A New 2,3-Dimethyl Butenolide from the Brittle Star *Ophiomastix mixta*

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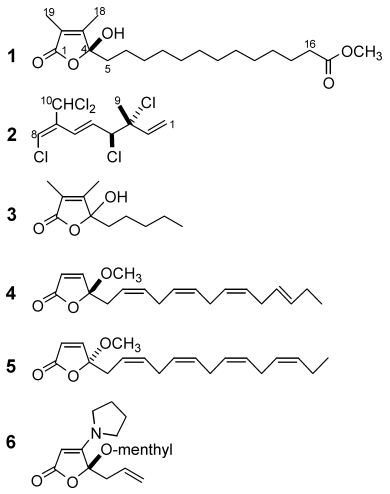
A new butenolide (1) was isolated, along with a known acyclic polyhalogenated monoterpene (2), from the brittle star *Ophiomastix mixta*. The structures were defined by analysis and comparision of the spectral data with those in the literature. The 2,3-dimethyl butenolide (1) is uncommon and first encountered in a marine organism. The compounds were tested for cytotoxicity against a panel of five human solid tumor cell lines and displayed mild to significant activity.

Key words brittle star; *Ophiomastix mixta*; 2,3-dimethyl butenolide; halogenated monoterpene

The brittle stars (Ophiuroidea) are the largest group of echinoderms in the number of species, and they are probably the most abundant also.[2)] Most of the previously reported compounds from the brittle stars are sterols. Compared to other echinoderms such as starfish and sea cucumbers, brittle stars have shown a general lack of saponins. Only two steroidal glycosides were isolated from *Ophioderma longicaudum*,[3)] whereas several sulfated polyhydroxy sterols were isolated from most of species investigated.[4)] The minor groups of the isolated compounds from the brittle stars are two carotenoid sulfates from *Ophiocomina nigra*,[5)] three gangliosides from *Ophiocoma scolopendrina*,[6)] a brominated indole from *Ophiocoma erinaceus*,[7)] and ten terpenes and two phenylpropanoids from *Ophioplocus japonicus*.[8)] In our screening for the bioactive compounds, the MeOH extract of the brittle star *Ophiomastix mixta* exhibited toxicity to the brine shrimp lavae (LD_{50}, 262 μg/ml). There was a previous report on a sulfated sterol from the same genus, *Ophiomastix annulosa*,[9)] while there was no report on a chemical study of the brittle star *Ophiomastix mixta*. Guided by the ^{1}H-NMR monitoring and the brine shrimp lethality assay, we have isolated a new butenolide (**1**) and a known acyclic polyhalogenated monoterpene (**2**).

Butenolides are occasionally encountered among various marine organisms such as sponges,[10,11)] fungi,[12)] bacteria,[13)] and gorgonians.[14,15)] However, 2,3-dimethylated butenolide is unusual and this is the first report from a marine organism. The structure of compound **1** was similar to the butenolide (**3**) which was previously reported from butter fat[16)] and fermented tobacco leaves as an aroma constituent,[17)] from timothy chokes as an fungitoxic substance,[18)] from liverworts cell culture as an allelochemical,[19)] from cress seedlings as plant growth inhibitor,[20)] and from the leaves and twigs of *Litsea verticillata* as an anti-HIV substance.[21)] The polyhalogenated monoterpene (**2**) has been previously found in the red algae *Plocamium* sp.,[22)] the digestive gland of the seahare *Aplysia limacina*,[23)] marine hydroids,[24)] and the tunicate *Clavelina lepadiformis*.[25)] Halogenated monoterpenes have been found to exhibit various biological activities, including antifungal, antimicrobial, and molluscicidal activities.[26)]

Many brittle stars feed on detritus, while others are carnivorous and prey on worms, crustaceans, and bivalves; both animal and vegetable contents are digested. Although the monoterpene (**2**) is first encountered in brittle stars, there is a precedence of isolation of these compounds as algal and cyanobacterial metabolites.[8)] It might be regarded that **2** in this brittle star is obtained from dietary sources such as red alga.



Results and Discussion

Compound **1** was isolated as colorless oil. The molecular formula of **1** was established as $C_{20}H_{34}O_5$ on the basis of a pseudomolecular ion peak at *m*/*z* 377.2309 $[M+Na]^+$ (Calcd for $C_{20}H_{34}NaO_5$, 377.2304) and the NMR data. The fragment peaks were also observed at *m*/*z* 177, 219, 261, 275 and 303 in the FAB-CID tandem mass spectrum (Fig. 1). The ^{1}H-NMR spectrum suggested the presence of two methyl groups (δ 1.78, 1.92), a methoxyl group (δ 3.64), and methylene groups (δ 1.78, 1.92, 1.15, 1.28, 1.59, 2.30) (Table 1). In the COSY spectrum, H-15 (δ 1.59) showed correlations to H-14

