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STEROIDS FROM THE SPONGE *Clathria vulpina* AND THEIR CYTOTOXIC ACTIVITIES

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Abstract

Using combined chromatographic methods, three steroids were isolated from the methanol extract of the sponge *Clathria vulpina*. Their structures were elucidated to be 3β-hydroxycholest-5-ene-7-one (**1**), stigmast-4-ene-3,6-dione (**2**), and stigmast-4-ene-3-one (**3**) by 1D- and 2D-NMR spectroscopic methods and in comparison with those reported in the literature. All compounds were evaluated for cytotoxic activities on eight human cancer cell lines, HepG-2, KB, LU-1, MCF-7, LNCaP, SW-480, MKN-7, and HL-60. As the results, compound **3** exhibited moderate cytotoxic activity with the IC_{50} values ranging of 37.12÷45.19 µg/mL.

Keywords: *Clathria vulpina*, sponge, steroid, cytotoxic activities.

1. INTRODUCTION

Clathria vulpina (Lamarck, 1814) belongs to Microcionidae family. Chemical investigation of *Clathria* species led to the isolation of an anti-HIV-1 compound, clathsterol [1]. In addition, clathriol was found in *Clathria lissosclera* and showed anti-inflammatory activity [2]. One new compound, microcionamide from *Clathria abietina* exhibited significant cytotoxic activities in MCF-7 and SKBR-3 cell lines [3]. Moreover, four alkaloids, clathrynamides A-C and clathryimine A were isolated from *Clathria* sp. [4, 5]. However, investigation in chemical constituents and biological activity of *Clathria vulpina* has not been reported yet. Herein, we reported the isolation, structure elucidation and cytotoxic activities of three sterols, 3β-hydroxycholest-5-ene-7-one (**1**), stigmast-4-ene-3,6-dione (**2**), and stigmast-4-ene-3-one (**3**) from *C. vulpina*.

2. MATERIAL AND METHODS

2.1. Animal materials

The specimen of *Clathria vulpina* was collected in Hon Troc Island, Ba Ria-Vung Tau, Vietnam during April, 2011 and kept frozen until used. The scientific name was identified by Dr. Do Cong Thung, Institute of Marine Resources and Environment, VAST. A voucher specimen was deposited at Institute of Marine Biochemistry and Institute of Marine Resources and Environment, VAST.

2.2. General experimental procedures

All NMR spectra were recorded on a Bruker AM500 FT-NMR spectrometer (500 MHz for ^{1}H- and 125 MHz for ^{13}C-NMR), and chemical shifts (δ) are reported in ppm using TMS as an internal standard. Column chromatography (CC) was performed on silica gel 230÷400 mesh (0.040÷0.063 mm, Merck) or YMC RP-18 resins (30÷50 µm, Fujisilisa Chemical Ltd.). Thin layer chromatography was performed on DC-Alufolien 60 F_{254} (Merck 15715) or RP_{18} F_{254s} (Merck) plates. Compounds were visualized by spraying with aqueous 10% H_2SO_4 and heating for 5 minutes.

2.3. Extraction and isolation

Fresh frozen samples of the sponge *C. vulpina* (4.2 kg) were well grinded and extracted with hot MeOH three times and then concentrated under reduced pressure to give MeOH extract (CV, 101 g). This extract was suspended in water and then partitioned with *n*-hexane, chloroform, ethyl acetate,

and *n*-butanol to give the *n*-hexane (CV1, 20.0 g), chloroform (CV2, 32.0 g), ethyl acetate (CV3, 13.0 g), and *n*-butanol (CV4, 15.0 g) residues after removal of the solvents *in vacuo*.

The CV1 extracts (32.0 g) was chromatographed on a silica gel column and eluting with a gradient elution of *n*-hexane – acetone (40:1 → 0:1, v/v) to yield two sub-fractions, CV1A (10.5 g) and CV1B (6.5 g). The CV1B fraction was chromatographed on a silica gel column eluting with *n*-hexane – EtOAc (3:1, v/v) to give three smaller fractions, CV1B1 (3.2 g), CV1B2 (1.0 g), and CV1B3 (3.3 g). Compound **1** (8.0 mg) was obtained from CV1B2 by chromatography on YMC column using mobile phase, methanol – water (8:1, v/v).

