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Cytotoxic and Antifungal Terpenoids from Bornean Soft Coral, *Sinularia flexibilis*

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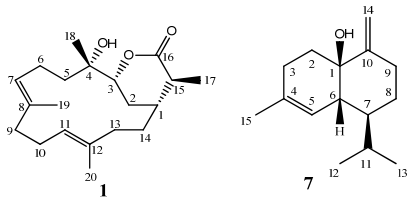
One new cembrane, *ent*-sinuflexibilin D (**1**), along with seven known compounds (**2-8**) were isolated from a population of Bornean soft coral *Sinularia flexibilis*. Their structures were elucidated on the basis of spectroscopic analyses. Cytotoxicity and antifungal activities of these compounds were evaluated *in vitro*. In addition, muurolene **7** was first isolates from a marine source.

Keywords: *Sinularia flexibilis*, Soft coral, Cembrane, Muurolene, Adult T-cell leukemia, Antifungal.

Soft coral genus *Sinularia* (Alcyonacea, Alcyoniidae) is known as one of the most prolific producers of secondary metabolites in the marine ecosystem [1]. These include terpenes that are believed to function as chemical defense against predators [2] and have shown various biological activities [3-13]. Despite the advances in the chemistry of soft corals, there is a shortage of information pertaining to chemical constituents of Bornean soft corals of the Sulu Sulawesi Coral Triangle. In our search of bioactive substances from marine resources, a population of *Sinularia flexibilis* has led to the isolation of one new cembrane, *ent*-sinuflexibilin D (**1**), along with seven known compounds, 14-deoxycrassin (**2**) [6], sinularin (**3**) [4], diepoxycembrene A (**4**) [7], 5-dehydrosinulariolide (**5**) [8], 11-*epi*-sinulariolide acetate (**6**) [6], muurola-4,10(14)-dien-1-ol (**7**) and scabralin A (**8**) [14]. It is worth to mention that muurolene **7** are previously reported from terrestrial plants *Cistus ladaniferus* [15]. However, this is the first report of its presence in the marine organism. To date, only partial data of this compound is available in the literatures. Herein we report the complete spectroscopic data for **1** and **7** (Figure 1) as well as cytotoxic and antifungal activities of **1**-**8**.

Compound **1** was isolated as colorless oil; IR (KBr) absorption at 3400 and 1721 cm^{-1}; and its molecular formula $C_{20}H_{32}O_3$ was determined by HRESIMS $[M+H]^+$ ion at *m/z* 321.2425 (calcd for $C_{20}H_{33}O_3$, 321.2424). The ^{13}C and ^1H NMR spectra (Table 1) of **1** were similar to those of sinuflexibilin D [16], with the exception of its antipodal rotation of $[\alpha]_D^{25}$: -11.9 (c 0.21, $CHCl_3$) in comparison with that of sinuflexibilin D for $[\alpha]_D^{25}$: +6.0 (c 0.01, $CHCl_3$), suggested **1** to be the enantiomer of sinuflexibilin D. There is noticeable chemical shifts difference between **1** (*ent*-sinuflexibilin D) compared to those of sinuflexibilin D at positions of C-2, C-6, C-10, C-13, C-19 and C-20 [16]. After careful assignment, it was found that the positions at C-2 with C-13, C-6 with C-10 and C-19 with C-20 are interchanged in sinuflexibilin D. This argument was supported by the chemical shift of these positions in **1** were consistent with **2** and **3**.



1 **7**

Figure 1: Structures of metabolites **1** and **7**.

Table 1: ^1H and ^{13}C NMR data (600 MHz and 150 MHz, $CDCl_3$) for **1**.

