

Steroids from the Cold Water Starfish *Ctenodiscus crispatus* with Cytotoxic and Apoptotic Effects on Human Hepatocellular Carcinoma and Glioblastoma Cells

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Chemical investigation on the methanol extract of the starfish *Ctenodiscus crispatus* resulted in the isolation of five steroids, (22*E*,24*ξ*)-26,27-bisnor-24-methyl-5 α -cholest-22-en-3 β ,5,6 β ,15 α ,25-pentol 25-*O*-sulfate (**1**), (22*E*,24*R*,25*R*)-24-methyl-5 α -cholest-22-en-3 β ,5,6 β ,15 α ,25,26-hexol 26-*O*-sulfate (**2**), (28*R*)-24-ethyl-5 α -cholesta-3 β ,5,6 β ,8,15 α ,28,29-heptaol-24-sulfate (**3**), (25*S*)-5 α -cholestane-3 β ,5,6 β ,15 α ,16 β ,26-hexaol (**4**), and Δ^7 -sitosterol (**5**). Their structures were identified by extensive spectroscopic analyses, including 1D, 2D NMR and MS and chemical methods. Compound **4** showed cytotoxicity against human hepatoma HepG2 and glioblastoma U87MG cells *via* inhibition of cell growth and induction of apoptosis. Induction of apoptosis by **4** was demonstrated by cell death, DNA fragmentation, increased Bax/Bcl-2 protein ratio and the activation of caspase-3, caspase-9 and poly (ADP-ribose) polymerase (PARP).

Key Words : Starfish, Steroids, *Ctenodiscus crispatus*, Anti-proliferation, Apoptosis

Introduction

Marine natural products are considered as a rich source of bioactive substances with high structural diversity. Since the first researches on marine natural product reported in 1960s, over 20,000 natural products have been discovered from marine organisms,¹ and a remarkable number of marine natural products are currently in different phases of clinical trials.^{2,3} Among marine organisms, the research on starfish has expanded rapidly over the past few years, which has been prompted by the discovery of a variety of unique structures and the interest in their pharmacological properties.⁴ Various secondary metabolites including steroids, steroidal glycosides, anthraquinones, alkaloids, phospholipids, peptides, and fatty acids were reported from starfish, in which polyhydroxysteroids are known as the predominant secondary metabolites.⁴ In this study, the starfish *Ctenodiscus crispatus* (Ctenodiscidae family) was collected at the Sea of Okhotsk. Previous investigations on chemical composition of *C. crispatus* have shown the presence of some polyhydroxylated steroids.^{5,6} As a part of our on-going search for bioactive secondary metabolites from marine organisms, this study describes the isolation and identification of five steroids (**1-5**) from the cold water starfish *C. crispatus*.

In this study, cytotoxic effects of five isolated steroids (**1-5**) were evaluated toward two human carcinoma cell lines, human hepatocellular carcinoma (HepG2) and human glioblastoma (U87MG). HepG2 is one of the most frequent malignancies worldwide and is associated with a high rate of

metastasis,⁷ and U87MG (or grade IV astrocytoma) is the most common primary malignant brain tumor that only weakly responsive to the current therapeutic strategies.^{8,9} Based on the result of this primary screening step, compound **4** was selected for further studies on molecular action mechanism of induction of apoptosis in HepG2 and U87MG cells.

Experimental

Extraction and Isolation. The starfish *C. crispatus* (09C-ST3-DR7), identified by Dr. Joung Han Yim, was collected at the Sea of Okhotsk (N 53°22.626'E 144°24.200') on January 28, 2009, and was stored in a freezer until being extracted. A voucher specimen is preserved in KOPRI. The frozen sample (122.0 g) was extracted with MeOH in room temperature. The MeOH extract was concentrated *in vacuo* to provide a residue (DR7, 2441.8 mg). The residue (DR7) was chromatographed over reversed phase (RP) YMC C₁₈ silica gel column, stepwise eluting with MeOH in H₂O (20%; 40%; 60%; 80%; and 100% v/v) to give 6 fractions, DR71-6. Fraction DR74 (162.9 mg) was subjected to C₁₈ silica gel column chromatography (CC), using MeOH-H₂O (13:10) to provide three subfractions DR741-3. Subfraction DR742 was then subjected to silica gel CC, eluted with EtOAc-MeOH-H₂O (35:10:1) to provide compound **3** (5.0 mg) and three subfractions, DR7421, DR7423, and DR7424. Subfraction DR7424 was further purified by silica gel CC, using CHCl₃-MeOH-H₂O (20:10:1) as eluents to give compounds **1** (2.0 mg) and **2** (5.0 mg). Fraction DR75 (514.4 mg) was sub-

jected to RP C₁₈ silica gel CC, eluting with MeOH-H₂O (4:1) to yield 2 subfractions, DR751 and DR752. Subfraction DR752 was subjected to RP C₁₈ CC, eluting with MeOH-H₂O (4:1) and further purified by silica gel CC, using CH₂Cl₂-MeOH-H₂O (40:10:1) as eluents to obtain compound **4** (5.0 mg). Fraction DR76 (203.4 mg) was separated by silica gel CC, eluting with n-hexane-EtOAc (4:1) and CH₂Cl₂-MeOH (7:1) to give 6 subfractions, DR761-6. From subfraction DR762, compound **5** (4.0 mg) was isolated by Sephadex LH-20 CC, using CH₂Cl₂-MeOH (10:1) as eluents.

