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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*,24ξ)-26,27-bisnor-24-methyl-5α-cholest-22-en-3β,5,6β,15α,25-pentol 25-*O*-sulfate (**1**), (22*E*,24*R*,25*R*)-24-methyl-5α-cholest-22-en-3β,5,6β,15α,25,26-hexol 26-*O*-sulfate (**2**), (28*R*)-24-ethyl-5α-cholesta-3β,5,6β,8,15α,28,29-heptaol-24-sulfate (**3**), (25*S*)-5α-cholestane-3β,5,6β,15α,16β,26-hexaol (**4**), and Δ7-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 C_{18} silica gel column, stepwise eluting with MeOH in H_2O (20%; 40%; 60%; 80%; and 100% v/v) to give 6 fractions, DR71-6. Fraction DR74 (162.9 mg) was subjected to C_{18} silica gel column chromatography (CC), using MeOH-H_2O (13:10) to provide three subfractions DR741-3. Subfraction DR742 was then subjected to silica gel CC, eluted with EtOAc-MeOH-H_2O (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 $CHCl_3$-MeOH-H_2O (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 C_{18} silica gel CC, eluting with MeOH-H_2O (4:1) to yield 2 subfractions, DR751 and DR752. Subfraction DR752 was subjected to RP C_{18} CC, eluting with MeOH-H_2O (4:1) and further purified by silica gel CC, using CH_2Cl_2-MeOH-H_2O (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 CH_2Cl_2-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 CH_2Cl_2-MeOH (10:1) as eluents.

(22*E*,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° (*c* 0.1, MeOH). HR-ESITOFMS *m/z* 515.2679 $[M]^-$ (calcd. for $C_{26}H_{43}O_8S$, 515.2679). ^{1}H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD): see Table 1.

(22*E*,24*R*,25*R*)-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° (*c* 0.1, MeOH). HR-ESITOFMS *m/z* 559.2926 $[M]^-$ (calcd. for $C_{28}H_{47}O_9S$, 559.2941). ^{1}H NMR (400 MHz, CD_3OD) 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, H_3-18), 1.16 (s, H_3-19), 1.01 (d, *J* = 6.4 Hz, H_3-21), 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, H_3-27), 0.99 (d, *J* = 6.4 Hz, H_3-28). ^{13}C NMR (100 MHz, CD_3OD) 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).

(28*R*)-24-Ethyl-5α-cholesta-3β,5,6β,8,15α,28,29-heptaol-24-sulfate (3): White, amorphous powder; $[\alpha]_D^{25}$ +32.0° (*c* 0.5, MeOH). HR-ESITOFMS *m/z* 591.3207 $[M]^-$ (calcd. for $C_{29}H_{51}O_{10}S$, 591.3203). ^{1}H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD): see Table 1. ^{1}H 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, H_3-18), 1.82 (s, H_3-19), 1.65 (m, H-20), 0.93 (d, *J* = 6.8 Hz, H_3-21), 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, H_3-26), 0.92 (d, *J* = 6.4 Hz, H_3-27), 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). ^{13}C 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).

(25*S*)-5α-Cholestane-3β,5,6β,15α,16β,26-hexaol (4): Colorless gum; $[\alpha]_D^{25}$ +10.1° (*c* 0.9, MeOH). HR-ESITOFMS *m/z* 513.3426 $[M + COOH]^-$ (calcd. for $C_{28}H_{49}O_8$, 513.3427). ESIMS *m/z* 433 $[M + H - 2H_2O]^+$. ^{1}H NMR (400 MHz, CD_3OD) 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, H_3-18), 1.17 (s, H_3-19), 0.96 (d, *J* = 6.8 Hz, H_3-21), 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, H_3-27). ^{13}C NMR (100 MHz, CD_3OD) 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° (*c* 0.7, $CHCl_3$). ESIMS *m/z* 415 $[M + H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.50 (m, H-3), 5.09 (br s, H-7), 0.47 (s, H_3-18), 0.72 (s, H_3-19), 0.86 (d, *J* = 6.0 Hz, H_3-21), 0.81 (d, *J* = 6.4 Hz, H_3-26), 0.80 (d, *J* = 6.4 Hz, H_3-27), 0.86 (d, *J* = 6.8 Hz, H_3-29). ^{13}C NMR (100 MHz, $CDCl_3$) 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 CH_2Cl_2-MeOH (4:1) to give **1a** (1.0 mg).

