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Cytotoxic Constituents of *Diadema setosum*

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5,8-Epidioxycholest-6-en-3-ol (**1**), cholesterol (**2**), glycerol 1-palmitate (**3**) and glycerol 1,3-dioleate-2-stearate (**4**) were isolated from the methanol extract of the sea urchin *Diadema setosum*, which was collected from the Halong sea, Vietnam. Chemical structures were established based on extensive 1D, 2D-NMR, FAB-MS, EI-MS spectroscopic data and GC-MS analysis. The NMR spectral data of compound **1** were reassigned by using HMQC and HMBC. Compound **1** was found to have strong cytotoxic effect against various cancer cell lines, such as KB (IC_{50}, 2.0 μg/mL), FL (IC_{50}, 3.93 μg/mL), and Hep-2 (IC_{50}, 2.4 μg/mL) by *in vitro* assay.

Key words: *Diadema setosum*, Sea urchin, 5,8-Epidioxycholest-6-en-3-ol

INTRODUCTION

Diadema setosum is a type of sea urchin, the name given to marine invertebrates belong to the phylum Echinodermata. Of the 600 species of sea urchins, approximately 80 species may be venomous to humans. The long spined or black sea urchin, *D. setosum* poses a threat because its brittle spines break off after penetrating the skin. Moreover, some sterols isolated from *Inonotus radiatus* and *Sinularia* species have been reported to have 5,8-epidioxy skeletons, and to be cytotoxic to various cancer cell lines (Kahlos *et al.*, 1989; Sheu *et al.*, 2000).

As part of our continuing research to identify bioactive compounds in the Vietnamese sea, we isolated two sterols and two lipids from the methanol extract of *D. setosum*. Their structures were identified as 5,8-epidioxycholest-6-en-3-ol (**1**), cholesterol (**2**), glycerol 1-palmitate (**3**), and glycerol 1,3-dioleate-2-stearate (**4**) spectroscopically, i.e., using combinations of ^{1}H-, ^{13}C-NMR, DEPT, HMQC, HMBC, FAB-MS, and GC-MS. Isolated compounds were tested for their cytotoxic effects against various cancer cell lines, such as KB (human epidermoid carcinoma), FL (fibrillary sarcoma of the uterus), and Hep-2 (human hepatocellular carcinoma) cells in an *in vitro* assay system.

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MATERIALS AND METHODS

General experimental procedures

FAB-MS was obtained using a JEOL JMS-DX 300 spectrometer. EI-MS was obtained using a Hewlett Packard 5989 B MS spectrometer. ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) were recorded on a Bruker AM500 FT-NMR spectrometer using TMS as the internal standard. Column chromatography (CC) was performed on silica gel (Kieselgel 60, 70-230 mesh and 230-400 mesh, Merck). GC-MS was obtained using a HP-6890 MSD Agilent 5973 instrument.

Marine material

D. setosum was collected in the Halong sea, Vietnam during December 2003 and identified by Dr. Do Cong Thung, Haiphong Institute of Oceanography, VAST, Vietnam. A voucher specimen (VN 338) was deposited at the herbarium of the Institute of Natural Product Chemistry, VAST, Vietnam.

Extraction and isolation

The samples were dissected to separate the body walls and intestines. Body walls (2.5 kg) were cut into small pieces and then extracted with methanol in a Soxhlet apparatus. The methanol extract (85.0 g) obtained was suspended in water and extracted with chloroform. The residue (32.0 g) from chloroform fraction was chromatographed on a silica gel column, using an increasing polarity of $CHCl_3$-MeOH (from 100 : 0 to 0 : 100) as eluent, which yielded five fractions (fr. A-E). Fraction C

(5.8 g) was chromatographed on a silica gel column using chloroform-methanol (100 : 1) as eluent to yield **1** (35 mg) and **2** (3500 mg) as white crystals. Fraction B (2.4 g) was chromatographed on a silica gel column using hexane-ethyl acetate (100 : 1) yielding the colorless oils **3** (120 mg) and **4** (87 mg).

5,8-Epidioxycholest-6-en-3-ol (1)
White crystals, mp. 102-105 °C, $[\alpha]_D^{25}$ - 30° ($CHCl_3$, *c* 0.5); IR $^{KBr}\nu_{max}$ cm^{-1}: 3410 (br, OH), 1050 (C-O-C); positive FAB-MS (*m/z*) 439 $[M+Na]^+$; The ^{13}C-NMR (125 MHz) and ^1H-NMR (500 MHz): see Table I.