(22E,24ξ)-26,27-Bisnor-24-methyl-5α-cholest-22-en-3β,5,6β,15α,25-pentol 25-O-sulfate (1): White, amorphous powder; $[\alpha]_D^{25} +4.8^\circ$ (*c* 0.1, MeOH). HR-ESITOFMS m/z 515.2679 [M]⁻ (calcd. for C₂₆H₄₃O₈S, 515.2679). ¹H NMR (400 MHz, CD₃OD) and ¹³C NMR (100 MHz, CD₃OD): see Table 1.

(22E,24R,25R)-24-Methyl-5α-cholest-22-en-3β,5,6β,15α,25,26-hexol 26-O-sulfate (2): White, amorphous powder; $[\alpha]_D^{25} +10.5^\circ$ (*c* 0.1, MeOH). HR-ESITOFMS m/z 559.2926 [M]⁻ (calcd. for C₂₈H₄₇O₉S, 559.2941). ¹H NMR (400 MHz, CD₃OD) δ 4.00 (m, H-3), 3.45 (br s, H-6), 1.12 (d, *J* = 10.4 Hz, H-14), 3.93 (m, H-15), 1.44 (m, H-17), 0.74 (s, H₃-18), 1.16 (s, H₃-19), 1.01 (d, *J* = 6.4 Hz, H₃-21), 5.29 (m, H-22), 5.42 (dd, *J* = 8.4, 15.6 Hz, H-23), 2.27 (m, H-24), 3.84 (d, *J* = 10.0 Hz, H-26a), 3.94 (dd, *J* = 10.0 Hz, H-26b), 1.14 (s, H₃-27), 0.99 (d, *J* = 6.4 Hz, H₃-28). ¹³C NMR (100 MHz, CD₃OD) δ 31.6 (C-1), 33.5 (C-2), 68.3 (C-3), 41.4 (C-4), 76.6 (C-5), 76.4 (C-6), 35.2 (C-7), 31.2 (C-8), 46.6 (C-9), 39.4 (C-10), 22.1 (C-11), 41.5 (C-12), 44.8 (C-13), 63.6 (C-14), 74.2 (C-15), 42.3 (C-16), 54.9 (C-17), 14.0 (C-18), 17.4 (C-19), 41.1 (C-20), 21.1 (C-21), 138.8 (C-22), 130.4 (C-23), 45.3 (C-24), 74.3 (C-25), 74.6 (C-26), 22.3 (C-27), 15.4 (C-28).

(28R)-24-Ethyl-5α-cholesta-3β,5,6β,8,15α,28,29-heptaol-24-sulfate (3): White, amorphous powder; $[\alpha]_D^{25} +32.0^\circ$ (*c* 0.5, MeOH). HR-ESITOFMS m/z 591.3207 [M]⁻ (calcd. for C₂₉H₅₁O₁₀S, 591.3203). ¹H NMR (400 MHz, CD₃OD) and ¹³C NMR (100 MHz, CD₃OD): see Table 1. ¹H NMR (400 MHz, pyridine-*d*₅) δ 4.88 (m, H-3), 4.29 (br s, H-6), 1.71 (H-14), 4.87 (m, H-15), 1.49 (m, H-17), 1.28 (s, H₃-18), 1.82 (s, H₃-19), 1.65 (m, H-20), 0.93 (d, *J* = 6.8 Hz, H₃-21), 1.65 (m, H-22), 1.35 (m, H-23), 1.61 (m, H-24), 1.96 (m, H-25), 1.02 (d, *J* = 6.4 Hz, H₃-26), 0.92 (d, *J* = 6.4 Hz, H₃-27), 4.45 (m, H-28), 4.75 (dd, *J* = 8.4, 10.4 Hz, H-29a), 4.58 (dd, *J* = 3.6, 10.4 Hz, H-29b). ¹³C NMR (100 MHz, pyridine-*d*₅) δ 31.8 (C-1), 34.4 (C-2), 67.3 (C-3), 42.5 (C-4), 75.9 (C-5), 78.0 (C-6), 42.0 (C-7), 76.7 (C-8), 48.8 (C-9), 39.1 (C-10), 19.5 (C-11), 42.5 (C-12), 44.9 (C-13), 66.4 (C-14), 69.3 (C-15), 40.9 (C-16), 55.0 (C-17), 15.6 (C-18), 18.2 (C-19), 35.9 (C-20), 19.0 (C-21), 35.9 (C-22), 23.3 (C-23), 47.5 (C-24), 29.4 (C-25), 20.7 (C-26), 20.6 (C-27), 71.3 (C-28), 72.5 (C-29).

(25S)-5α-Cholestane-3β,5,6β,15α,16β,26-hexaol (4): Colorless gum; $[\alpha]_D^{25} +10.1^\circ$ (*c* 0.9, MeOH). HR-ESITOFMS m/z 513.3426 [M + COOH]⁻ (calcd. for C₂₈H₄₉O₈, 513.3427). ESIMS m/z 433 [M + H - 2H₂O]⁺. ¹H NMR (400 MHz,

