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TETRAHEDRON

Novel Norsesterterpenes, Which Inhibit Gastrulation of the Starfish Embryo, from the Marine Sponge *Rhopaloeides* sp.

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Abstract: Two new norsesterterpenes, rhopaloic acids B (**2**) and C (**3**), have been isolated from the marine sponge *Rhopaloeides* sp. together with the known rhopaloic acid A (**1**). Their structures have been elucidated by spectroscopic methods. Compounds **1**, **2**, and **3** potently inhibited gastrulation of the starfish (*Asterina pectinifera*) embryo, whereas hydrogenation or esterification resulted in loss of the inhibitory activity. Furthermore, methacrylic acid (**9**) did not affect embryonic development. These results suggest that the α-exo-methylene carboxyl functionality attached to a tetrahydropyran ring is essential for the inhibitory activity of **1**-**3**. © 1998 Elsevier Science Ltd. All rights reserved.

Keywords: Biologically active compounds; Marine metabolites; Structure-activity; Terpenes and terpenoids

INTRODUCTION

During the course of our screening program for inhibitors of starfish (*Asterina pectinifera*) embryonic development from marine organisms,[1-6] we found that the MeOH extract of the marine sponge *Rhopaloeides* sp. inhibited starfish embryogenesis specifically at the stage prior to gastrulation. A preliminary examination led to the isolation of an unusual norsesterterpene designated rhopaloic acid A (**1**) which inhibited gastrulation of the starfish embryo and exhibited potent cytotoxicities against human myeloid K-562 cells, human MOLT-4 leukemia cells, and murine L1210 leukemia cells.[7] Further investigation of the extract of *Rhopaloeides* sp. led to the isolation of related metabolites, rhopaloic acids B (**2**) and C (**3**). This paper deals with the isolation, structure elucidation, and biological activities of these compounds.

RESULTS AND DISCUSSION

The MeOH extract of *Rhopaloeides* sp. was partitioned between EtOAc and water. The EtOAc layer, which showed inhibitory activity against gastrulation of the starfish embryo, was separated by silica gel chromatography, followed by ODS HPLC to afford two bioactive fractions. Of these fractions, the major fraction was subjected to recycling ODS HPLC to afford **1** (24 mg) and **2** (12 mg) as viscous colorless oils. The minor fraction was further purified by repeated ODS HPLC to afford **3** (1.4 mg) as a viscous colorless oil.

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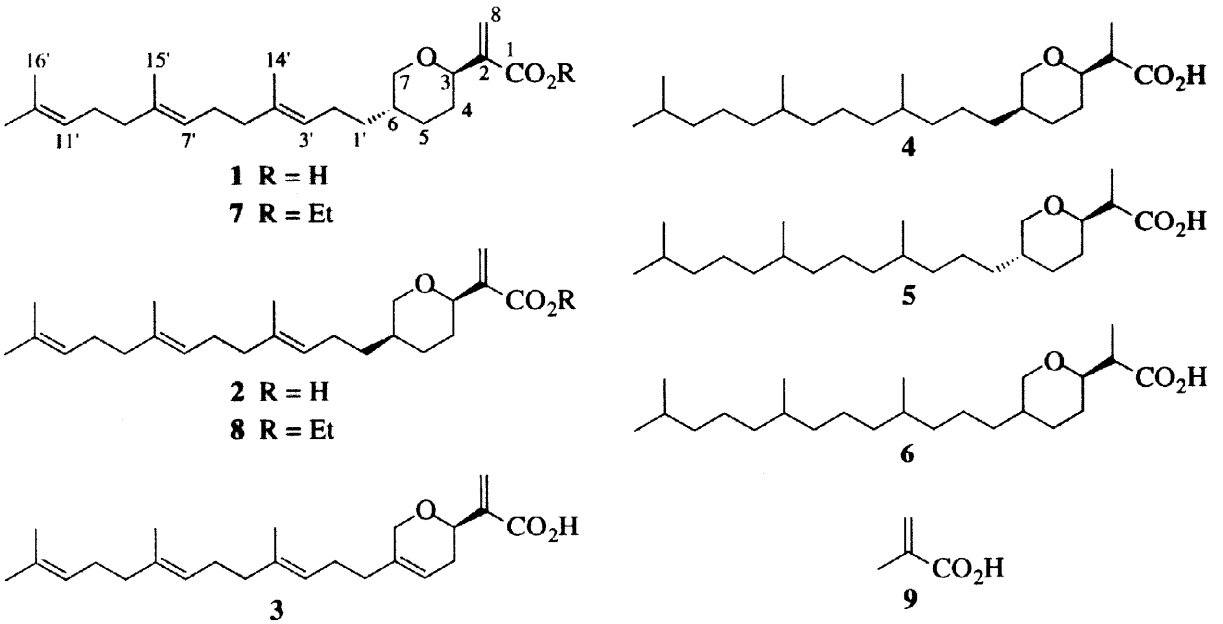

