6.36 (1H, dt, $J = 7.0$ and 7.0 Hz)
16	106.3 s	–	107.6 d	6.12 (1H, dt, $J = 7.0$ and 1.2 Hz)
OMe	52.2 q	3.71 (1H, s)	52.2 q	3.71 (3H, s)

[a] Obtained from HMBC spectrum.

(7.0 Hz). As negative optical rotation values were observed for *R*-2H-azirine-2-carboxylic esters [11,12,17,18], it was suggested that **2** has *R*-configuration. *S*-configuration of dysidazirine and antazirines showed optical rotation with $[\alpha]_D^{25}$ value from $+8.9$ to $+96.0$. On the other hand, the *R*-configuration showed optical rotation with $[\alpha]_D^{25}$ value from -165 to -4 [11,12,17,18]. Compounds **1** and **2** showed moderate cytotoxicity against NBT-T2 cells with IC$_{50}$ values of 8.3 and 4.7 μg ml^{-1}, respectively.

3. Experimental

3.1 *General experimental procedures*

Optical rotations were measured on a JASCO P-1010 (JASCO, Tokyo, Japan) using 3.5 mm × 50 mm column in methanol. IR spectra were recorded on a JASCO FT/IR-6100. HR-ESI-MS were measured on a JEOL JMS-T100LP (JEOL, Tokyo, Japan) using reserpine as an internal standard. Low resolution fast atom bombardment was performed on JMS-SX-102/JMA-DA 6000 Data System (JEOL). ^{1}H and ^{13}C NMR spectra were measured on JEOL

alpha-500 and Bruker AVANCE III-500 (Bruker, Billerica, MA, USA) in CDCl$_3$ with tetramethylsilane as an internal standard. The proton and carbon chemical shifts were given in ppm, while coupling constants were given in Hz. Methanol (MeOH) used for extraction was of technical grade. Guaranteed reagent grade solvents (Wako, Tokyo, Japan) were utilized for isolating the compounds. Open column chromatography was performed on Si-60 (70–230 mesh) (Merck, NJ, USA). Thin layer chromatography was performed on Si-60 F$_{254}$ (Merck). HPLC was performed either on a Waters model 510 (Waters, Milford, MA, USA) equipped with a Waters 486 UV detector and a Shodex RI-101 (Shodex, Tokyo, Japan) or a Hitachi L-6000 (Hitachi, Tokyo, Japan) equipped with a Hitachi L-4000 UV detector and a Shodex R-101 on a Mightysil Si-60 (10 mm × 250 mm) column (Kanto, Tokyo, Japan) or Wakosil 5SIL (4.6 mm × 250 mm) (Wako).

3.2 *Animal material*

The sponge *Dysidea* sp. was collected by hand using SCUBA diving at 20–35 m

depths in Biak, Papua-Indonesia on August 2008. The sample was kept frozen until extracted. The sponge was identified by Nicole J. de Voogd. The sponge voucher was kept in National Museum of Natural History, Leiden, the Netherlands with registration number RMNH POR 7741. The specimens were also kept at Tanaka laboratory of University of the Ryukyus and a marine pharmaceutical laboratory of Diponegoro University.

3.3 Extraction and isolation

After cutting into small pieces, 4 kg of *Dysidea* sponge was soaked with MeOH for three times. Then, the solvent was concentrated under vacuum to obtain a crude extract. A part of the methanolic extract (29.1 g) was triturated with ethyl acetate (EtOAc) to give a fraction showing cytotoxicity at $10 \, \mu g \, ml^{-1}$. The EtOAc extract was subjected to a silica gel column using stepwise gradient solvents (hexane/EtOAc 5:1, 2:1, 1:2, 0:1; EtOAc/MeOH 10:1) to afford 12 fractions. The fifth fraction (209.1 mg) was purified by repetitive silica HPLC using solvent mixtures as hexane/CH_2Cl_2/EtOAc 10:10:1 followed by hexane/EtOAc 8:1 to give compound **1** (1.5 mg). The fourth fraction (77.6 mg) was purified by repetitive silica HPLC (hexane/EtOAc 1.75:1; hexane/EtOAc 12:1) followed by reversed-phase HPLC (MeOH/H_2O 10:1) to give compounds **2** (6.7 mg) and antazirine (**3**, 2.6 mg). Purification of the second fraction (301.9 mg) with silica HPLC (hexane/EtOAc 7.5:1) afforded dysidazirine (**4**, 77.4 mg). A part of the seventh fraction (128.3 mg) was purified with silica HPLC (CH_2Cl_2/EtOAc 50:1) to afford neoavarol (**5**, 23.5 mg) and frondosin (**6**, 10.0 mg).

3.3.1 Compound 1

White solid, $[\alpha]_D^{25} + 20$ ($c = 0.05$, MeOH). IR ν_{max} (neat) 1786 and $1203 \, cm^{-1}$. For ^1H and ^{13}C NMR spectral data, see Table 1. HR-ESI-MS: *m/z* 321.1597 and 323.1568 $[M + Na]^+$

(calcd for $C_{17}H_{27}ClO_2$, 321.1597 and 323.1574).

3.3.2 Compound 2

White solid, $[\alpha]_D^{25} - 20$ ($c = 0.05$, MeOH). IR ν_{max} (neat) 2924, 2850, 1747, 1728, 1620, 1435, 1334, 1264, and $1195 \, cm^{-1}$. For ^1H and ^{13}C NMR spectral data, see Table 2. HR-ESI-MS: *m/z* 378.1042 and 380.1022 $[M + Na]^+$ (calcd for $C_{17}H_{26}BrNNaO_2$, 378.1045 and 380.1024).

3.4 Cytotoxicity assay

NBT-T2 cells were utilized for screening and evaluating the cytotoxicity. NBT-T2 is a cell line derived from chemically induced rat bladder carcinoma cells [19]. The extract and fractions were tested at concentrations of 10^{-1}, 10^0, and $10^1 \, \mu g \, ml^{-1}$ in triplicate. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) in the presence of antibiotic-antimycotic (Sigma Chemical, St. Louis, MO, USA), fetal bovine serum (Biowest, Miami, FL, USA), minimum essential media, nonessential amino acid (Gibco, Pensacola, FL, USA) on a 24-well or 48-well plate (Falcon, Pittsburgh, PA, USA). After adding the extract or fractions, the cells were incubated for 24 h under 5% CO_2 at 36°C. Then, the cells were observed under a microscope to check the toxicity of the extract or fractions.

To evaluate the cytotoxicity of purified material, a series of concentrations (0, 0.1, 0.25, 0.5, 0.75 1, 2.5, 5, and $10 \, \mu g \, ml^{-1}$) were prepared in triplicates. The cells were incubated in a 96-well plate with approximate cell density $1 \times 10^4 \, cells \, ml^{-1}$ in DMEM media as described above. After 24-h incubation, the media were replaced with $20 \, \mu l$ of $5 \, g \, ml^{-1}$ 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution in phosphate buffered saline (PBS), and the cells were incubated for 3.5 h. After removal of the

PBS solution, 150 μl of dimethyl sulfoxide was added and the cells were re-incubated for 15 min prior to measurement with Tecan microplate reader (Wako) at 590 nm with a reference filter at 620 nm [20].

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