CD₃OD) δ 3.33 (m, H-3), 3.47 (br s, H-6), 1.26 (m, H-14), 3.74 (dd, *J* = 2.0, 10.0 Hz, H-15), 3.97 (dd, *J* = 2.0, 8.0 Hz, H-16), 0.99 (m, H-17), 0.90 (s, H₃-18), 1.17 (s, H₃-19), 0.96 (d, *J* = 6.8 Hz, H₃-21), 1.57 (H-25), 4.02 (dd, *J* = 6.4, 10.8 Hz, H-26a), 3.43 (dd, *J* = 6.4, 10.8 Hz, H-26b), 0.91 (d, *J* = 6.8 Hz, H₃-27). ¹³C NMR (100 MHz, CD₃OD) δ 31.6 (C-1), 33.5 (C-2), 68.3 (C-3), 41.4 (C-4), 76.5 (C-5), 76.3 (C-6), 35.1 (C-7), 31.1 (C-8), 46.5 (C-9), 39.3 (C-10), 21.9 (C-11), 41.9 (C-12), 44.7 (C-13), 60.9 (C-14), 85.0 (C-15), 82.9 (C-16), 59.9 (C-17), 15.0 (C-18), 17.4 (C-19), 30.9 (C-20), 18.6 (C-21), 37.4 (C-22), 24.8 (C-23), 34.9 (C-24), 36.9 (C-25), 68.4 (C-26), 17.2 (C-27).

Δ7-Sitosterol (5): White, amorphous powder; $[\alpha]_D^{25} +5.5^\circ$ (*c* 0.7, CHCl₃). ESIMS m/z 415 [M + H]⁺. ¹H NMR (400 MHz, CDCl₃) δ 3.50 (m, H-3), 5.09 (br s, H-7), 0.47 (s, H₃-18), 0.72 (s, H₃-19), 0.86 (d, *J* = 6.0 Hz, H₃-21), 0.81 (d, *J* = 6.4 Hz, H₃-26), 0.80 (d, *J* = 6.4 Hz, H₃-27), 0.86 (d, *J* = 6.8 Hz, H₃-29). ¹³C NMR (100 MHz, CDCl₃) δ 37.1 (C-1), 31.1 (C-2), 70.7 (C-3), 37.6 (C-4), 40.2 (C-5), 29.6 (C-6), 117.4 (C-7), 139.6 (C-8), 49.5 (C-9), 34.2 (C-10), 21.5 (C-11), 39.5 (C-12), 43.4 (C-13), 55.0 (C-14), 23.0 (C-15), 27.9 (C-16), 56.1 (C-17), 11.8 (C-18), 13.0 (C-19), 36.2 (C-20), 18.8 (C-21), 33.9 (C-22), 26.2 (C-23), 45.8 (C-24), 29.1 (C-25), 19.8 (C-26), 19.0 (C-27), 22.9 (C-28), 11.9 (C-29).

Solvolysis of Compound 1. Compound **1** (3.0 mg) was dissolved in a 2 mL of dioxane-pyridine (1:1 v/v). The solution was stirred and heated at 100 °C for 5 h. The reaction mixture was concentrated *in vacuo*, and the dry residue was chromatographed over preparative TLC using CH₂Cl₂-MeOH (4:1) to give **1a** (1.0 mg).

(22E,24ξ)-24-Methyl-26,27-bisnor-5α-cholest-22-en-3β,5,6β,15α,25-pentol (1a): white, amorphous powder, $[\alpha]_D^{25} +12.6^\circ$ (*c* 0.06, MeOH). HR-ESITOFMS m/z 481.3181 [M + COOH]⁻ (calcd. for C₂₇H₄₅O₇, 481.3165). ¹H NMR (400 MHz, CD₃OD) and ¹³C NMR (100 MHz, CD₃OD): see Table 1.

Preparation of the (S)- and (R)-MTPA Esters. Compound **3** (2.0 mg) was treated with (*R*)-(-)- or (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (10 μL) in dry pyridine (400 μL) for 24 h at room temperature. The reaction was monitored by silica gel TLC and stopped when the original spot had disappeared. The reaction product was purified by preparative silica gel TLC using CH₂Cl₂-MeOH (5:1) as eluent to give (*S*)- (**3a**) and (*R*)-MTPA esters (**3b**), respectively. The ¹H NMR spectra of the esters were recorded in pyridine-*d*₅ and the assignments were done by ¹H-¹H COSY spectra. Selected ¹H NMR (pyridine-*d*₅, 400 MHz) of (**3a**): 6.18 (H-3), 6.13 (H-28), 5.94 (H-15), 4.76 (H-29), 4.26 (H-6), 3.71 (OCH₃), 3.64 (OCH₃), 3.59 (OCH₃), 1.87 (H-25), 1.67 (H-24), 1.42 (H-20), 1.36 (H-23), 0.96 (d, *J* = 6.8 Hz, H₃-21 and H₃-26), 0.92 (d, *J* = 6.8 Hz, H₃-27). Selected ¹H NMR (pyridine-*d*₅, 400 MHz) of (**3b**): 6.13 (H-3), 5.99 (H-28), 5.86 (H-15), 4.78 (H-29), 3.91 (H-6), 3.89 (OCH₃), 3.75 (OCH₃), 3.65 (OCH₃), 1.72 (H-25), 1.58 (H-24), 1.27 (H-20), 1.27 (H-23), 0.90 (d, 6.8 Hz, H₃-21), 0.85 (d, *J* = 6.8 Hz, H₃-26), 0.81 (d, *J* = 6.8 Hz, H₃-27).