Compound **2** had a molecular formula of $C_{24}H_{38}O_3$, as determined by high resolution electron impact (HREI) mass spectrometry (*m/z* 374.2818 $[M]^+$, Δ -0.3 mmu). The EI mass spectra of **2** and its hydrogenated derivative **4** were identical to those of **1** and its hydrogenated derivative **5**, respectively. The ^1H and ^{13}C NMR spectra of **2** were similar to those of **1**, except for signals of the C-4, C-5, C-7 and C-1' methylene, and C-6 methine moieties (Table 1), suggesting that **2** is an epimer of **1**. This was supported by the NMR data of ^1H-^1H COSY, ^{13}C-^1H COSY, and HMBC[8] experiments as shown below.

The vicinal spin-spin coupling constants ($J_{3,4\beta}$ = 10.1 Hz), the NOESY correlations between H-3, H-5$_\alpha$ and H-7$_\alpha$, and the long-range coupling between H-5$_\beta$ and H-7$_\beta$ implied that the tetrahydropyran ring (C-3–C-7) is in a chair conformation and that H-3, H-5$_\alpha$ and H-7$_\alpha$ are 1,3-diaxial, as shown in Figure 1. The small coupling constants ($J_{6,7\alpha}$ = 2.7 Hz and $J_{6,7\beta}$ < 1 Hz) indicate H-6 to be equatorial so that the homofarnesyl

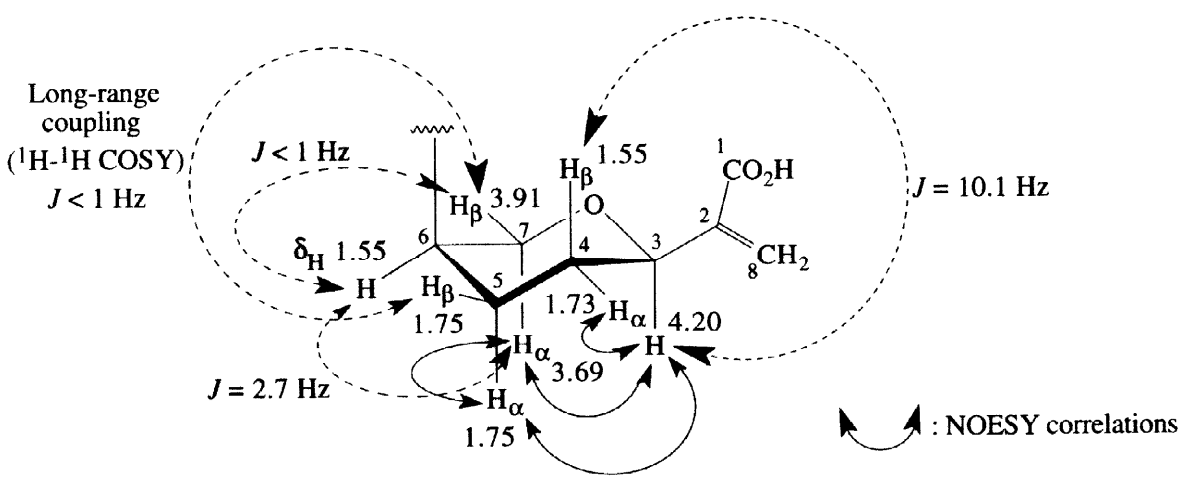

Figure 1. Stereochemistry of the tetrahydropyran moiety in rhopaloic acid B (**2**).