Position	δ_C	δ_H (mult., *J* in Hz)
1	36.3	1.29 m
2	26.8	2.07 m
		1.30 m
3	84.3	4.02 dd (11.0, 2.1)
4	74.4	
5	37.8	1.75 ddd (14.4, 8.9, 2.8)
		1.62 m
6	22.6	2.26 m
		1.88 m
7	124.6	5.04 t (8.3)
8	134.5	
9	39.4	2.17 m
		1.97 ddd (13.8, 9.6, 3.4)
10	23.9	2.23 m
		2.09 m
11	126.5	5.10 t (6.9)
12	132.3	
13	36.5	2.12-2.15 m
14	30.3	1.87 m
		1.10 m
15	42.0	2.08 m
16	175.2	
17	16.3	1.31 d (6.9)
18	24.8	1.39 s
19	15.3	1.56 s
20	14.1	1.56 s

The relative configurations of four chiral centers at C-1, C-3, C-4 and C-15 of **1** were determined by NOE correlations, molecular modeling, vicinal proton-proton coupling constant values and optical rotation. The configurations of H-1 and H-3 were determined in β-orientation based on dihedral angle between H-3

and H_2-2 in a fixed conformation of δ-lactone ring. This was carried out, upon investigation of the vicinal proton coupling between H-3 to H_2-2 ($^3J_{2\text{-}3}$ = 11.0, 2.1 Hz) in **1** in comparison to those of related structure of **2** ($^3J_{2\text{-}3}$ = 11.7, 1.4 Hz), 11-acetylsinuflexolide ($^3J_{2\text{-}3}$ = 11.5 Hz), 11-acetyldihydrosinuflexolide ($^3J_{2\text{-}3}$ = 11.5, 2.5 Hz) [3]. Additional argument for β-assignments of H-1, H-3 and H_3-18 were associated with the (-) sign of antipodal rotation for **1** $[\alpha]_D^{25}$: -11.9 (*c* 0.21, $CHCl_3$), **2** $[\alpha]_D^{25}$: -19.1 (*c* 0.22, $CHCl_3$) and **3** $[\alpha]_D^{25}$: -98.7 (*c* 1.14, $CHCl_3$) obtained from the same organism. Because the assistance of these findings, the NOE cross peaks were assumed between H-3/H_2-2β, H-3/H-1 and H-3/H_3-17. Based on these findings, structure of **1** was identified as *ent*-sinuflexibilin D.

Compound **7** was isolated as colorless oil; $[\alpha]_D^{25}$ –20.9 (*c* 0.28, $CHCl_3$); IR (KBr) absorption at 3416, 2956, 1457, 1367, and 891 cm^{-1}; and molecular formula $C_{15}H_{24}O$ determined by HRESIMS ion $[M+H\text{-}H_2O]^+$ at *m/z* 203.1794 (calcd. for $C_{15}H_{23}$, 203.1794). The NMR data (Table 2) revealed the presence of 15 carbon signals including one hydroxyl carbon at δ_C 73.7.

The relative configurations of three successive chiral centers at C-1, C-6 and C-7 were deduced by NOESY spectrum and proton-proton vicinal coupling constants. In the NOESY spectrum, H-6 showed no NOE correlation to H-7 indicated H-6 and H-7 has a *trans* relationship. This deduction was further confirmed by the large coupling constants $^3J_{6\text{-}7}$ = 11.7 Hz. The NOE correlations of H-6 to H-11 and H_3-12 suggested H-6 and the isopropyl group were located on the same side of the molecule, reflecting on β-orientation. While, H_2-14 showed NOE correlations to H_2-2β and H_2-9β has led to assumption that the hydroxyl group on β-orientation. Thus, the structure of **7** was unambiguously determined.

Table 2: ^{1}H and ^{13}C NMR data (600 MHz and 150 MHz, $CDCl_3$) for **7**.

Position	δ_C	δ_H (mult., *J* in Hz)
1	73.7	
2	30.5	2.10 m
		1.43 ddd (13.8, 7.7, 1.3)
3	27.6	2.20 m, 2.03 m
4	134.4	
5	122.5	5.50 dq (5.4, 1.8)
6	49.0	1.80 dd (11.7, 5.4)
7	50.2	1.33 tt (11.7, 3.2)
8	26.2	1.65 dq (12.1, 3.2)
		1.08 qd (12.1, 4.4)
9	33.8	2.37 dt (14.2, 3.2), 2.10 m
10	154.9	
11	27.8	2.03 m
12	16.0	0.78 d (6.9)
13	22.4	0.91 d (6.9)
14	106.6	5.11 s, 4.77 s
15	26.2	1.73 s

The cytotoxicity of compounds **1**-**8** is shown in Table 3. The result showed **1**, **2**, **3**, **5** and **6** were active against adult T-cell leukemia (ATL), S1T cells. The ATL is a lethal disease and outlook of this disease was remained dismal [17]. In addition, compounds **1**-**8** were screened against three strains of marine fungi *Exophiala* sp. NJM 1551, *Lagenidium thermophilum* IPMB 1401 and *Haliphthoros sabahensis* IPMB 1402 as shown in Table 4. These marine fungi

Table 3: Cytotoxicity of **1**-**8** against S1T cell line.