Results and Discussion

Compound **1** was isolated as white amorphous powder. The ^1H and ^{13}C NMR spectra contained signals characteristic of a common $3\beta,5\alpha,6\beta,15\alpha$ -tetrahydroxy-substituted cholestane nucleus (Table 1). The ^1H and ^{13}C spectra further showed the presence of one oxymethylene group (C-25) and one double bond at C-22 of the side chain of **1**. The downfield chemical shift of the oxymethylene group (δ 73.5) suggested location of a sulfate group at C-25. By analyzing the HMQC, HMBC, and ^1H - ^1H COSY spectra, the positions of the functionalities and side chain of **1** were fully assigned. The molecular formula of **1** was established as $\text{C}_{26}\text{H}_{43}\text{O}_8\text{S}^-$ based on the NMR data along with a molecular anion peak at m/z 515.2679 [515.2679 required, Δ 0.0 mmu] in the HR-ESITOFMS (negative ion mode). Based on these analyses, in combination with the comparison of the NMR data of **1** with those of the reported steroid,⁶ the structure of **1** was established as (22*E*,24*\xi*)-26,27-bisnor-24-methyl-5 α -cholest-

22-en-3 β ,5,6 β ,15 α ,25-pentol 25-*O*-sulfate (Figure 1).

Upon solvolysis in a dioxane-pyridine mixture followed

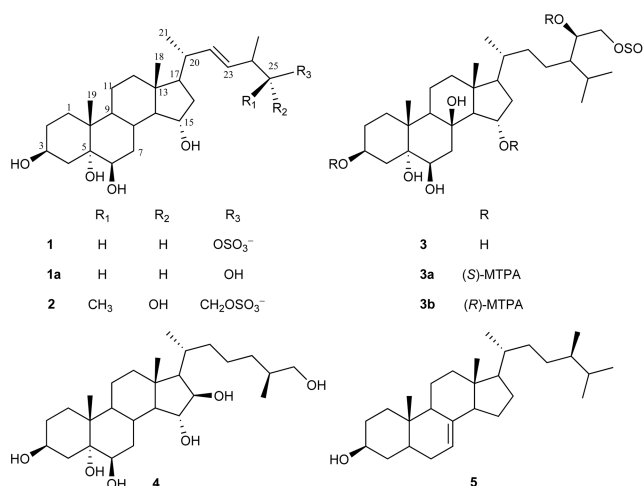


Figure 1. Structures of compounds **1-5**, **1a**, **3a**, and **3b**.

Table 1. ^1H and ^{13}C NMR data for compounds **1**, **1a**, and **3**

Position	1		1a		3	
	$\delta_{\text{H}}^{a,b}$	$\delta_{\text{C}}^{a,c}$	$\delta_{\text{H}}^{a,b}$	$\delta_{\text{C}}^{a,c}$	$\delta_{\text{H}}^{a,b}$	$\delta_{\text{C}}^{a,c}$
1		31.6		31.6		30.9
2		33.5		33.5		34.4
3	4.00 (m)	68.3	3.98 (m)	68.3	4.07 (m)	68.2
4		41.4		41.4		41.7
5		76.6		76.6		76.4
6	3.45 (br s)	76.4	3.44 (br s)	76.4	3.57 (br s)	77.9
7		35.2		35.2		40.4
8		31.2		31.2		77.2
9		46.6		46.6		49.0
10		39.3		39.3		39.1
11		22.1		22.1		19.7
12		41.5		41.5		42.9
13		44.8		44.8		45.5
14	1.12 (m)	63.7	1.10 (m)	63.8	1.25 (d, 9.6)	66.4
15	3.93 (m)	74.1	3.81 (ddd, 3.2, 9.2, 9.6)	74.1	4.25 (dt, 2.8, 9.6)	70.0
16		41.9		41.9		41.1
17	1.44 (m)	54.7	1.44 (m)	54.8	1.36 (m)	55.6
18	0.74 (s)	14.0	0.72 (s)	14.0	0.94 (s)	15.3
19	1.16 (s)	17.4	1.14 (s)	17.2	1.29 (s)	18.0
20		40.9		40.9	1.35 (m)	36.7
21	1.01 (d, 6.4)	21.0	0.99 (d, 6.4)	21.1	0.93 (d, 6.8)	18.9
22	5.35 (dd, 8.0, 15.6)	138.2	5.31 (dd, 8.0, 15.2)	137.7	1.42 (m)	36.7
23	5.29 (dd, 6.4, 15.6)	130.5	5.29 (dd, 6.4, 15.2)	131.5	1.07 (m)	23.6
24		37.7		40.4	1.41 (m)	47.8
25	3.73 (dd, 8.0, 9.2)	73.5	3.27 (m)	68.4	1.18 (m)	30.0
26	3.88 (dd, 6.0, 9.2)		3.40 (dd, 6.0, 10.4)		1.81 (m)	
27	1.02 (d, 6.8)	17.4	0.95 (d, 6.8)	17.3	0.95 (d, 7.2) ^d	20.6
28					0.93 (d, 7.2) ^d	20.6
29					3.95 (br s)	71.5
30					3.95 (br s)	72.2