The chloroform extracts (CV2, 32.0 g) was chromatographed on a silica gel column eluting with a gradient elution of chloroform – methanol (100:1 → 1:1, v/v) to give four fractions CV2A (15.5 g), CV2B (3.8 g), CV2C (3.7 g), and CV2D (4.5 g). The CV2B fraction (3.8 g) was chromatographed on a silica gel column using chloroform – ethyl acetate (5:1, v/v) to give three smaller fractions, CV2B1 (1.4 g), CV2B2 (0.8 g), and CV2B3 (1.2 g). The CV2B2 was chromatographed on an YMC column using acetone – water (4:1, v/v) to yield **2** (17.0 mg). The CV2C fraction (3.7 g) was chromatographed on a silica gel column using chloroform – methanol (10: 1, v/v) to give three fractions, CV2C1 (1.5 g), CV2C2 (0.7 g), and CV2C3 (1.3 g). The CV2C2 fraction was chromatographed on a silica gel column eluting with chloroform – ethyl acetate (6:1, v/v) to yield compound **3** (35 mg).

3β-hydroxycholest-5-ene-7-one (1): White crystal; melting point: 171-172 °C; optical rotation: $[\alpha]_D^{25}$: -50.6 (*c* = 0.1, MeOH); molecular formula: $C_{27}H_{44}O_2$; molecular weight: 400.7; ^{1}H- and ^{13}C-NMR data, see table 1.

Stigmast-4-ene-3,6-dione (2): White crystal; melting point: 170-172 °C; optical rotation: $[\alpha]_D^{25}$: -60.5 (*c* = 1.0, $CHCl_3$); molecular formula: $C_{29}H_{46}O_2$; molecular weight: 426.7; ^{1}H- and ^{13}C-NMR data, see table 1.

Stigmast-4-ene-3-one (3): White crystal; melting point: 171-172°C; optical rotation: $[\alpha]_D^{25}$: -50.6 (*c*=0.51, $CHCl_3$); molecular formula: $C_{29}H_{48}O$; molecular weight: 412.7; ^{1}H- and ^{13}C-NMR data, see table 1.

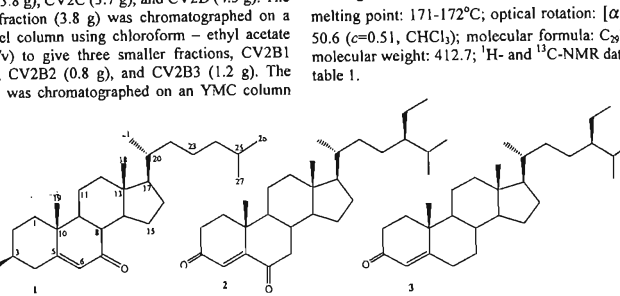

Figure 1: Chemical structures of compounds **1–3**

2.4. Cytotoxic assay

Effects of **1–3** on the growth of human cancer cells were determined by measuring the cytotoxic activity using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Eight human cancer cell lines, including Hep-G2 (hepatocellular carcinoma), KB (oral carcinoma), LU-1 (lung carcinoma), MCF-7 (breast carcinoma), LNCaP (prostatic carcinoma), SW-480 (colon carcinoma), MKN-7 (gastric carcinoma) and HL-60 (promyelocytic leukemia) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/mL and 100 g/mL, respectively) at 37°C in a humidified 5% CO_2 atmosphere. The exponentially growing cells were used throughout the experiments. The MTT assays were performed as follows: human cancer cells ($1.5 \times 2.5 \times 10^5$ cells/mL) were treated for 3 days with 1, 10, 30 and 100 μg/mL of compounds. Ellipticine was used to final concentrations of 1, 3, 10, and 20 μg/mL as a reference compound. After incubation, 0.1 mg (50 μL of a 2 mg/mL solution) MTT (Sigma, Saint Louis MO, USA) was added to each well and the cells were then incubated at 37°C for 4 h. The plates were centrifuged at 1000 rpm for 5 min at room temperature and the media was then carefully aspirated. Dimethylsulfoxide (150 μL) was then added to each well to dissolve the formazan crystals. The plates were read immediately at 540 nm on a microplate reader (Amersham Pharmacia Biotech., USA). All the experiments were performed three times and the mean absorbance values were calculated. The results are expressed as the percentage of inhibition by

produced a reduction in the absorbance by the treatment of crude extract or solvent fractions compared to the untreated controls. A dose-response curve was generated and the inhibitory concentration of 50% (IC_{50}) was determined for each compound as well as each cell line.