Compound	IC_{50} (μg/mL)
1	5.27
2	4.39
3	3.15
4	44.60
5	5.80
6	7.78
7	48.40
8	46.80

Positive control: etoposide with IC_{50} of 0.05 μg/mL.

Table 4: MIC of **1**-**8** against three strains of marine fungi.

	MIC (μg/mL)							
Strains	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Exophiala sp.	25	12.5	50	25	50	50	100	100
L. thermophilum	25	25	12.5	25	25	25	100	100
H. sabahensis	50	50	25	50	50	50	100	100

Positive control: itraconazole with MIC 3.2 μg/mL.

are known to cause fungal infection in aquatic organisms, especially in fishes and mangrove crabs [18]. Therefore, new antifungal agent against these fungi could prevent lethal infection of these marine fungi. It is worth to mention that *H. sabahensis* was a new fungal species described in 2017 [19]. Hence, this work provided valuable information to facilitate searching of potent antifungal agent against this species and cytotoxic substance on adult T-cell leukemia.

Experimental

General: Optical rotations were measured on an AUTOPOL IV automatic polarimeter (Rudolph Research Analytical). ^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz) spectra were recorded with a JEOL ECA 600 instrument. HR-ESI-MS spectrum was obtained with LCMS-IT-TOF (Shimadzu). Preparative TLC silica gel plates (Merck, Kieselgel 60 F_{254}) and silica gel column chromatography (Merck, Kieselgel 60, 70-230 mesh) were used for isolation.

Biological Material: Specimen of *S. flexibilis* was collected from Mengalum Island, Sabah (06°10'7.44''N, 115°34'32.94''E), on September 2014. The voucher specimen (BORMI0002) was deposited in the BORNEENSIS Collection of Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah.

Extraction and Isolation: The fresh soft coral (1 kg wet wt) was extracted in MeOH. The resulting MeOH extract was concentrated and partitioned between EtOAc/H_2O. The EtOAc fraction was further partitioned with hexane/90% MeOH. Both crudes (1.0 g) were subjected to column chromatography eluting with a gradient of hexane and EtOAc (9:1 to 5:5) in an increasing polarity to obtain six fractions. A portion of fraction 2 (85.0 mg) of hexane crude was subjected to preparative TLC with toluene to isolate **4** (43.4 mg), **7** (10.8 mg) and **8** (10.6 mg). Purification of fraction 4 (89.4 mg) yielded **5** (12.5 mg) via repeated preparative TLC with $CHCl_3$-EtOAc (85:15) and toluene-EtOAc (8:2). Compounds **1** (16.9 mg), **3** (3.1 mg) and **6** (10.7 mg) were afforded from fraction 5 (100.0 mg) by repeat preparative TLC with $CHCl_3$-EtOAc (85:15) followed by various solvent systems; $CHCl_3$-EtOAc (8:2) and toluene-EtOAc (73:23) for **1**; toluene-EtOAc (1:1) for **3**; toluene-EtOAc (1:1) and hexane-EtOAc (45:55) for **6**. While fraction 5 (114.0 mg) of 90% MeOH has afforded **2** (6.2 mg) through repeat preparative TLC with $CHCl_3$-EtOAc (85:15).

Cytotoxic Assay: The assay was performed according to previously described procedures [20,21].

Antifungal activity: The minimum inhibitory concentration (MIC) of fungistatic effect on hyphae were performed by incorporating the pure compound solutions (100, 50, 25 and 12.5 μg/mL) onto PYGS agar in petri dish followed inoculation of three tested fungal strains [18]. The MIC was determined visually as the lowest concentration showing no hyphal growth when they were incubated at 25 °C for 7 days.

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