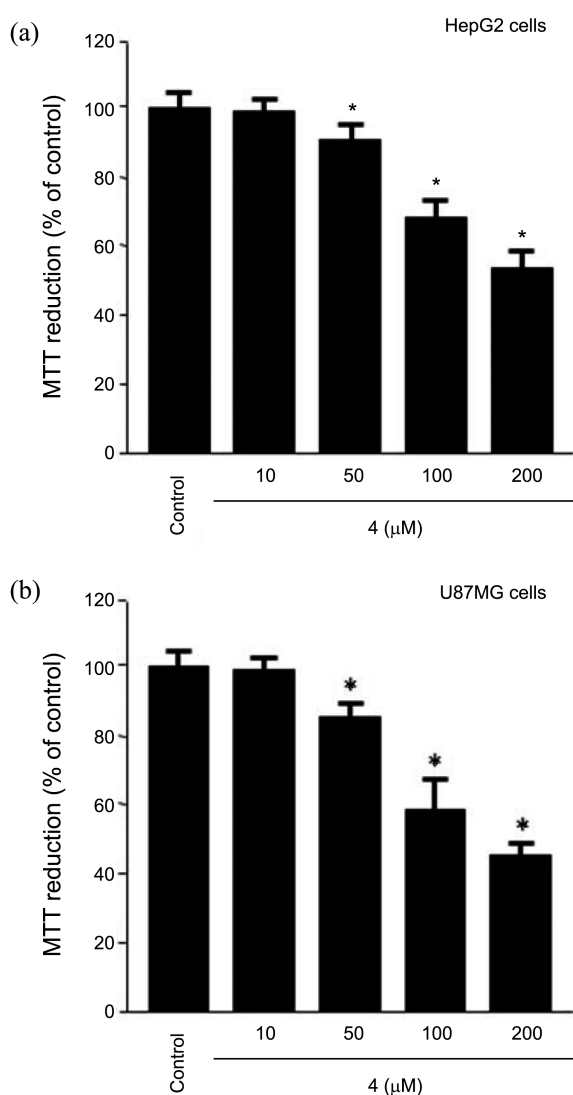
^aSpectra were recorded in CD₃OD. ^b400 MHz. ^c100 MHz. ^dSignals are interchangeable.

Table 2. ^1H NMR chemical shift differences ($\Delta\delta$) for the MTPA esters of **3**

Position	$\Delta\delta^a$
20	+0.147
21	+0.062
23	+0.091
24	+0.086
25	+0.153
26	+0.119
27	+0.115
29	-0.017

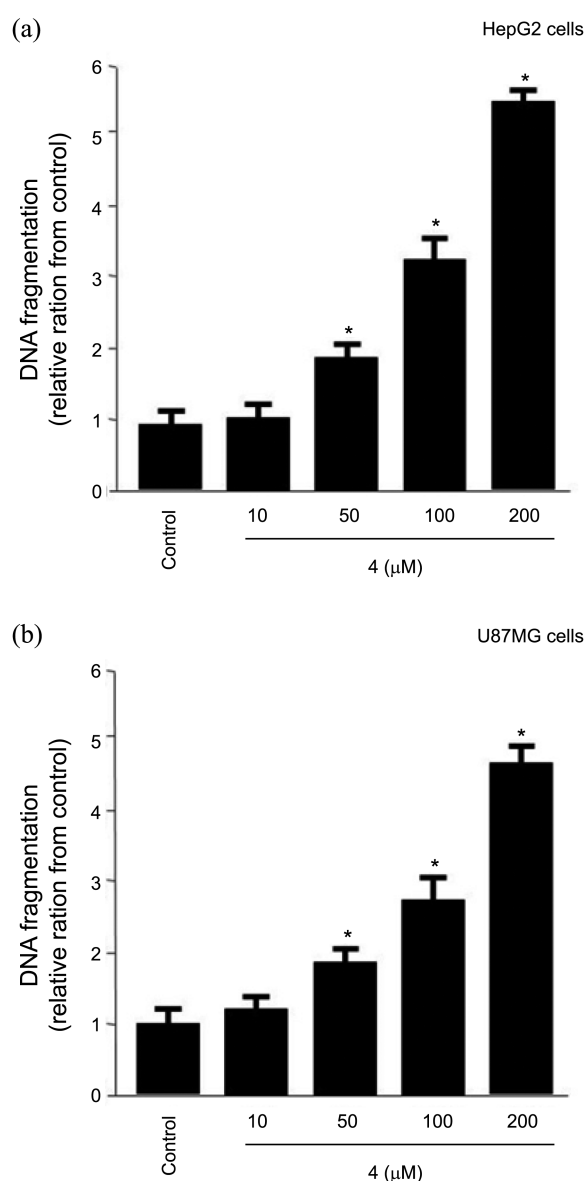
$$^a\Delta\delta \text{ (ppm)} = \delta_S - \delta_R$$

by preparative TLC, compound **1** afforded a desulfated derivative **1a**. The ^1H and ^{13}C NMR spectra of **1a** were identical to those of **1**, except for the upfield shifted chemical

**Figure 2.** Effects of compound **4** on cell viability. HepG2 and U87MG cells were incubated for 24 h with various concentrations of compound **4** (10–200 μM). Cell viability was determined by MTT assay. Bar represents the mean \pm S.D. of three independent experiments. *P < 0.05 vs. control.

shifts of the oxymethylene (δ 3.27 and 3.40/68.4, C-25), showing that the sulfate group at C-25 has been removed (Table 1). Furthermore, the molecular formula of **1a** was established as $\text{C}_{26}\text{H}_{44}\text{O}_5$ by a pseudomolecular ion m/z 481.3181 [$\text{M} + \text{COOH}$] $^-$ (481.3165 required, Δ -1.6 mmu) in the HR-ESITOFMS. Thus, the structure of **1a** was identified as (22*E*,24*ξ*)-24-methyl-26,27-bisnor-5*α*-cholest-22-en-3*β*,5,6*β*,15*α*,25-pentol, representing a new steroidal derivative (Figure 1).

