Polyoxygenated Sterols from Freshwater Clam

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Two new biologically active polyoxygenated sterols, 3,5,9-trihydroxycholest-7-en-6-one (**1**) and cholest-7-ene-3,6,9-triol (**3**), together with one known sterone, topsentisterol D3 (**2**), were isolated from freshwater clam (*Corbicula fluminea* MULLER; an important cultured edible shellfish in Taiwan). The structure elucidation of sterols **1**–**3** were accomplished by ^{1}H- and ^{13}C-NMR, HMQC, and HMBC, and MS analyses. The sterols **1**–**3** displayed cytotoxicities against the human hepatoma Hep G2 cells (IC_{50}: 6.04 ± 0.07, 40.78 ± 4.28, and 10.57 ± 0.51 μg/ml, resp.).

Introduction. – Freshwater clam (*Corbicula fluminea* MULLER) is an important cultured shellfish in Taiwan. Freshwater clam soup is a popular home remedy for hepato-protection in Taiwan. Recently, its hot-water extract (so-called freshwater clam essences) has been marketed in Taiwan as a nutritional supplement. Some studies have shown that the extracts of *Corbicula fluminea* possess a broad range of biological activities including hepato-protective [1][2], antioxidant [3], anticancer [4][5], antihypertension [6], and hypocholesterolemic effects [7–9]. However, the active constituents of *Corbicula fluminea* have not been studied extensively.

A bioactive glycoprotein, CFp-a, purified from the fresh *C. fluminea*, exhibited antitumor activity by inducing the apoptosis of human hepatoma BEL7404 cells [4]. On the other hand, we found significant growth inhibitory activity of the MeOH extract of *Corbicula fluminea* on human leukemia HL-20 cells [5]. Bioassay-guided isolation based on cytotoxicity against a human hepatoma cell-line was used in the present study to afford two new active sterols and one known sterone.

Results and Discussion. – 1. *Structure Elucidation.* The high-resolution (HR)-ESI-MS analysis of **1** showed a *pseudo*-molecular-ion ($[M+Na]^+$) peak at m/z 455.3135 which suggested the molecular formula, $C_{27}H_{44}O_4$. The ^{13}C-NMR spectrum of **1** (*Table*), together with the information from a DEPT spectrum, displayed 27 signals which were assigned to five Me, ten CH_2, and six CH groups; one olefinic, and one geminal to an O-atom, and to six nonprotonated C-atoms (one CO, one olefinic, and two geminal to an O-atom). The ^{13}C- and DEPT-NMR data were consistent with an unsaturated sterone having three OH groups.

The ^{1}H-NMR spectrum exhibited two Me *singlet*s at δ(H) 0.57 and 0.96, and two Me *doublet*s at δ(H) 0.90 (d, J = 6.6) and 0.85 (d, J = 6.6, 6 H) which suggested **1** has a

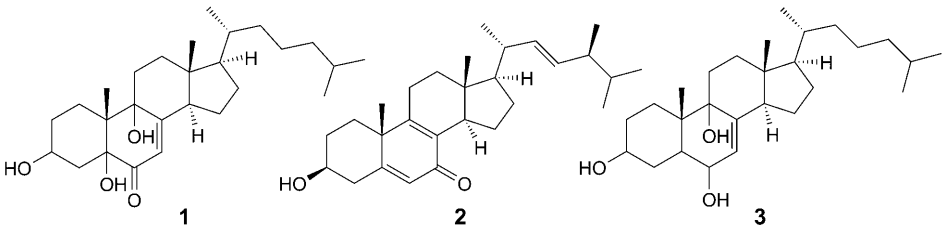

Table. *^{13}C-NMR Chemical Shifts of Sterols* **1** *and* **3**

	1	**3**		**1**	**3**
C(1)	26.7 (CH_2)	31.75 (CH_2)	C(15)	23.5 (CH_2)	24.08 (CH_2)
C(2)	31.0 (CH_2)	33.91 (CH_2)	C(16)	29.3 (CH_2)	28.93 (CH_2)
C(3)	67.8 (CH)	68.39 (CH)	C(17)	57.7 (CH)	57.54 (CH)
C(4)	40.6 (CH_2)	40.49 (CH_2)	C(18)	12.3 (Me)	12.51 (Me)
C(5)	80.9 (C)	44.35 (CH)	C(19)	20.6 (Me)	19.37 (Me)
C(6)	200.2 (C)	74.24 (CH)	C(20)	37.2 (CH)	37.46 (CH)
C(7)	120.1 (CH)	119.05 (CH)	C(21)	19.2 (Me)	18.90 (Me)
C(8)	165.1 (C)	143.81 (C)	C(22)	37.2 (CH_2)	37.25 (CH_2)
C(9)	76.11 (C)	76.95 (C)	C(23)	24.9 (CH_2)	24.94 (CH_2)
C(10)	42.8 (C)	38.13 (C)	C(24)	48.8 (CH_2)	40.68 (CH_2)
C(11)	28.8 (CH_2)	23.03 (CH_2)	C(25)	29.1 (CH)	29.15 (CH)
C(12)	36.3 (CH_2)	40.86 (CH_2)	C(26)	23.2 (Me)	23.17 (Me)
C(13)	46.4 (C)	44.81 (C)	C(27)	23.0 (Me)	22.93 (Me)
C(14)	52.8 (CH)	55.85 (CH)			

