Hydroperoxysterols from the Tunicate *Eudistoma* sp.

Ping-Jyun Sung,*[,a,b] Mei-Ru Lin,[a] Jih-Jung Chen,[c] Shen-Fan Lin,[a] Yang-Chang Wu,[d] Tsong-Long Hwang,[e] and Lee-Shing Fang[a,f]

[a] *National Museum of Marine Biology and Aquarium; Checheng, Pingtung 944, Taiwan, R.O.C.:* [b] *Institute of Marine Biotechnology, National Dong Hwa University; Checheng, Pingtung 944, Taiwan, R.O.C.:* [c] *Department of Pharmacy, Tajen University; Pingtung 907, Taiwan, R.O.C.:* [d] *Graduate Institute of Natural Products, Kaohsiung Medical University; Kaohsiung 807, Taiwan, R.O.C.:* [e] *Graduate Institute of Natural Products, Chang Gung University; Kweishan, Taoyuan 333, Taiwan, R.O.C.: and* [f] *Cheng Shiu University; Niaosong, Kaohsiung 833, Taiwan, R.O.C.*

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Two 27C hydroperoxysterols, 7β-hydroperoxycholesterol (1) and its stereoisomer 7α-hydroperoxycholesterol (2), were isolated from the lipophilic extracts of a Formosan tunicate belonging to the genus *Eudistoma*. The structures of sterols 1 and 2 were elucidated on the basis of extensive spectral data analyses. Cytotoxicity of sterols 1 and 2 against a limited panel of cancer cell lines is also described. Sterol 1 show weak inhibitory effects on human neutrophil elastase release.

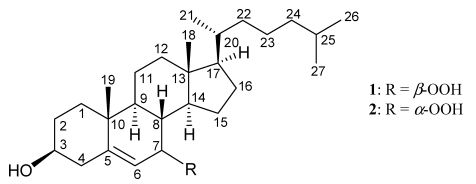
Key words tunicate; *Eudistoma*; hydroperoxysterol; cytotoxicity; human neutrophil elastase

Tunicates have been well-recognized as marine organisms containing large quantities of secondary metabolites that exhibit varying degrees of biological activities.[1,2] In connection with our continuing investigations of bioactive substances from marine organisms, a tunicate *Eudistoma* species (subphylum Urochordata, class Ascidiacea, order Enterogona, suborder Aplousobranchiata, family Polycitoridae) was selected for study,[3,4] as the EtOAc extract of this organism was found to exhibit cytotoxicity against MCF-7 (human breast cancer), NCI-H460 (human non-small cell lung cancer), and SF-268 (human CNS cancer) cells. In this paper, we report the isolation, structure elucidation, and biological activity of two hydroperoxysterols, 7β-hydroperoxycholesterol (**1**) and its stereoisomer 7α-hydroperoxycholesterol (**2**), from a Formosan tunicate identified as *Eudistoma* sp.

Results and Discussion

In the previous studies, a series of interesting hydroperoxysterols were obtained from various marine organisms, such as green algae,[5] brown algae,[6—8] red algae,[9,10] soft corals,[11] and tunicate.[12] During the course of further searching for bioactive components from Formosan marine organisms, the tunicate *Eudistoma* sp., collected in Feb. 2004, was freezed-dried and extracted with the mixtures of MeOH/DCM (1 : 1). The residue was partitioned between EtOAc and H_2O. Metabolites **1** and **2** were isolated from the EtOAc layer by silica gel column chromatography.