Compound **3** was isolated as white, amorphous powder. The molecular formula of **3** was identified as $\text{C}_{29}\text{H}_{51}\text{O}_{10}\text{S}^-$ by analysis of 1D and 2D NMR data and a molecular anion m/z 591.3207 [591.3203 required, Δ -0.4 mmu] in the negative ion mode of HR-ESITOFMS. Comparative analysis indicated

**Figure 3.** Effects of compound **4** on DNA fragmentation in HepG2 and U87MG cells. Cells were incubated for 24 h with various concentrations of compound **4** (10–200 μM). DNA fragmentation was evaluated using the Cellular DNA fragmentation ELISA kit. Bar represents the mean \pm S.D. of three independent experiments. *P < 0.05 vs. control.

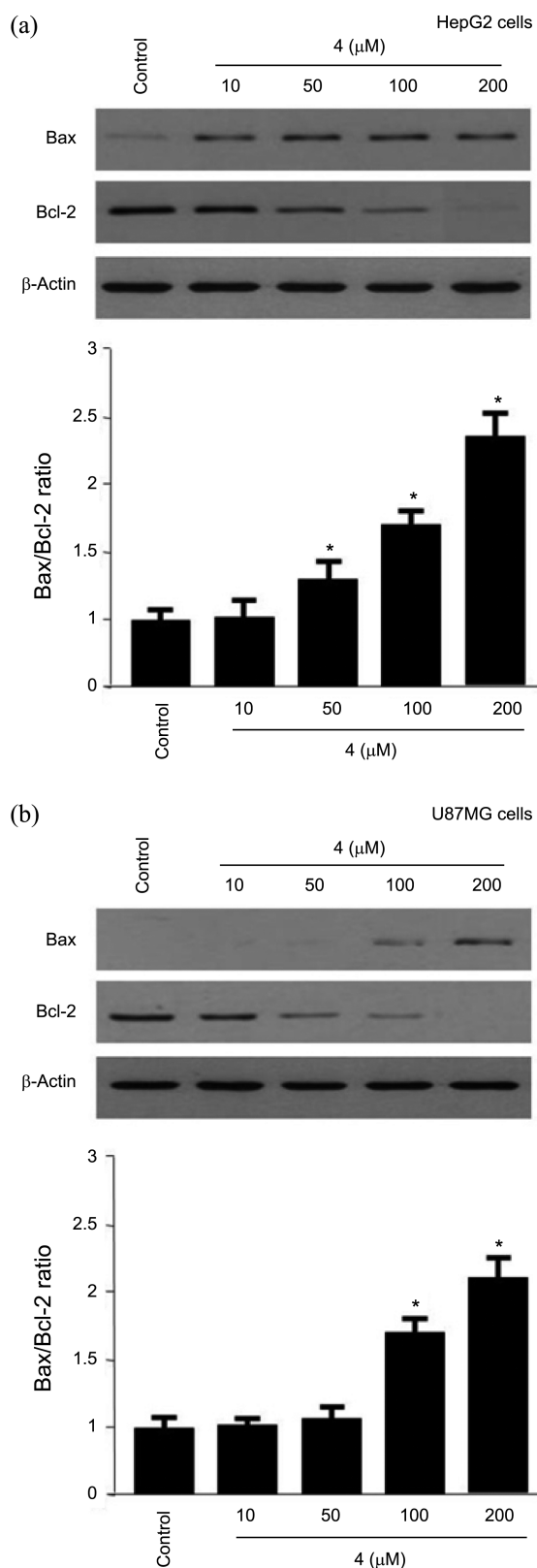


Figure 4. Effects of compound **4** on the expression of Bcl-2 family proteins in HepG2 and U87MG cells. Cells were incubated for 24 h with various concentrations of compound **4** (10–200 μM). Western blot analyses for proteins expression were performed as described in Experimental. Representative blots of three independent experiments are shown. Relative changes of Bax/Bcl-2 ratio were quantitated using an image program. * $P < 0.05$ vs. control.

that the NMR data of **3** was identical with those of the known steroid, 24-ethyl-5 α -cholesta-3 β ,5,6 β ,8,15 α ,28,29-heptaol-24-sulfate.⁵ Since the absolute configuration at C-28 has not been determined,⁵ the Mosher's method has been applied to determine the stereochemistry at this position. The (*S*)- and (*R*)-MTPA esters of **3** (**3a** and **3b**, respectively) were prepared from the corresponding (*R*)- and (*S*)-MTPA chlorides, respectively. Analysis of ¹H NMR and COSY spectra allowed the assignment of the proton chemical shifts for the two diastereomeric esters **3a** and **3b** in proximity of the esterified carbon (C-28). Calculation of $\Delta\delta$ values ($\delta_S - \delta_R$) for protons neighboring C-28 led to the assignment of