cholestane skeleton. In addition, the ^{1}H-NMR spectrum displayed signals for one olefinic H-atom at δ(H) 5.59 and a carbinol H-atom at δ(H) 3.94.

Interpretation of the ^{1}H-detected heteronuclear multiple-bond connectivity (HMBC) spectrum revealed correlations from H–C(7) to C(5), C(9), and C(14); H–C(14) to C(13) and C(18); Me(18) to C(12), C(13), C(14), and C(17); Me(19) to C(1), C(5), C(9) and C(10); H–C(20) to C(13) and C(18); Me(21) to C(17) and C(22); and Me(27) to C(25) and C(26). The HMBC correlation of the Me(19) to C(5) and C(9) (both are nonprotonated C-atoms geminal to an O-atom) indicated that the OH groups are at C(5) and C(9) (*Fig. 1*). The HMBC correlation of H–C(7) (olefinic) with C(9) and C(14) suggested that the structure of compound **1** was a cholesterol skeleton with a C(7)=C(8) bond. The structure of **1** was confirmed as 3,5,9-trihydroxycholest-7-en-6-one based on comparison of the NMR data with those published for 3,5,9-trihydroxyergost-7-en-6-one (isolated from fungi [10]) and cholesterol [11] (*Table*).

The HR-ESI-MS of **2** showed a *pseudo*-molecular-ion ($[M+Na]^+$) peak at *m/z* 433.3102 suggesting the molecular formula, $C_{28}H_{42}O_2$. The ^{13}C-NMR spectrum of **2**, together with the information from a DEPT spectrum, showed 28 signals which were ascribed to six Me, seven CH_2, and nine CH groups (three olefinic, and one geminal to an O-atom), and six nonprotonated C-atoms (one CO, three olefinic). The ^{13}C-NMR spectrum of **2** was consistent with the data published for topsentisterol D3 [12][13].

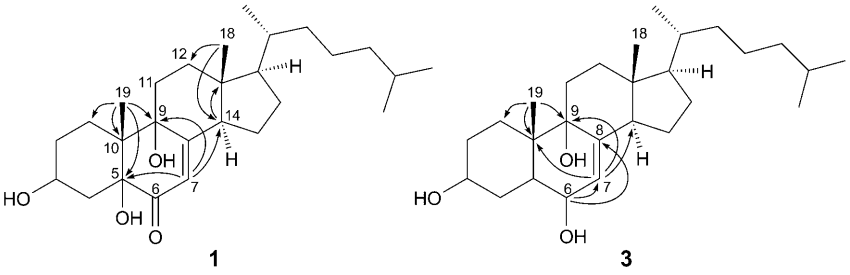

Fig. 1. *HMBCs for sterols* **1** *and* **3**

The HR-ESI-MS of **3** showed a *pseudo*-molecular-ion ($[M+Na]^+$) peak at *m/z* 441.3147 suggesting the molecular formula, $C_{27}H_{46}O_3$. The ^{13}C-NMR spectrum of **3** (*Table*), together with the information from a DEPT spectrum, showed 27 signals which were attributed to five Me, ten CH_2, and eight CH groups (one olefinic, and two geminal to an O-atom), and four nonprotonated C-atoms (one olefinic and one geminal to an O-atom). The ^{13}C- and DEPT-NMR data were consistent with the structure of polyhydroxycholesterol having three OH groups.

The ^{1}H-NMR spectrum contained two Me *singlet*s at δ(H) 0.62 and 1.05, three Me *doublet*s at δ(H) 0.87 ($d, J = 6.6$), 0.88 ($d, J = 6.6$), and 0.95 ($d, J = 6.4$), suggesting that **3** has a cholestane skeleton. In addition, the ^{1}H-NMR spectrum displayed signals for one olefinic H-atom at δ(H) 5.5, and two carbinol H-atoms at δ(H) 3.97 and 3.26.