Sterol **1** was isolated by normal-phase HPLC eluted with the mixtures of *n*-hexane and EtOAc (stepwise, 3 : 1—2 : 1) and obtained as an amorphous white powder. This sterol was recognized as a hydroperoxysterol by the presence of the characteristic signal for the hydroperoxy proton at δ 7.76 ppm in the ^{1}H-NMR spectrum of **1** (Table 1).[5—14] An olefinic proton signal in the ^{1}H-NMR spectrum appeared at δ 5.58 (1H, dd, J=2.0, 2.0 Hz) was ascribed to H-6. The hydroperoxy- and hydroxy-bearing methines showed signals at δ 4.13 (1H, ddd, J=8.8, 2.0, 2.0 Hz, H-7) and 3.56 (1H, m, H-3), respectively. A doublet at δ 0.86 (6H, J=6.8 Hz) was attributed to the H_3-26 and H_3-27 methyl protons. The coupling constant between H-6 and H-7 (J=2.0 Hz) revealed that the dihedral angle between these two related C–H bonds is close to 90° and suggested the presence of a β-equatorial hydroperoxy group at C-7. A doublet at δ 0.91 (3H, J=6.4 Hz) was due to the C-21 methyl protons. Two singlets of H_3-18 and H_3-19 methyls appeared at δ 0.68 and 1.04 ppm, respectively. The ^{13}C and DEPT spectra of **1** displayed the signals of twenty-seven carbons, including five methyls, ten methylenes, nine methines, and three quaternary carbons (Table 1). Two olefinic carbons (δ 140.0, s, C-5; 121.6, d, CH-6), a hydroxy-bearing methine carbon (δ 71.3, CH-3), and a hydroperoxy-bearing methine carbon (δ 86.5, CH-7) were further identified. Moreover, the ^{13}C-NMR spectrum of **1** showed that the chemical shift of C-7 appeared at δ 86.5 ppm, which significantly different with that reported previously for (24*S*)-24-ethyl-7α-hydroperoxycholesta-5,25-dien-3β-ol (δ 78.5, CH-7),[5] and further suggested the β-configuration of the hydroperoxy group attached at C-7 of **1**. Based on above findings, the structure of **1** was established as 7β-hydroperoxycholesterol.



Further elution with *n*-hexane/EtOAc (2 : 1) led to the isolation of sterol **2**. Its ^{1}H-NMR spectrum showed a broad singlet at δ 7.68 ppm which revealed the presence of a hydroperoxy group.[5—14] Based on detailed spectral data analysis (IR, ^{1}H-, and ^{13}C-NMR), it was found that the structure of **2** was similar to that of **1**. Comparison of the ^{13}C-NMR chemical shift of C-7 of **1** (δ 86.5, d) with those of **2** (δ 78.5, d) and the known hydroperoxysterol (24*S*)-24-ethyl-7α-hydroperoxycholesta-5,25-dien-3β-ol (δ 78.5, d),[5] showed that the configuration of C-7 in **2** should be existed in α-oriented.

The hydroperoxysterols **1** and **2** had been prepared from cholesterol in 1973[15] and 1958,[16] respectively. The mechanism of the rearrangements of allylic hydroperoxides also have been proven.[17—22] However, to the best of our knowledge, hydroperoxysterols **1** and **2** have not been isolated previously from any natural sources. The detailed assignments