Table 1: The ^{1}H- and ^{13}C-NMR data of **1–3** and reference compounds

	1			**2**			**3**		
	δ_C[a]	δ_C[a,b]	δ_H[a,c] (mult., J = Hz)	δ_C[#]	δ_C[a,b]	δ_H[a,c] (mult., J = Hz)	δ_C[5]	δ_C[d,b]	δ_H[d,c] (mult., J = Hz)
1	36.4	36.40	1.24 (m)/1.95 (m)	35.5	35.81	2.09 (m)	35.7	36.77	1.74 (m)/2.11 (m)
2	31.2	31.29	1.62 (m)/1.93 (m)	34.0	34.26	2.45 (m)/2.57 (m)	34.0	34.71	2.35 (m)/2.50 (m)
3	70.6	70.56	3.68 (m)	199.5	201.16	-	198.9	202.37	-
4	41.9	41.86	2.50 (dd, 2.5, 13.5) 2.39 (m)	125.4	125.57	6.04 (s)	123.8	124.07	5.72 (s)
5	165.2	165.05	-	161.1	162.44	-	171.1	175.38	-
6	126.2	126.15	5.69 (s)	202.3	203.54	-	33.0	34.00	2.35 (m)/2.50 (m)
7	202.4	202.21	-	46.8	47.14	2.08 (m) 2.56 (dd, 4.0, 16.0)	32.1	33.36	1.07 (m)/1.91 (m)
8	45.5	45.46	2.24 (t, 11.0)	34.2	34.70	1.89 (m)	35.7	36.89	1.64 (m)
9	50.0	50.04	1.34 (m)	51.0	51.38	1.36 (m)	53.9	55.39	1.01 (m)
10	38.3	38.31	-	39.8	40.38	-	38.7	39.97	-
11	21.3	21.27	1.58 (m)	20.9	21.29	1.60 (m)	21.1	22.12	1.55 (m)/1.60 (m)
12	39.5	39.51	1.13 (m)	39.1	39.56	1.17 (m)/2.03 (m)	39.7	41.02	1.23 (m)/2.11 (m)
13	41.9	43.15	-	42.5	42.99	-	42.4	43.58	-
14	50.0	50.02	1.51 (m)	56.5	56.29	1.12 (m)	56.0	57.40	1.18 (m)
15	26.4	26.34	1.25 (m)/2.39 (m)	24.0	24.34	1.05 (m)/1.60 (m)	24.2	25.17	1.21 (m)/1.69 (m)
16	28.6	28.54	1.27 (m)/1.88 (m)	28.0	28.39	1.26 (m)/1.86 (m)	28.2	29.29	1.35 (m)/1.91 (m)
17	54.9	54.89	1.11 (m)	55.9	56.96	1.12 (m)	56.1	57.29	1.08 (m)
18	12.0	11.99	0.68 (s)	11.9	12.16	0.67 (s)	12.0	12.38	0.78 (s)
19	17.4	17.33	1.20 (s)	17.5	17.65	1.10 (s)	17.4	17.71	1.25 (s)
20	35.8	35.72	1.38 (m)	36.0	36.57	1.33 (m)	36.2	37.51	1.44 (m)
21	18.9	18.90	0.90 (d, 6.0)	18.7	19.03	0.87 (d, 7.0)	18.7	19.30	0.98 (d, 6.5)
22	36.2	36.23	1.01 (m)	33.8	34.22	1.00 (m)/1.38 (m)	34.0	35.08	1.03 (m)/1.46 (m)
23	23.9	23.86	1.15 (m)/1.36 (m)	26.0	26.77	1.32 (m)	26.2	27.49	1.12 (m)/1.40 (m)
24	38.8	38.87	2.02 (br d, 13.0)	45.8	46.54	0.86 (m)	45.9	47.52	0.98 (m)
25	28.0	28.01	1.53 (m)	29.1	29.40	1.62 (m)	29.3	30.75	1.45 (m)
26	22.6	22.66	0.86 (d, 6.5)	19.0	19.19	0.74 (d, 7.0)	18.8	19.38	0.86 (d, 6.5)
27	22.8	22.79	0.86 (d, 6.5)	19.8	19.78	0.75 (d, 7.0)	20.2	19.96	0.88 (d, 6.5)
28				23.1	23.42	1.05 (m)/1.29 (m)	23.1	24.12	1.20 (m)/1.40 (m)
29				12.0	12.49	0.78 (t, 7.5)	12.0	12.65	0.90 (t, 7.5)