The HMBC correlation of the Me(19) H-atoms to a nonprotonated C-atom geminal to an O-atom suggested that the OH group was at C(9). The HMBC correlations of H–C(6) (a C-atom geminal to an O-atom) with C(7) and C(8) (both olefinic C-atoms) suggested the presence of an allylic alcohol moiety (*Fig. 1*). The HMBC correlations of the H–C(7) (olefinic C-atoms) to C(8), C(9), and C(14) suggested the OH groups are at C(6) and at C(9). The structure of compound **3** was established as cholest-7-ene-3,6,9-triol based on comparison of the NMR data compared with those published for 3,5,9-trihydroxyergost-7-en-6-one (isolated from fungi [10]) and cholest-7-ene-3β,5,6β,9-tetraol [14] (*Table*).

2. *Cytotoxicity.* The sterols **1**–**3** described in the present study displayed cytotoxicities against the Hep G2 hepatoma cell line (IC_{50}: 6.04 ± 0.07, 40.78 ± 4.28, and 10.57 ± 0.51 μg/ml, resp.). Although sterol **2** has been isolated from different sources such as the sponge *B. fortis*, *Clathrina clathrus*, *Topsentia* sp., and the marine-derived fungus *Rhizopus* sp., this is the first report to show that it also occurred in freshwater calm. The sterol **2** has been reported to possess cytotoxic activity against P388, A549, HL-60, and BEL-7420 cell lines with IC_{50} values of 3.0, 4.2, 23.8, and 34.2 μM, respectively [13]. However, the cytotoxicity of sterol **2** on the Hep G2 hepatoma cell lines was moderate (IC_{50}: 40.78 ± 4.28 μg/ml).

Experimental Part

General. All org. solvents (HPLC grade), silica gel *60* (SiO_2; 230–400 mesh), and TLC plates (*RP-18 F254* and silica *60 F254*) were obtained from *Merck Co.* (D-Darmstadt). MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) was purchased from *Sigma Chemical Co.*

(St. Louis, MO). *Dulbecco*'s modified *Eagle*'s medium, trypsin-EDTA, and fetal bovine serum were purchased from *GibCo* (CA, USA). ^{1}H-, ^{13}C- and 2D-^{1}H,^{13}C-HSQC, ^{1}H,^{13}C-HMBC NMR experiments: at 500 and 125 MHz for ^{1}H and ^{13}C, resp., on a *Bruker Avance 500* instrument (CH-Fällanden); the ^{13}C and the ^{1}H signals of the solvent ((D$_6$)acetone, CDCl$_3$) were used as references. MS: *Finnigan MAT-95S* spectrometer.

Material. Corbicula fluminea MULLER was provided by *LiChuan Aquariculture Farm* (Hualiang, Taiwan).

Extraction and Isolation (see *Fig. 2*). The whole freshwater clam was briefly boiled to facilitate opening the shells. The residual meat (18 kg) was homogenized with deionized H_2O and then freeze-dried to powder form. After lyophilization, 4.8 kg of powder was obtained (26.7%). The clam powder (500 g) was extracted with abs. EtOH (2 l) at r.t. overnight. After filtration, the extraction was repeated. The org. phases were combined, and the solvent was removed under reduced pressure. The EtOH extract (FE = freshwater clam extracted with EtOH) was obtained as a dark-brown syrup (66.17 g, 13.2%).