* To whom correspondence should be addressed. e-mail: pjsung@nmmba.gov.tw

Table 1. ^{1}H- and ^{13}C-NMR Data for Steroids **1** and **2**

C/N	**1**		**2**	
	^{1}H	^{13}C	^{1}H	^{13}C
1	1.82 (m)	36.8 (t)	1.62 (m)	37.1 (t)
1′	1.05 (m)		1.13 (m)	
2	1.86 (m)	31.5 (t)	1.84 (m)	31.3 (t)
2′	1.49 (br d, 6.8)		1.54 (m)	
3	3.56 (m)	71.3 (d)	3.62 (m)	71.4 (d)
4	2.39 (ddd, 13.2, 4.8, 2.0)	41.8 (t)	2.41 (dd, 12.8, 4.8)	42.2 (t)
4′	2.28 (m)		2.32 (br t, 12.8)	
5		140.0 (s)		148.9 (s)
6	5.58 (dd, 2.0, 2.0)	121.6 (d)	5.72 (dd, 5.2, 1.6)	119.9 (d)
7	4.13 (ddd, 8.8, 2.0, 2.0)	86.5 (d)	4.16 (m)	78.5 (d)
8	1.63 (m)	34.5 (d)	1.13 (m)	37.4 (d)
9	1.16 (m)	56.0 (d)	1.40 (m)	43.5 (d)
10		36.4 (s)		36.7 (s)
11	1.53 (ddd, 6.4, 4.8, 2.8)	21.3 (t)	1.48 (m)	20.9 (t)
11′	1.46 (dd, 12.4, 4.0)		1.38 (m)	
12	2.01 (ddd, 12.8, 3.6, 3.6)	39.5 (t)	1.95 (br d, 12.8)	39.0 (t)
12′	1.20 (m)		1.19 (m)	
13		42.8 (s)		42.3 (s)
14	1.09 (m)	48.7 (d)	1.43 (m)	49.0 (d)
15	1.78 (m)	25.9 (t)	1.86 (m)	24.4 (t)
15′	1.35 (m)		1.10 (m)	
16	1.81 (m)	28.3 (t)	1.89 (m)	28.3 (t)
16′	1.26 (m)		1.27 (m)	
17	1.06 (m)	55.6 (d)	1.16 (m)	55.7 (d)
18	0.68 (s)	11.9 (q)	0.66 (s)	11.3 (q)
19	1.04 (s)	18.8 (q)	0.99 (s)	18.2 (q)
20	1.35 (m)	35.8 (d)	1.34 (m)	35.8 (d)
21	0.91 (d, 6.4)	18.8 (q)	0.92 (d, 6.4)	18.7 (q)
22	1.31 (m)	36.2 (t)	1.33 (m)	36.1 (t)
23	1.38 (m)	23.9 (t)	1.32 (m)	23.7 (t)
23′	1.19 (m)		1.19 (m)	
24	1.12 (m)	39.6 (t)	1.11 (m)	39.5 (t)
25	1.47 (m)	28.0 (d)	1.52 (m)	28.0 (d)
26	0.86 (d, 6.8)	22.8 (q)	0.86 (d, 6.4)	22.8 (q)
27	0.86 (d, 6.8)	22.5 (q)	0.86 (d, 6.4)	22.6 (q)
7-OOH	7.76 (br s)		7.68 (br s)	

Table 2. Cytotoxic Data of Hydroperoxysterols **1** and **2**

Compounds	Cell lines IC_{50} (μg/ml)[a]				
	Hep3B	A549	HepG2	MCF7	MDA-MB231
1	15.6	17.4	>20	>20	>20
2	14.6	>20	15.7	11.2	11.2
Doxorubicin[b]	0.27	0.52	0.17	0.32	0.15

a) Concentration of compound required to inhibit cell growth by 50%, as determined by MTT assay (see Experimental). Data are expressed as means±S.E.M. of three independents. *b*) Doxorubicin was used as a reference compound.

of NMR (^{1}H and ^{13}C) chemical shifts for sterols **1** and **2** are disclosed (Table 1) for the first time by extensive 2D-NMR spectral data analysis.

Cytotoxicity of metabolites **1** and **2** toward a limited panel of cancer cell lines was evaluated (Table 2). The result showed that hydroperoxysterol **1** exhibited moderate cytotoxicity against the growth of Hep3B (human hepatoma cells) and A549 (human lung adenocarcinoma). Also, hydroperoxysterol **2** exhibited moderate cytotoxicity toward Hep3B, HepG2 (human hepatocellular carcinoma), MCF7 (human breast carcinoma), and MDA-MB-231 (human breast carcinoma) cells. Sterol **1** was found to show weak activity to inhibit the superoxide anion generation and human neutrophil elastase release at 10 μg/ml (Table 3).

Table 3. Inhibitory Effects of Sterol **1** on Superoxide Anion Generation and Elastase Release by Human Neutrophils in Response to fMet-Leu-Phe/Cytochalasin B

Compound	Superoxide anion	Elastase
	Inh %	Inh %
1	16.00±3.06[a]	21.56±3.56[b]

Percentage of inhibition (Inh %) at 10 μg/ml concentration. Results are presented as average±S.E.M. (n=4). *a*) $p<0.01$; *b*) $p<0.001$ compared with the control.