[a]Recorded in $CDCl_3$, [b]125MHz, [c]500MHz, [d]Recorded in CD_3OD, [#]δ_C of 3β-hydroxycholest-5-en-7-one [6], [5]δ_C of stigmast-4-ene-3,6-dione [7], [5]δ_C of stigmast-4-ene-3-one in $CDCl_3$ [8].

3. RESULTS AND DISCUSSION

Compound **1** was obtained as a white crystal. The ^{1}H-NMR spectrum of **1** showed the signals for five methyl groups at δ_H 0.68 (s, H-18), 0.86 (d, J = 6.5 Hz, H-26/H-27), 0.90 (d, J = 6.0 Hz, H-21), and 1.20 (s, H-19), one oxymethine proton at δ_H 3.68 (m, H-3), and one olefinic proton at δ_H 5.69 (s, H-6). The ^{13}C-NMR and DEPT spectra of **1** exhibited the signals for 27 carbons, including one carbonyl, three quaternary, eight methine, ten methylene, and five methyl carbons and its NMR data were similar to those of 3β-hydroxycholest-5-ene-7-one [6]. The HMBC correlations between H-26/H-27 (δ_H 0.86) and C-24 (δ_C 38.87)/C-25 (δ_C 28.01); H-21 (δ_H 0.90) and C-17 (δ_C 54.89)/C-20 (δ_C 35.72)/C-22 (δ_C 36.23); H-18 (δ_H 0.68) and C-12 (δ_C 39.51)/C-13 (δ_C 43.13)/C-14 (δ_C 50.02)/C-17 (δ_C 54.89) indicated the position of two methyl groups at C-13 and C-20 and two other methyl groups at C-25. The HMBC correlations from H-19 (δ_H 1.20) to C-1 (δ_C 36.40), C-5 (δ_C 165.05), C-9 (δ_C 50.04), and C-10 (δ_C 38.31) suggested the methyl group at C-10. In addition, the HMBC correlations from H-9 (δ_H 1.34 and 1.51), H-8 (δ_H 2.24) and H-6 (δ_H 5.69) to C-7 (δ_C 202.21) confirmed the carbonyl group at C-7 and the double bond at C-5/C-6. Based on the above evidence, compound **1** was determined to be a known compound, 3β-hydroxycholest-5-ene-7-one.

The ^{1}H-NMR of **2** showed the signals for six methyl groups at δ_H 0.66 (s, H-18), 0.74 (d, J = 7.0 Hz, H-26), 0.75 (d, J = 7.0 Hz, H-27), 0.78 (t, J = 7.5 Hz, H-29), 0.87 (d, J = 7.0 Hz, H-21), and 1.10 (s, H-19), one olefinic proton at δ_H 6.04 (s, H-4).