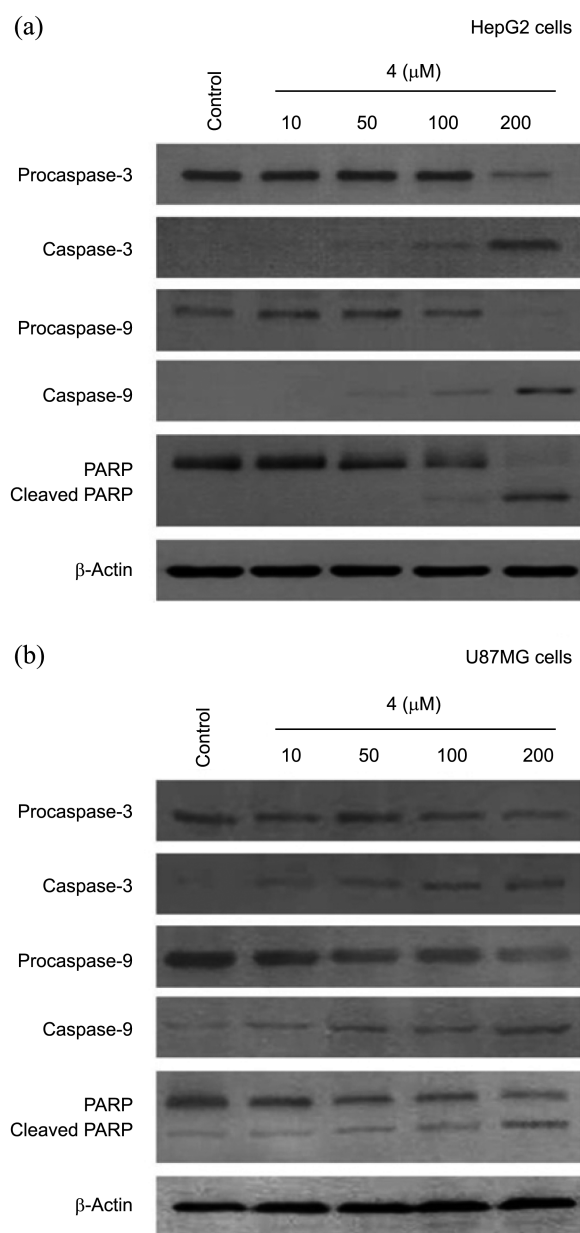


Figure 5. Effects of compound **4** on caspase-3, caspase-9, and PARP activation in HepG2 and U87MG cells. Cells were incubated for 24 h with various concentrations of compound **4** (10–200 μM). Western blot analyses for proteins expression were performed as described in Experimental. Representative blots of three independent experiments are shown.

the 28R configuration (Table 2). Finally, the structure of **3** was established as (28R)-24-ethyl-5 α -cholesta-3 β ,5,6 β ,8,15,28,29-heptaol-24-sulfate (Figure 1).

Three remaining sterols were determined to be (22E,24R,25R)-24-methyl-5 α -cholest-22-en-3 β ,5,6 β ,15 α ,25,26-hexol 26-O-sulfate (**2**),⁶ (25S)-5 α -cholestane-3 β ,5,6 β ,15 α ,16 β ,26-hexaol (**4**),¹⁰ and Δ 7-sitosterol (**5**)¹¹ by comparing their NMR and MS data with those reported in the literature (Figure 1).

The toxic potentials of five steroids (**1-5**) were evaluated toward the viability of HepG2 and U87MG cells using MTT assay (see Supporting information). As shown in Figure 2(a) and 2(b), cell viability was decreased in a dose-dependent manner after treatment with varying concentration of **4** for 24 h, whereas other steroids had no effect (data not shown). From this primary result, compound **4** was selected for further studies to elucidate the underlying mechanism of cell death induction in HepG2 and U87MG cells.

Apoptosis, a process of programmed cell death, is a physio-

logical mechanism, characterized by specific morphological and biochemical changes such as chromatin condensation, DNA fragmentation and protein cleavage.¹² To evaluate whether the cytotoxic effect of **4** against HepG2 and U87MG cells was mediated *via* apoptosis, we initially performed DNA fragmentation assay using ELISA (see Supporting information). After the treatment of **4** (10–200 μ M) with HepG2 and U87MG cells for 24 h, the amount of fragmented DNA increased by 1.1, 1.8, 3.9, and 6.6-fold and 1.3, 2.6, 3.1, and 5.1-fold compared with the control, respectively (Figure 3). This result revealed that the cytotoxic effect of **4** might be derived from the induction of apoptosis.

Apoptosis is tightly regulated by the complex interplay of individual members of the Bcl-2 family.¹³ To further determine the molecular mechanisms of apoptotic induction of **4**, we next examined the expression of Bcl-2 family proteins in HepG2 and U87MG cells (see Supporting information). The Bcl-2 family proteins, consisting of anti-apoptotic (Bcl-2)