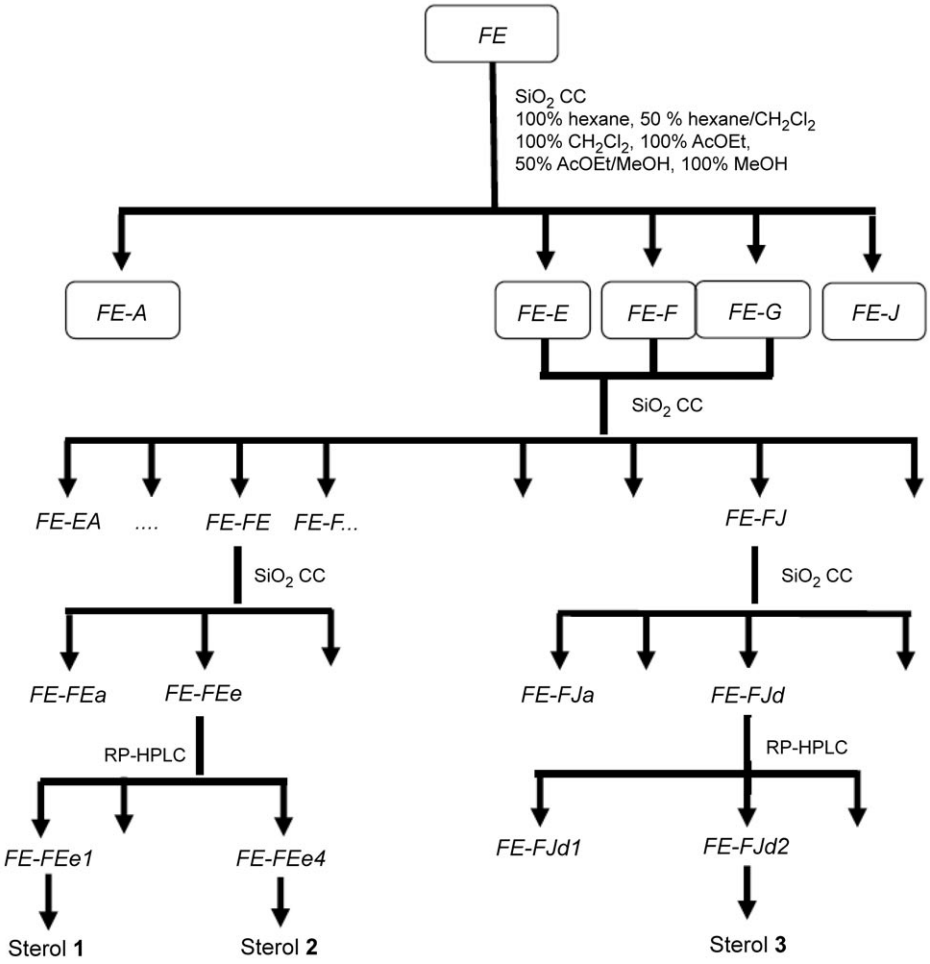

Fig. 2. *Purification scheme of sterols* **1**, **2**, *and* **3**

The FE (66.17 g) was chromatographed on a SiO_2 column with a stepwise gradient of hexane, hexane/CH_2Cl_2 1:1 (*v*/*v*), CH_2Cl_2, AcOEt, AcOEt/MeOH 1:1, and MeOH as eluents to yield fractions *FE-A* (34.03 g), *FE-B* (0.21 g), *FE-C* (11.56 g), *FE-D* (6.78 g), *FE-E* (3.50 g), *FE-F* (0.31 g), *FE-G* (0.81 g), and *FE-H* (8.96 g).

The combined active fractions *FE-E*, *FE-F*, and *FE-G* (4.61 g; IC_{50} values for Hep G2: 38, >50, and 50 μg/ml, resp.) were chromatographed on a SiO_2 column successively eluted with a stepwise gradient of hexane/AcOEt (9:1 → 1:9) and finally 100% MeOH. The fifth fraction, *FE-FE* (IC_{50} for Hep G2: 11 μg/ml) was chromatographed on SiO_2 with a stepwise gradient of hexane/AcOEt (9:1 → 1:9) and 100% MeOH. The active fraction, *FE-FEe* (IC_{50} for Hep G2: 34 μg/ml) was purified by RP-HPLC (*C18 Vercopack ODS-3* column, 10 mm × 250 mm, flow rate: 3.5 ml/min) with MeCN/H_2O 95:5 as eluent. The active fraction, *FE-FEe-2* (IC_{50} for Hep G2: 9 μg/ml) was purified by HPLC (*Vercopack Inersil 5μ Phenyl*, 10 mm × 250 mm, eluent: MeCN/H_2O 50:50, flow rate: 5.0 ml/min) to yield sterol **1** as a white amorphous powder (m.p. 203–205°).

The active fraction *FE-FEe-4* (IC_{50} for HepG2: 11.5 μg/ml) was purified (*Vercopack Inersil 5μ Phenyl*, 10 mm × 250 mm, eluent: MeCN/H_2O 55:45, flow rate: 5.0 ml/min) to yield sterol **2**.

The active fraction *FE-FJ* (IC_{50} for Hep G2: 50 μg/ml) was chromatographed on a SiO_2 column eluted with a stepwise gradient (CH_2Cl_2/MeOH 98:2 → CH_2Cl_2/MeOH 80:2). The fraction *FE-FJd* was further purified by RP-HPLC (*C18 Vercopack ODS-3* column, 10 mm × 250 mm, flow rate: 3.5 ml/min) with MeOH/H_2O 90:10 as eluent. The active fraction *FE-FJd-2* (IC_{50} for Hep G2: 24 μg/ml) was further purified by RP-HPLC (*Vercopack Inersil 5μ Phenyl*, 10 mm × 250 mm, eluent: MeCN/H_2O 50:50, flow rate: 5.0 ml/min) to yield sterol **3** as white amorphous powder (m.p. 184–185°).

Cell-Survival Assay The human hepatoma cell line (Hep G2) obtained from *Bioresource Collection and Research Center* (BCRC, Hsinchu) was used. The cells were grown in *Eagle*'s minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS) in a humidified incubator (37°, 5% CO_2). Cell viability was assayed with MTT. Hepatoma cells were plated at a density of 1×10^5 cells/ml into 24-well plates. After overnight growth, cells were treated with different concentrations of samples dissolved in DMSO for 48 h. At the end of treatment, MTT was added, and cells were incubated for a further 4 h. Cell viability was determined by scanning with an ELISA reader using a 570-nm filter.

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