Table 1. **NMR** Spectral Data for Compounds **1-3** in $CDCl_3$.

	1		**2**		**3**	
Carbon no.	δ_C and multiplicity	δ_H and multiplicity [a]	δ_C and multiplicity	δ_H and multiplicity [a]	δ_C and multiplicity	δ_H and multiplicity [a]
1	170.7 s		169.9 s		168.1 s	
2	141.9 s		141.0 s		140.2 s	
3	76.0 d	4.11 d (10.1)	76.1 d	4.20 d (10.1)	72.3 d	4.35 dd (8.7, 2.8)
4	32.1 t	1.30 m	27.2 t	1.55 m	30.7 t	2.07 m
		1.94 m		1.73 m		2.31 m
5	30.4 t	1.22 m	27.2 t	1.75 m	117.6 d	5.56 d (4.6)
		1.91 m		1.75 m		
6	35.3 d	1.60 m	32.6 d	1.55 m	136.2 s	
7	73.9 t	3.15 t (11.0)	71.7 t	3.69 dd (11.0, 2.7)	68.8 t	4.20 d (15.6)
		4.04 ddd (11.0, 3.8, 1.6)		3.91 d (11.0)		4.24 d (15.6)
8	125.8 t	5.88 br s	127.0 t	5.94 br s	126.9 t	5.94 br s
		6.31 br s		6.39 br s		6.40 br s
1'	32.5 t	1.15 m	30.1 t	1.49 m	32.9 t	1.97 m
		1.15 m		1.64 m		1.97 m
2'	25.0 t	2.00 m (2H)	25.8 t	2.03 m (2H)	26.2 t	2.07 m (2H)
3'	124.2 d [b]	5.10 m	124.4 d [e]	5.10 m	123.5 d [h]	5.09 m
4'	135.0 s [c]		135.0 s [f]		135.0 s [i]	
5'	39.7 t	1.99 m (2H)	39.7 t	1.99 m (2H)	39.7 t	1.97 m (2H)
6'	26.6 t [d]	2.06 m (2H)	26.6 t [g]	2.07 m (2H)	26.6 t [j]	2.07 m (2H)
7'	124.2 d [b]	5.10 m	124.2 d [e]	5.10 m	124.1 d [h]	5.09 m
8'	135.3 s [c]		135.4 s [f]		135.8 s [i]	
9'	39.7 t	1.99 m (2H)	39.7 t	1.99 m (2H)	39.7 t	1.97 m (2H)
10'	26.8 t [d]	2.06 m (2H)	26.8 t [g]	2.07 m (2H)	26.8 t [j]	2.07 m (2H)
11'	124.4 d [b]	5.10 m	124.4 d [e]	5.10 m	124.4 d [h]	5.09 m
12'	131.2 s		131.2 s		131.3 s	
13'	25.7 q	1.68 s (3H)	25.7 q	1.68 s (3H)	25.7 q	1.68 s (3H)
14'	16.0 q	1.60 s (3H)	16.0 q	1.60 s (3H)	16.1 q [k]	1.60 s (3H)
15'	16.0 q	1.60 s (3H)	16.0 q	1.60 s (3H)	16.0 q [k]	1.60 s (3H)
16'	17.7 q	1.60 s (3H)	17.7 q	1.60 s (3H)	17.7 q	1.60 s (3H)

[a] Coupling constants, $J_{H\text{-}H}$ (in Hz), are given in parentheses.

[b-k] Signals with identical letters may be interchanged.