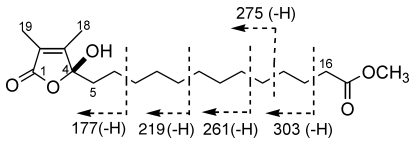


Fig. 1. Fragmentations of the $[M+Na]^+$ Ion of **1** in FAB-CID MS/MS

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Table 1. ^{1}H- and ^{13}C-NMR Data of Compound **1**

Position	$^{1}H^{a)}$ (in CD_3OD)	$^{1}H^{a)}$ (in $CDCl_3$)	^{13}C (in CD_3OD)$^{b)}$
1			173.2
2			159.2
3			125.7
4			108.1
5	1.78 m	1.78 ddd (14.0, 10.5, 4.5)	36.9
	1.92 m	2.00 ddd (14.0, 10.5, 4.5)	
6	1.15 m	1.21 m	24.1
7—14	1.28 m	1.27 m	30.64
			30.57
			30.54
			30.51
15	1.59 m	1.63 quint (7.5)	26.0
16	2.30 t (7.5)	2.32 t (7.5)	34.8
17			176.1
18	1.78 s	1.84 s	8.2
19	1.92 s	1.96 s	10.8
$-OCH_3$	3.64 s	3.69 s	52.0

a) δ values in ppm and coupling constants (in parentheses) in Hz, measured at 500 MHz. *b*) Measured at 75 MHz.

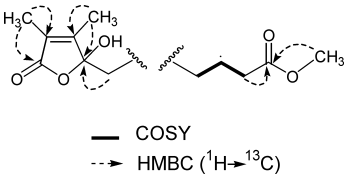

Fig. 2. Key COSY and HMBC Correlations of Compound **1**

(δ 1.28) and H-16 (δ 2.30). The HSQC data of **1** showed correlations among the proton signals at δ 1.78 and 1.92 and three carbon signals at δ 8.2, 10.8, and 36.9. These data indicated that H-5 (δ 1.78, 1.92) were overlapped with H-18 (δ 1.78) and H-19 (δ 1.92). The overlapped proton signals of H-5, H-18, and H-19 were resolved (δ 1.78, 2.00, H-5; δ 1.84, H-18; δ 1.96, H-19) when the ^{1}H-NMR was measured in $CDCl_3$. The ^{13}C-NMR spectrum indicated the presence of two carbonyl groups (δ 173.2, 176.1), two olefinic carbons (δ 125.7, 159.2), a methoxyl carbon (δ 52.0), and two methyl carbons (δ 8.2, 10.8). In the HMBC spectrum, H-18 (δ 1.78) and H-19 (δ 1.92) showed correlations to C-1 (δ 173.2), C-2 (δ 159.2), C-3 (δ 125.7), and C-4 (δ 108.1). HMBC correlations between H-5 (δ 1.78, 1.92) and C-4 (δ 108.1) were also observed. The H-15 (δ 1.59), H-16 (δ 2.30), and methoxyl protons (δ 3.64) showed correlations to C-17 (δ 176.1) (Fig. 2). Henceforth, an α,β-unsaturated-γ-lactone moiety and a methoxycarbonyl side chain were confirmed, and the structure of **1** was defined as 13-(2-hydroxy-3,4-dimethyl-5-oxo-2,5-dihydrofuran-2-yl) tridecanoic acid methyl ester. The stereochemistry of the hydroxyl group was defined by optical rotation and CD spectroscopy. The model compound **4** with *S* configuration was reported to show a positive optical rotation ($[\alpha]_D$ +14°). Compound **1** also showed a positive optical rotation ($[\alpha]_D$ +5.7°). Furthermore, the CD spectrum of **1** showed a positive Cotton effect at 213 nm (π–π*). This pattern of Cotton effect was similar to that of the γ-methoxy-γ-alkyl-disubstituted butenolides (+0.37, 205 nm, **4**; +14.0, 204 nm, **6**).[10,27] The red-shift in the CD spectrum of **1**, compared to that of **4**, might be due to 2,3-dimethyl substitution.[28] In contrast, **1** showed opposite Cotton effect to **5** (−0.58, 206 nm, **5**), which has opposite stereochemistry to **4**