Cholesterol (2)
White crystals, mp. 148-149 °C, $[\alpha]_D^{25}$ - 47° ($CHCl_3$, *c* 0.5); ^1H-NMR (500 MHz, $CDCl_3$) δ: 3.52 (1H, m, H-3), 5.35 (1H, br d, 5.0 Hz, H-6), 1.00 (3H, s, H-19), 0.91 (3H, s, H-21), 0.86 (6H, d, 6.6 Hz, H-26, 27) and 0.68 (3H, s, H-18); The ^{13}C-NMR (125 MHz, $CDCl_3$): see Table I.

Table I. ^1H- and ^{13}C-NMR data for **1** and **2**

C	δ_C of **1**[a,b]	δ_H of **1**[a,c]	HMBC of **1** (C to H)	δ_C [a,b] of **2**
1	39.5 (t)	1.95/2.00 (m)		37.2 (t)
2	30.1 (t)	1.52/1.82 (m)	H-4	31.6 (t)
3	66.5 (d)	4.00 (1H, m)	H-4	71.8 (d)
4	36.8 (t)	1.90/2.12 (m)	H-3	42.2 (t)
5	82.1 (s)	-	H-19, H-6, H-7	140.7 (s)
6	135.4 (d)	6.25 (d, 8.5 Hz)	H-7	121.7 (d)
7	130.8 (d)	6.55 (d, 8.5 Hz)	H-6	31.8 (t)
8	79.5 (s)	-	H-6, H-7	31.8 (d)
9	51.1 (d)	1.50 (m)	H-19	50.1 (d)
10	36.9 (s)	-	H-4	36.5 (s)
11	20.6 (t)	1.01/1.61 (m)		21.1 (t)
12	39.5 (t)	1.21 (m)		39.8 (t)
13	44.7 (s)	-	H-18	42.3 (s)
14	51.6 (d)	1.58 (d, 3.5 Hz)	H-18	56.7 (d)
15	23.4 (t)	1.21/1.50 (m)		24.3 (t)
16	28.2 (t)	1.42*		28.3 (t)
17	56.4 (d)	1.20*	H-18, H-21	56.1 (d)
18	12.6 (q)	0.80 (3H, s)		11.9 (q)
19	18.6 (q)	0.90 (3H, s)		19.4 (q)
20	35.2 (d)	1.65 *		35.7 (d)
21	18.1 (q)	0.90 (3H, d, 6.5 Hz)		18.7 (q)
22	35.9 (t)	1.70 (m)		36.1 (t)
23	23.8 (t)	1.32*		23.8 (t)
24	39.5 (t)	1.21*	H-26, H-27	39.5 (t)
25	28.0 (d)	1.42 (m)	H-26, H-27	28.0 (d)
26	22.5 (q)	0.85 (3H, d, 6.5 Hz)		22.5 (q)
27	22.8 (q)	0.85 (3H, d, 6.5 Hz)		22.8 (q)

[a]In $CDCl_3$, [b]125 MHz, [c]500 MHz, *Overlap signals

Glycerol 1-palmitate (3)
Colorless oil, $[\alpha]_D^{25}$ - 5.0° ($CHCl_3$, *c* 1.0); EI-MS (*m/z*) 330 $[M]^+$, $C_{19}H_{38}O_4$; ^1H-NMR (500 MHz, $CDCl_3$) δ: 4.23 (2H, m, H-1), 3.90 (1H, m, H-2), 3.60 and 3.70 (2H, m, H-3), 2.35 (2H, t, 6.5 Hz, H-2'), 1.65 (2H, m, H-3'), 1.30 (24H, H-4' to H-15') and 0.90 (3H, t, 5.0 Hz, H-16'); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 174.5 (s, C-1'), 70.5 (d, C-2), 65.4 (t, C-1), 63.4 (t, C-3), 34.2 (t, C-2), 32.1 (t, C-14'), 30.0 (t, C-4' to C-13'), 25.0 (t, C-3'), 22.7 (t, C-15') and 14.0 (q, C-16').