Table 2. Inhibitory Effect of Compounds **1-9** on Gastrulation of Starfish Embryos.

Compound	Minimum effective concentration (μM)
Rhopaloic acid A (**1**)	0.5
Rhopaloic acid B (**2**)	0.4
Rhopaloic acid C (**3**)	0.2
Hydrogenated derivative of **2** (**4**)	>25
Hydrogenated derivative of **1** (**5**)	>25
Hydrogenated derivative of **3** (**6**)	>25
Ethyl ester of **1** (**7**)	>25
Ethyl ester of **2** (**8**)	>25
Methacrylic acid (**9**)	>25

group (C-1'–C-16') at C-6 is axial. Compound **2** exhibited a positive Cotton effect ($\Delta\varepsilon_{205}$ +1.3±0.3) at around 205 nm due to the $\pi\rightarrow\pi^*$ transition of the α,β-unsaturated carboxyl chromophore, which is in good agreement with that of **1** ($\Delta\varepsilon_{205}$ +1.4±0.3). This indicates that the C-3 chirality of **2** is same as that of **1**. Compound **1** has been synthesized and its absolute configuration has been established to be 3*R*,6*S*.[9] Therefore, the structure of **2** was determined to be (3'*E*,7'*E*)-(3*R*,6*R*)-2-methylene-6-(4,8,12-trimethyl-3,7,11-tridecatrienyl)-3,7-epoxyheptanoic acid.

Compound **3** had a molecular formula of $C_{24}H_{36}O_3$, which was determined by HREI mass spectrometry (*m/z* 372.2664 $[M]^+$, Δ -0.1 mmu). The IR spectra were nearly identical to those of **2**, although an additional degree of unsaturation is present in **3**. The ^1H and ^{13}C NMR spectra of **3** were nearly identical to those of **2** except that **3** contains an sp^2 methine (δ_C 117.6) and a quaternary sp^2 carbon (δ_C 136.2) instead of an sp^3 methylene and an sp^3 methine carbon (Table 1). The ^1H -^1H COSY, HMQC, and HMBC spectra indicated the presence of the $\Delta^{5,6}$ double bond. Upon hydrogenation using palladium carbon as a catalyst, **3** afforded **6**, whose EI mass spectrum was identical to that of **4** or **5**. These findings indicate that **3** is the C-5/C-6 dehydrogenated compound of **1** and **2**. Therefore, the structure of **3** was determined to be (3'*E*,7'*E*)-2-methylene-6-(4,8,12-trimethyl-3,7,11-tridecatrienyl)-3,7-epoxy-5-heptenoic acid. Due to the paucity of material, the absolute configuration at C-3 has not been experimentally determined. However, because **1-3** were derived from the same origin, it is likely that **3** shares the same configuration (3*R*) at the oxymethine as that of **1** and **2**.

When fertilized *A. pectinifera* eggs were cultured from fertilization in the presence of **2** and **3**, they blastulated normally after passing through a rapid cleavage period, and hatched on schedule; however, gastrulation was selectively inhibited. The minimum inhibitory concentrations of **2** and **3** were 0.4 and 0.2 μM, respectively (Table 2). Furthermore, **2** or **3** affected neither fertilization of the starfish gametes nor early embryonic development of fertilized eggs up to the pre-gastrula stage even at the concentration of 25 μM. The hydrogenated derivatives of **1-3** (**4-6**), ethyl ester of **1** (**7**), ethyl ester of **2** (**8**), or methacrylic acid (**9**) did not affect gastrulation of the starfish embryo even at the concentration of 25 μM. These findings indicate a significant contribution of the α-exo-methylene carboxyl functionality attached to a tetrahydropyran ring to the development inhibitory activity. Compounds containing such a functionality have not been encountered among sponge metabolites.[10] Although brevetoxin B3[11] obtained from the greenshell mussel *Perna canaliculus* contains such functionality, its biological activities have not been reported.