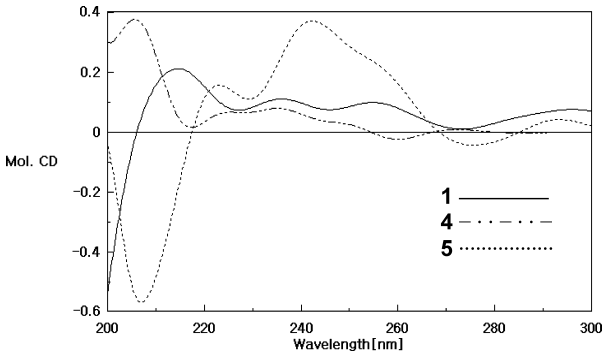

Fig. 3. CD Spectra of Compounds **1**, **4**, and **5**

Table 2. Cytotoxicity Data of Compounds **1** and **2**$^{a)}$

Compound	A549	SK-OV-3	SK-MEL-2	XF498	HCT15
1	10.0	10.1	10.5	15.2	30.2
2	1.15	11.7	5.81	0.99	4.56
Doxorubicin	0.02	0.02	0.01	0.01	0.04

a) Data as expressed in ED_{50} values (μg/ml). A549: human lung cancer; SK-OV-3: human ovarian cancer; SK-MEL-2: human skin cancer; XF498: human central nervous system (CNS) cancer; HCT15: human colon cancer.

(Fig. 3).[10] Therefore, an *S* configuration at C-4 was proposed for compound **1**.

Compound **2** was isolated as colorless oil. The molecular formula of **2** was established as $C_{10}H_{11}Cl_5$ on the basis of a molecular ion cluster at *m/z* 305.9234/307.9359/309.9330 $[M]^+$ (Calcd for $C_{10}H_{11}{}^{35}Cl_5$: 305.9303, $C_{10}H_{11}{}^{35}Cl_4{}^{37}Cl$: 307.9274, $C_{10}H_{11}{}^{35}Cl_3{}^{37}Cl_2$: 309.9244) in the EI-MS and by comparison of the NMR data with those reported.[22] The major four isotope peaks were in the ratio of 3 : 5 : 3 : 1 (*m/z* 306/308/310/312) as expected for a pentachloro compound. The stereochemistry of the Δ^5 was assigned as (*E*) on the basis of the characteristic coupling constant (J=16.0 Hz). Chemical shifts of H-5 to H-10 matched well to those reported for the (*E*) configuration of Δ^7.[22] But the signal assignment of H-8 and H-10 were erroneously switched in the earlier paper.[22] The negative optical rotation of **2**, together with the characteristic chemical shifts of the methyl carbon, C-9 at δ 25.1 ($CDCl_3$), indicated that **2** should be an *erythro* derivative (expected values for *erythro* and *threo* series are δ_C 25 and δ_C 28, respectively).[29] Thus, **2** was identified as (5*E*,7*E*)-3,4-*erythro*-3,4,8-trichloro-7-dichloromethyl-3-methylocta-1,5,7-triene, which was previously reported from the Pacific red alga *Plocamium cartilagineum*.[22]

Compounds **1** and **2** were evaluated for cytotoxicity against a panel of five human solid tumor cell lines and showed mild to significant activity (Table 2). Compound **2** exhibited considerable cytotoxicity against all five cell lines, with a substantial degree of selectivity to XF498 and A549 (ED_{50}, 0.99 and 1.15 μg/ml, respectively). Compound **1** showed mild activity against five human solid tumor cell lines.

Experimental

General Optical rotation was recorded with a JASCO DIP-370 digital polarimeter. CD spectra were measured using a JASCO J-715 spectropolarimeter (sensitivity 50 mdeg, resolution 0.2 nm). ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AC200, Varian Unity Plus 300 and Varian Inova 500 instruments. Chemical shifts were reported with reference to the respec-

tive residual solvent or deuterated solvent peaks (δ_H 3.30 and δ_C 49.0 for CD_3OD, δ_H 7.26 and δ_C 77.0 for $CDCl_3$). FAB-MS data were obtained on a JEOL JMS-700 double focusing (B/E configuration) instrument. EI-MS data were obtained on a Shimadzu QP5050 instrument. HPLC was performed with a C18-5E Shodex packed column (250×10 mm i.d., 5 μm, 100 Å) and C18-10E Shodex packed column (250×10 mm i.d., 5 mm, 100 Å) using a Shodex RI-71 detector.

Animal Material The brittle star was collected in May 2004, off the coast of Jeju Island, Korea. The specimen was identified by Prof. Sook Shin, Sahmyook University, Seoul, Korea. The voucher specimen (J04M-A) of the brittle star was deposited at the Marine Natural Product Chemistry Laboratory, Pusan National University, Busan, Korea.