Glycerol 1,3-dioleate-2-stearate (4)
Colorless oil, $[\alpha]_D^{25}$ - 12.5° ($CHCl_3$, *c* 1.0); ^1H-NMR (500 MHz, $CDCl_3$) δ: 5.34 (4H, m, 2×H-9' and H-10'), 5.23 (1H, m, H-2), 4.20 and 4.30 (4H, H-1, 3), 2.35 (6H, 2×H-2' and H-2"), 2.00 (4H, 2×H-8' and H-11'), 1.65 (6H, 2×H-3' and H-3"), 1.30 (28 CH_2) and 0.89 (9H, 2×H-18' and H-18"); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 173.3 (s, 2×C-1'), 172.9 (s, C-2"), 130.0 (d, 2×C-9' and 2×C-10'), 68.9 (d, C-2), 62.1 (t, C-1 and C-3), 34.5 (t, 2×C-2' and C-2"), 33.0 (t, 2×C-8' and 2×C-11'), 32.0 (t, 2×C-16' and C-16"), 29.5 (t, 25×CH_2), 27.5 (t, 2×C-15', C-15"), 24.5 (t, 2×C-3' and C-3"), 22.8 (t, 2×C-17' and C-17") and 14.0 (2×C-18' and C-18").

RESULTS AND DISCUSSION

Repeated silica gel column chromatography of the methanol extract of *D. setosum* yielded **1** and **2** as white crystals, and **3** and **4** as colorless oils. Compound **1** had a hydroxyl absorption peak at 3410 cm^{-1} and C-O-C absorption peak at 1050 cm^{-1}. The positive FAB-MS showed a molecular ion at *m/z* 439 $[M+Na]^+$, corresponding to the molecular formula of $C_{27}H_{44}O_3$. The ^{13}C- and ^1H-NMR spectra revealed the presence of 27 sterol-like carbons, including 4 quaternary, 8 methine, 10 methylene, and 5 methyl carbons. Of which, one double bond -CH=CH- was confirmed at δ_H 6.25 (d, 8.5 Hz)/ 6.55 (d, 8.5 Hz) and δ_C 135.4 (d)/130.8 (d); two oxygen bearing quaternary carbons at δ_C 82.1 (s)/79.5 (s) and one oxygen bearing methine carbon at δ_C 66.5 (d) (Table I). The assignments of the chemical shifts of **1** were made by HMQC and HMBC, and compared with those of ergosterol peroxide (Kahlos *et al.*, 1989) and 5α,8α-epidioxysterol (Sheu *et al.*, 2000). The chemical shifts obtained by ^1H-, ^{13}C-NMR, and HMBC are shown in Table I. Long-range C-H correlations between proton H-3 (δ_H 4.00) and C-4 (δ_C 36.8)/C-5 (82.1), between proton H-6 (δ_H 6.25) and carbons C-5 (82.1)/C-7 (130.8)/C-8 (79.5), and between proton H-7 (δ_H 6.55) and carbons C-5/C-6 (135.4)/C-8 were observed in the HMBC spectrum. This confirmed the locations of the hydroxyl group at C-3, one double bond at C-6/C-7, and the presence of an epidioxy group attached to C-5 and C-