EXPERIMENTAL SECTION

General. ^1H and ^{13}C NMR spectra were recorded on a JEOL GSX500 spectrometer (500 MHz for ^1H, 125 MHz for ^{13}C). NMR chemical shifts were referenced to solvent peaks: δ_H 7.26 (residual $CHCl_3$) and δ_C 77.0 for $CDCl_3$. HMBC spectra were optimized for a $^nJ_{CH}$ value of 8 Hz. A spectral width of approximately 3000 Hz was employed with the HMBC spectra acquired as 512 data points in t_2 for 256 data points in t_1 increments and zero-filled to 1024 and 512 points in t_2 and t_1, respectively, prior to the Fourier transformation. NOESY experiments were performed with mixing times of 0.75 sec. Multiplicities of the ^{13}C signals were determined by DEPT experiments. EIMS and HREIMS were measured on a JEOL SX102A spectrometer. UV and IR spectra were recorded on a Shimadzu UV-3000 and a JASCO FT/IR-5300 spectrometer, respectively. Optical rotations were measured on a JASCO DIP-370 digital polarimeter. CD spectra were recorded with a JASCO J-600 circular dichroic spectropolarimeter. Compound **9** was purchased from Tokyo Chemical Industry Co., Ltd., and freshly distilled under reduced pressure prior to use. 1-Methyladenine was purchased from Sigma, St. Louis, Missouri, U.S.A.

Bioassays. Specimens of *Asterina pectinifera* were collected from the coastal waters off Japan during their breeding season and kept in sea water at 15 °C in laboratory aquaria. Oocytes and sperm were removed from ovarian and testicular fragments, respectively. Experiments were performed at 20 °C and artificial sea water (Jamarin Laboratory, Osaka, Japan) was used throughout. Oocytes were induced to mature by the treatment with 1 μM 1-methyladenine.[12] Maturing oocytes were fertilized at 40 min after the start of 1-methyladenine treatment.

Stock solutions of compounds to be tested in DMSO were added to the suspensions of fertilized eggs to give final concentrations of DMSO less than 0.2% in sea water. DMSO at the concentrations used had no effect on embryonic development. To assay for the developmental arrest of embryos, a small number of fertilized eggs were added to serially diluted sample solutions within 30 min after insemination. They were periodically observed for any cytological changes.

Animal Material, Extraction, and Isolation. *Rhopaloeides* sp. (1 kg, wet weight) was collected off the coast of Sada-misaki, Ehime Prefecture, Japan, and immediately frozen after collection. The marine sponge was identified by Professor Patricia R. Bergquist, The University of Auckland, New Zealand. A voucher specimen is kept in the laboratory of one of the authors (S.O.). The methanolic extract (27 g) was partitioned between EtOAc and water. The EtOAc layer (1.0 g) was subjected to chromatography on a silica gel column using 0→100% acetone in $CHCl_3$ as the eluent. The bioactive fraction (20→30% acetone in $CHCl_3$) was fractionated by HPLC on ODS (95% MeOH) to afford two bioactive fractions. The major fraction was subjected to recycling HPLC on ODS (95% MeOH containing 0.05% TFA) to afford **1** (24 mg; 2.4×10^{-3}% wet weight) and **2** (12 mg; 1.2×10^{-3}% wet weight) as viscous colorless oils. The minor fraction was further separated by repeated HPLC on ODS (95% MeOH) to afford **3** (1.4 mg; 1.4×10^{-4}% wet weight) as a viscous colorless oil.

Rhopaloic acid B (2): a viscous colorless oil; $[\alpha]^{25}_D$ +55° (*c* 0.23, $CHCl_3$); UV (CH_3CN) λ_{max} (log ε) 202 nm (4.32); IR (film) ν_{max} 2930, 1700, 1630 cm^{-1}; ^1H and ^{13}C NMR: see Table 1; EIMS *m/z* (rel. intensity) 374 $[M]^+$ (15), 305 (5), 137 (13), 136 (23), 81 (43), 69 (100); HREIMS *m/z* 374.2818 (calcd. for $C_{24}H_{38}O_3$, 374.2821); $\Delta\varepsilon_{205}$ +1.3±0.3 (5.6×10^{-5} M, CH_3CN).