Extraction and Isolation The frozen brittle star (3 kg) was extracted with MeOH at room temperature. Guided by brine shrimp lethality assay, the MeOH extract was partitioned between H_2O and CH_2Cl_2. The CH_2Cl_2 layer was further partitioned between aqueous MeOH and *n*-hexane to afford aqueous MeOH-soluble and *n*-hexane-soluble fractions. The aqueous MeOH fraction was subjected to reversed-phase flash column chromatography (YMC Gel ODS-A, 60 Å, 500/400 mesh) and eluted with a step gradient solvent system of 50 to 100% MeOH/H_2O to afford 12 fractions (1—12). Fraction 6 was very active in the brine shrimp lethality assay (LD_{50}, 31 μg/ml) and was further separated by normal-phase MPLC (Silica gel 60, 400/230 mesh), eluted with a solvent system of 0 to 50% MeOH/$CHCl_3$ to afford 9 fractions. Compound **1** (1.12 mg) was obtained by the separation of subfraction 2 on a reversed-phase HPLC (C18-5E Shodex packed, 250×10 mm i.d., 5 μm, 100 Å) column eluting with 75% MeOH, followed by purification on a C18-10E Shodex packed (250×10 mm i.d., 5 μm, 100 Å) column eluting with 75% MeOH. Compound **2** (2.36 mg) was obtained by the separation of subfraction 1 on a reversed-phase HPLC (C18-5E Shodex packed, 250×10 mm i.d., 5 μm, 100 Å) column eluting with 75% MeOH, followed by purification on a C18-10E Shodex packed (250×10 mm i.d., 5 μm, 100 Å) column eluting with 75% MeOH.

Compound **1**: Colorless oil; $[\alpha]_D$ +5.7° (c=0.03, MeOH); CD (c=3×10^{-4} M, MeOH) $\Delta\varepsilon$ (nm) −0.5 (200), 0 (204), +0.3 (213), +0.13 (236), +0.1 (255); ^{1}H-NMR (500 MHz, CD_3OD, $CDCl_3$): see Table 1; ^{13}C-NMR (75 MHz, CD_3OD): see Table 1; HR-FAB-MS (+ve) *m/z*: 377.2309 ($[M+Na]^+$, Calcd for $C_{20}H_{34}NaO_5$: 377.2304).

Compound **2**: Colorless oil; $[\alpha]_D$ −57.2° (c=0.79, MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.80 (3H, s, H-9), 4.60 (1H, d, J=8.5 Hz, H-4), 5.32 (1H, d, J=11.0 Hz, H-1), 5.43 (1H, d, J=17.0 Hz, H-1), 6.11 (1H, dd, J=11.0, 17.0 Hz, H-2), 6.41 (1H, s, H-10), 6.50 (1H, dd, J=8.5, 16.0 Hz, H-5), 6.64 (1H, d, J=16.0 Hz, H-6), 6.77 (1H, s, H-8); ^{1}H-NMR (500 MHz, CCl_4) δ: 1.78 (3H, s, H-9), 4.55 (1H, d, J=8.5 Hz, H-4), 5.30 (1H, d, J=10.5 Hz, H-1), 5.42 (1H, d, J=17.0 Hz, H-1), 6.08 (1H, dd, J=10.5, 17.0 Hz, H-2), 6.39 (1H, s, H-10), 6.47 (1H, dd, J=8.5, 16.0 Hz, H-5), 6.63 (1H, d, J=16.0 Hz, H-6), 6.79 (1H, s, H-8); ^{13}C-NMR (50 MHz, $CDCl_3$) δ: 25.1 (s, C-9), 69.39 (s, C-4), 69.43 (s, C-10), 71.7 (s, C-3), 116.6 (s, C-1), 123.9 (s, C-6), 124.2 (s, C-8), 131.9 (s, C-5), 136.1 (s, C-7), 139.5 (s, C-2); HR-EI-MS *m/z*: 305.9234, 307.9359, 309.9330 ($[M]^+$, Calcd for $C_{10}H_{11}{}^{35}Cl_5$: 305.9303, $C_{10}H_{11}{}^{35}Cl_4{}^{37}Cl$: 307.9274, $C_{10}H_{11}{}^{35}Cl_3{}^{37}Cl_2$: 309.9244).

Evaluation of Cytotoxicity The sulforhodamine B (SRB) assay, developed for measuring the cellular protein content of the cultures, is applied for the measurement of the cytotoxicity of the compounds against tumor cells. The rapidly growing cells were harvested, counted, and inoculated at the appropriate concentrations (1—2×10^4 cells/well) into 96-well microtiter plates. After incubation for 24 h, the compounds dissolved in culture medium were applied to the culture wells in triplicate, followed by incubating for 48 h at 37 °C under a 5% CO_2 atmosphere. The cultures fixed with cold TCA were stained by 0.4% SRB dissolved in 1% acetic acid. After solubilizing the bound dye with 10 mM unbuffered tris base by gyratory shaker, the absorbance at 520 nm was measured with a microplate reader (Dynatech Model MR 700). Fifty percent inhibitory concentration (ED_{50}) was defined as the concentration which reduced absorbance by 50% of compared to the control level in the untreated wells.

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