Experimental

General Experimental Procedures Melting points were determined using a Fargo melting point apparatus. Optical rotation values were measured on a Jasco P-1010 automatic digital polarimeter at 25 °C. IR spectra were recorded on a Varian Diglab FTS 1000 FT-IR spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded at 400 and 100 MHz, respectively, on a Varian Mercury Plus 400 FT-NMR spectrometer in $CDCl_3$. Proton chemical shifts were referenced to the residual $CHCl_3$ signal (δ 7.26 ppm). ^{13}C-NMR spectra were referenced to the center peak of $CDCl_3$ at δ 77.1 ppm. Column chromatography was performed on silica gel (230—400 mesh, Merck, Darmstadt, Germany). TLC was carried out on precoated Kieselgel 60 F_{254} (0.25 mm, Merck, Darmstade, Germany) and spots were visualized by spraying with 10% H_2SO_4 solution followed by heating. HPLC was performed by using a system comprised of a Hitachi L-7100 pump, a Hitachi photo diode array detector L-7455, and a Rheodyne 7725 injection port. A preparative column (Hibar 250—25 mm, LiChrospher Si60, 5 μm) was used for preparative HPLC.

Animal Material Specimens of the tunicate *Eudistoma* sp. were collected by hand at a depth of −1 m from the pool of the 3rd Nuclear Power Plant located at southern Taiwan coast in Feb. 2004. The sample was frozen immediately after collection. The species was identified from description.[4] The voucher samples were deposited at the National Museum of Marine Biology & Aquarium (NMMBA).

Extraction and Isolation The animal material (962 g, dry weight) was extracted with the mixtures of MeOH and DCM (1 : 1). The residue was partitioned between EtOAc and H_2O. The EtOAc layer (25.2 g) was separated on Sephadex LH-20 and eluted with the mixture of MeOH and DCM (3 : 1) to yield five fractions 1—5. Fraction 2 (5.38 g) was chromatographed on Si gel 60 using *n*-hexane/EtOAc (stepwise, 0—100% EtOAc), the fraction which was eluted with 9—11% EtOAc (2.19 g) was subjected to silica gravity chromatography and the new fraction 9—13% EtOAc (983 mg) was purified by normal phase HPLC, using the mixtures of *n*-hexane and EtOAc as a mobile phase to afford steroids **1** (stepwise, 3 : 1—2 : 1, 13.3 mg) and **2** (2 : 1, 0.8 mg).

7β-Hydroperoxycholesterol (**1**): White amorphous powder, ^{1}H- and ^{13}C-NMR data, see Table 1. The physical and spectral data of **1** are in full agreement with those reported previously.[15,19]

7α-Hydroperoxycholesterol (**2**): White amorphous powder, ^{1}H- and ^{13}C-NMR data, see Table 1. The physical and spectral data of **2** are in full agreement with those reported previously.[16,19]

Cytotoxicity Assays Steroids **1** and **2** were assayed for cytotoxicity against Hep3B, HepG2, A549, MCF7, and MDA-MB-231 tumor cells using the MTT method.[23] Freshly trypsinized cell suspensions were seeded in 96-well microtitre plates at densities of 5000—10000 cells per well with tested compound added from DMSO-diluted stock. After 3 d in culture, attached cells were incubated with MTT and subsequently solubilized in DMSO. The absorbency at 550 nm was then measured using a microplate reader. The IC_{50} is the concentration of agent that reduced cell growth by 50% under the experimental conditions.

Human Neutrophil Superoxide Generation and Elastase Release Human neutrophil were obtained by means of dextran sedimentation and Ficoll centrifugation. Superoxide generation and elastase release were carried out according to the procedure described previously.[24,25] Briefly, superoxide anion production was assayed by monitoring the superoxide dismutase-inhibitable reduction of ferricytoxhrome *c*. Elastase release experiments were performed using MeO-Suc-Ala-Ala-Pro-Valp-nitroanilide as the elastase substrate.

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