Rhopaloic acid C (3): a viscous colorless oil; $[\alpha]^{25}_D$ +84° (*c* 0.03, $CHCl_3$); UV (CH_3CN) λ_{max} (log ε) 203 nm (4.23); IR (film) ν_{max} 2920, 1700, 1630 cm^{-1}; ^1H and ^{13}C NMR: see Table 1; EIMS *m/z* (rel.

intensity) 372 $[M]^+$ (10), 219 (15), 167 (35), 69 (100); (-) HREIMS *m/z* 372.2664 (calcd. for $C_{24}H_{36}O_3$, 372.2665); $\Delta\varepsilon_{205}$ +12.7±0.3 (7.5×10^{-5} M, CH_3CN).

Hydrogenation of 2. To a suspension of palladium carbon (10 mg) in EtOH (5 ml) was added a solution of **2** (2 mg) in EtOAc (0.5 ml). The slurry was stirred at room temperature under 1 atm of H_2 for 1 h. Removal of the palladium catalyst by filtration through Celite, followed by purification by silica gel column chromatography (EtOAc-hexane, EtOAc: 10→40%) afforded **4** (2 mg): EIMS *m/z* (rel. intensity) 382 $[M]^+$ (5), 309 (100). The ^1H NMR spectrum of **4** was very complicated because **4** is a mixture of diastereomers.

Hydrogenation of 1 and 3. Following the method similar to that used for the hydrogenation of **2**, **1** (2 mg) and **3** (0.2 mg) were converted into hydrogenated compounds **5** (2 mg) and **6** (0.1 mg), respectively. **5**: EIMS *m/z* (rel. intensity) 382 $[M]^+$ (5), 309 (100). **6**: EIMS *m/z* (rel. intensity) 382 $[M]^+$ (5), 309 (100).

Ethylation of 1. To a solution of **1** (5 mg) in EtOH (1 ml) was added ethyl iodide (0.5 ml) and sodium carbonate (100 mg). After being refluxed for 2 h, the reaction mixture was filtered through a short silica gel column. The filtrate was evaporated and subjected to silica gel column chromatography (EtOAc-hexane, EtOAc: 2→20%) to afford **7** (5 mg): $[\alpha]^{25}_D$ +46° (*c* 0.09, $CHCl_3$); IR (film) ν_{max} 1717, 1630 cm^{-1}; ^1H NMR ($CDCl_3$) δ_H 1.30 (3H, t, *J* = 7.3 Hz, $-OCH_2\underline{C}H_3$), 4.22 (2H, q, *J* = 7.3 Hz, $-OC\underline{H}_2CH_3$); ^{13}C NMR ($CDCl_3$) δ_C 14.2 ($-OCH_2\underline{C}H_3$), 60.6 ($-O\underline{C}H_2CH_3$), 166.0 (C-1); EIMS *m/z* (rel. intensity) 402 $[M]^+$ (40), 69 (100).

Ethylation of 2. Following the method similar to that used for the ethylation of **1**, **2** (4 mg) was converted into **8** (4 mg): $[\alpha]^{25}_D$ +51° (*c* 0.06, $CHCl_3$); IR (film) ν_{max} 1717, 1630 cm^{-1}; ^1H NMR ($CDCl_3$) δ_H 1.30 (3H, t, *J* = 7.3 Hz, $-OCH_2\underline{C}H_3$), 4.21 (2H, q, *J* = 7.3 Hz, $-OC\underline{H}_2CH_3$); ^{13}C NMR ($CDCl_3$) δ_C 14.2 ($-OCH_2\underline{C}H_3$), 60.6 ($-O\underline{C}H_2CH_3$), 166.0 (C-1); EIMS *m/z* (rel. intensity) 402 $[M]^+$ (20), 69 (100).

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