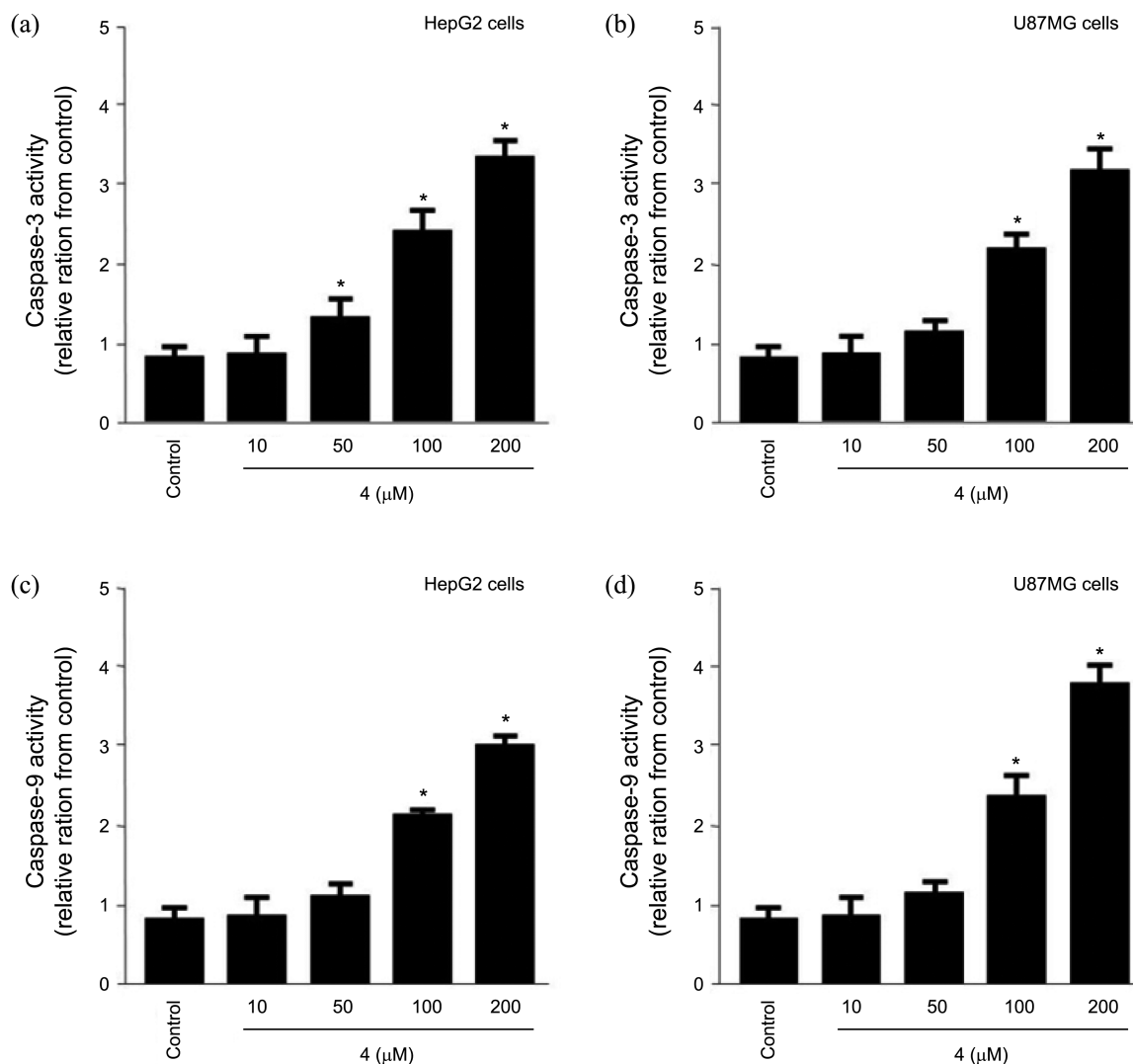


Figure 6. Effects of compound **4** on caspase-3 and caspase-9 activity in HepG2 and U87MG cells. Cells were incubated for 24 h with various concentrations of compound **4** (10–200 μ M). Caspase-3 and caspase-9 activities were measured by a colorimetric assay. Bar represents the mean \pm S.D. of three independent experiments. * $P < 0.05$ vs. control.

and pro-apoptotic (Bax) proteins, play crucial roles in the regulation of apoptosis. After the treatment with **4**, the expression of Bcl-2 proteins was decreased dose-dependently, whereas the expression of Bax protein accumulated with increasing concentrations (Figure 4). Densitometric tracing showed that the ratio of Bax/Bcl-2 exhibited a dose-dependent increase in both HepG2 and U87MG cells. This data indicated that **4** induced apoptosis by interfering with the expression of Bcl-2 family proteins resulting in up-regulation of Bax:Bcl-2 ratio (Figure 4).

An increase in the ratio of Bax:Bcl-2 can stimulate the release of cytochrome *c* from mitochondria into the cytosol, resulting in the activation of caspases and PARP.^{14,15} Activated caspase-3 is the key executioner of apoptosis that leads to the cleavage of key cellular proteins including PARP, which serves as a marker of apoptosis when cleaved.¹⁶ Therefore, we further examined the effects of **4** on the cleavage of caspases and the proteolytic cleavage of PARP in HepG2 and U87MG cells (see Supporting information). The treatment with **4** (10-200 μ M) for 24 h significantly increased the cleavage of caspase-3, caspase-9 and PARP in a dose-dependent manner (Figure 5). A colorimetric assay was further confirmed the increased caspase-3 and caspase-9 activity in HepG2 and U87MG cells treated with **4** as shown in Figure 6. These results strongly indicate that the observed anti-tumoric effects of **4** were mediated through the apoptotic process.

Conclusion

In the present study, five known steroids, including three sulfated steroids, were isolated from the cold water starfish *C. crispatus*. In addition, a new polyhydroxylated steroidal derivative, (22*E*,24*\xi*)-24-methyl-26,27-bisnor-5 α -cholest-22-en-3 β ,5,6 β ,15 α ,25-pentol (**1a**) was prepared from desulfation of compound **1**. The modified Mosher's method was applied to assign the absolute configuration at C-28 of the unusual stigmastane side chain of compound **3**. The cytotoxic effects of five isolated steroids (**1-5**) were evaluated against two human carcinoma cell lines (HepG2 and U87MG). Among the isolated compounds, only compound

4 induced apoptosis of HepG2 and U87MG cells through the enhanced expression of Bax and the activation of caspase pathways.

Supporting Information. ¹H and ¹³C NMR spectra of compounds **1**, **1a**, **3**, and **4**, and experimental information, including general procedures, chemical and reagents, cell culture, MTT assay, DNA fragmentation assay, caspase activity assay, Western blot analysis, and statistical analysis.

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