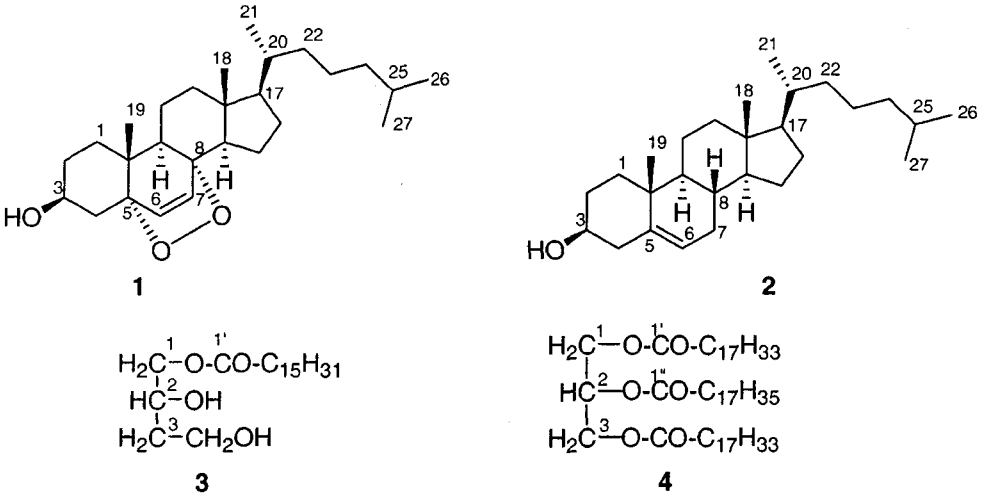

Fig. 1. Structures of compounds **1-4**

8. The chemical shift at C-3 (δ_C 66.5) is typical for a 3β-OH stereochemistry at C-3 (Sheu *et al.*, 2000). In addition, the HMBC spectrum showed correlations between protons H-4 (δ_H 1.90/ 2.12) and carbon C-10 (δ_C 36.9), especialy between protons H-19 (δ_H 0.90) and carbons C-5 (δ_C 82.1)/C-9 (51.1), and between protons H-4 (δ_H 1.90) and carbon C-6 (δ_C 135.4). By comparisons with reference data (Kahlos *et al.*, 1989; Sheu *et al.*, 2000) of the DEPT and 2D-NMR of **1**, the carbon chemical shifts at C-4, C-5, C-6, C-7, C-8 and C-9 were reassigned to δ_C 36.8, 82.1, 135.4, 130.8, 79.5 and 51.1, respectively. Based on above data, **1** was determined to be 5,8-epidioxycholest-6-en-3-ol, which was first identified in *Diadema* species.

The ^{1}H- and ^{13}C-NMR spectrums of **2** also revealed the presence of sterol-like 27 carbon skeleton, and included 3 quaternary, 8 methine, 11 methylene, and 5 methyl carbons. One hydroxyl group bearing methine signal occurred at δ_H 3.52 (1H, m, H-3)/δ_C 71.8. One double bond >C=CH- resonances at δ_C 140.7/121.7 and δ_H 5.35 (1H, br d, J = 5.0 Hz) assigned to be C-5/C-6 and H-6, respectively. The NMR data of **2** was compared with that of cholesterol, and found to match (Goad *et al.*, 1997).

Compound **3** was obtained as white oil. The ^{1}H-, ^{13}C-NMR and DEPT spectrums of **3** confirmed the presence of one ester carbonyl group (δ_C 174.5), one oxygen bearing methine (δ_C 70.5/δ_H 3.90), two oxygen bearing methylene carbons (δ_C 65.4/δ_H 4.23 and δ_C 63.4/δ_H 3.60, 3.70), and a long chain fatty acid. From the ^{1}H-NMR spectrum, a long chain was assumed to be a palmitoyl moiety. Furthermore, the EI-MS spectrum of **3** showed a molecular ion peak at *m/z* 330 corresponding to the molecular formula of $C_{19}H_{38}O_4$. Thus, **3** was identified as glycerol 1-palmitate.

Compound **4** was identified as a lipid component from its ^{1}H-, ^{13}C-NMR, and DEPT spectra. Three ester carbonyl groups (δ_C 173.3×2, 172.9), two double bonds [δ_C overlap at 130.0/ δ_H 5.34 (4H, m)], two oxygen bearing methylene carbons [δ_H 4.20/4.30 (4H) and δ_C 62.1 (t, C-1, C-3)] and one oxygen bearing methine carbon [δ_H 5.23 and δ_C 68.9 (t, C-2)] were present in the NMR spectrums of **4**. Concerning the intensity of the NMR peaks and the above data, **4** was suggested to have the same chain at C-1 and C-3, which contained a double bond. In order to confirm the structure of **4**, it was hydrolyzed with KOH and the hydrolysates were methylated with CH_2CN_2 (Dale *et al.*, 1992). GC-MS analysis (column HP-5 MS 30m×0.25× 0.25 μm, carrier gas Helium, nominal initial 1.0 mL/min) then showed octadeca-9-enoic acid methyl ester and octadecaoic acid methyl ester at 20.85 and 20.89, respectively. Therefore, **4** was identified as glycerol 1,3-dioleate-2-stearate.

Cytotoxicity

The cytotoxic activities of the identified compounds were assayed on KB (human epidermoid carcinoma), FL (fibrillary sarcoma of the uterus), and Hep-2 (human hepatocellular carcinoma) cells by SRB method (Lee *et al.*, 2003; Likhitwitayawuid *et al.*, 1993). As a result, **1** was found to be strongly cytotoxic to several cancer cell lines KB (human epidermoid carcinoma, IC_{50}, 2.0 μg/mL), FL (fibrillary sarcoma of the uterus, IC_{50}, 3.93 μg/mL), and Hep-2 (human hepatocellular carcinoma, IC_{50}, 2.4 μg/mL) cells in an *in vitro* assay system.

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