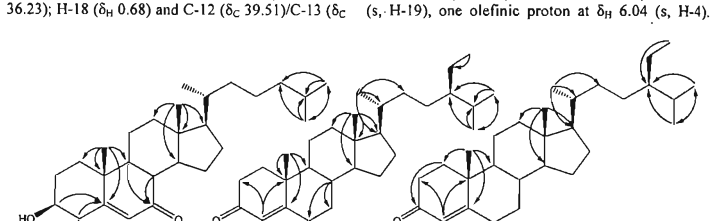

Figure 2: The important HMBC correlations of **1**–**3**

The ^{13}C-NMR of **2** showed the signals for 29 carbons, including two carbonyl groups at δ_C 201.16 and 203.54, two olefinic carbons at δ_C 125.57 (C-4) and δ_C 162.44 (C-5). The ^{1}H and ^{13}C-NMR data of **2** were similar to those of stigmast-4-ene-3,6-dione [7]. The HMBC correlations between H-4 (δ_H 6.04) and C-3 (δ_C 201.16)/C-5 (δ_C 162.44)/C-6 (δ_C 203.54); H-19 (δ_H 1.10) and C-1 (δ_C 35.81)/C-5 (δ_C 162.44)/C-10 (δ_C 40.38) suggested two carbonyl groups and the double bond were at C-3, C-7, and C-5/C-6, respectively. In addition, the HMBC correlations from H-26 (δ_H 0.74)/H-27 (δ_H 0.75) to C-24 (δ_C 46.54)/C-25 (δ_C 29.40); from H-29 (δ_H 0.78) to C-24 (δ_C 46.54) and C-28 (δ_C 23.42), confirming the ethyl group at C-24 and two methyl groups at C-25. Consequently, compound **2** was elucidated to be a known compound, stigmast-4-ene-3,6-dione [7].

Compound **3** was also obtained as a white crystal. The ^{1}H-NMR spectrum of **3** showed the presence of one olefinic proton at δ_H 5.72 (s, H-4), two tertiary methyl groups at δ_H 0.78 (s, H-18) and 1.18 (s, H-19), three secondary methyl groups at δ_H 0.86 (d, J = 6.5 Hz, H-26), 0.88 (d, J = 6.5 Hz, H-27) and 0.98 (d, J = 6.5 Hz, H-21) and one primary methyl group at δ_H 0.90 (t, J = 7.5 Hz, H-29). The ^{13}C-NMR and DEPT spectra of **3** showed the signals for 29 carbons including one carbonyl, three quaternary, eight methine, eleven methylene, and six methyl carbons. Comparing the ^{13}C-NMR data of **3** with the corresponding data of **2** confirmed the disappearence of one carbonyl group at C-6. In addition, the NMR data of **3** were similar to those of stigmast-4-ene-3-one [8]. The HMBC correlations between H-19 (δ_H 1.25) and C-1 (δ_C 36.77)/C-5 (δ_C 175.38)/C-9 (δ_C 55.39)/C-10 (δ_C 39.97); H-4 (δ_H 5.72) and C-2 (δ_C 34.71)/C-3 (δ_C 202.37)/C-5 (δ_C 175.38)/C-6 (δ_C 34.00) confirmed the carbonyl group at C-6 and the double bond at C-4/C-5. Based on the above evidence, compound **3** was also determined to be a known compound, stigmast-4-ene-3-one.

Compounds **1**–**3** were evaluated for cytotoxic activities on eight human cancer cell lines, HepG-2, KB, LU-1, MCF-7, LNCaP, SW-480, MKN-7, and HL-60. As the results, compound **3** exhibited moderate cytotoxic activity on eight cancer cell lines

with the IC_{50} values of 37.12÷45.19 μg/mL. Compound **1** exhibited weak cytotoxic activity on four cancer cell lines, HepG-2, KB, LU-1, and MCF-7. Meanwhile compound **2** showed weak cytotoxic activities on three cancer cell lines, LU-1, MCF-7, and LNCaP.

Table 2: The effects of compounds **1–3** on the growth of human cancer cells

Compound	IC_{50} (μg/mL)							
	HepG-2	KB	LU-1	MCF-7	LNCaP	SW-480	MKN-7	HL-60
1	63.22	69.03	69.04	74.05	>100	>100	>100	>100
2	>100	>100	79.86	75.66	80.17	98.84	95.38	97.02
3	41.62	41.62	43.40	42.79	45.19	37.12	42.26	39.18
Ellipticine	1.06	0.99	0.87	0.92	0.70	0.84	0.95	0.62

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