(22*E*,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° (*c* 0.06, MeOH). HR-ESITOFMS *m/z* 481.3181 $[M + COOH]^-$ (calcd. for $C_{27}H_{45}O_7$, 481.3165). ^{1}H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD): 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 CH_2Cl_2-MeOH (5:1) as eluent to give (*S*)- (**3a**) and (*R*)-MTPA esters (**3b**), respectively. The ^{1}H NMR spectra of the esters were recorded in pyridine-d_5 and the assignments were done by ^{1}H-^{1}H COSY spectra. Selected ^{1}H 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 (OCH_3), 3.64 (OCH_3), 3.59 (OCH_3), 1.87 (H-25), 1.67 (H-24), 1.42 (H-20), 1.36 (H-23), 0.96 (d, *J* = 6.8 Hz, H_3-21 and H_3-26), 0.92 (d, *J* = 6.8 Hz, H_3-27). Selected ^{1}H 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 (OCH_3), 3.75 (OCH_3), 3.65 (OCH_3), 1.72 (H-25), 1.58 (H-24), 1.27 (H-20), 1.27 (H-23), 0.90 (d, 6.8 Hz, H_3-21), 0.85 (d, *J* = 6.8 Hz, H_3-26), 0.81 (d, *J* = 6.8 Hz, H_3-27).

Results and Discussion

Compound **1** was isolated as white amorphous powder. The ^{1}H and ^{13}C NMR spectra contained signals characteristic of a common 3β,5α,6β,15α-tetrahydroxy-substituted cholestane nucleus (Table 1). The ^{1}H 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 ^{1}H–^{1}H COSY spectra, the positions of the functionalities and side chain of **1** were fully assigned. The molecular formula of **1** was established as $C_{26}H_{43}O_8S^-$ 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*,24ξ)-26,27-bisnor-24-methyl-5α-cholest-22-en-3β,5,6β,15α,25-pentol 25-*O*-sulfate (Figure 1).

Upon solvolysis in a dioxane-pyridine mixture followed

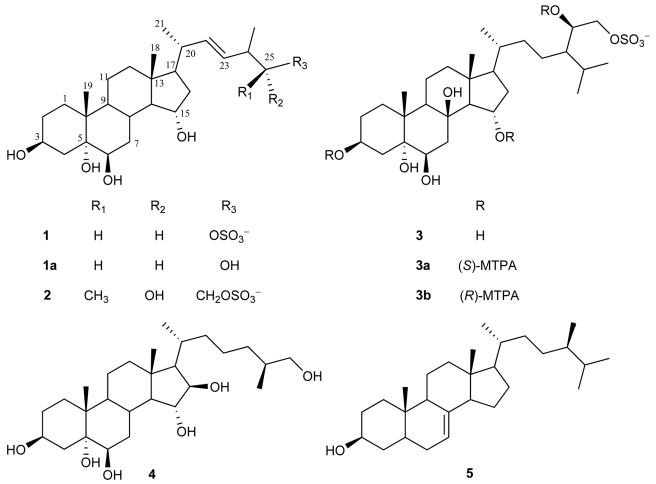

Figure 1. Structures of compounds **1**-**5**, **1a**, **3a**, and **3b**.

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

Position	**1**		**1a**		**3**	
	δ_H[a,b]	δ_C[a,c]	δ_H[a,b]	δ_C[a,c]	δ_H[a,b]	δ_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
					1.07 (m)	
23	5.29 (dd, 6.4, 15.6)	130.5	5.29 (dd, 6.4, 15.2)	131.5	1.41 (m)	23.6
					1.29 (m)	
24		37.7		40.4	1.18 (m)	47.8
25	3.73 (dd, 8.0, 9.2)	73.5	3.27 (m)	68.4	1.81 (m)	30.0
	3.88 (dd, 6.0, 9.2)		3.40 (dd, 6.0, 10.4)			
26	1.02 (d, 6.8)	17.4	0.95 (d, 6.8)	17.3	0.95 (d, 7.2)[d]	20.6
27					0.93 (d, 7.2)[d]	20.6
28					3.95 (br s)	71.5
29					3.95 (br s)	72.2

[a]Spectra were recorded in CD_3OD. [b]400 MHz. [c]100 MHz. [d]Signals are interchangeable.

Table 2. ^{1}H NMR chemical shift differences (Δδ) for the MTPA esters of **3**

Position	Δδ[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]Δδ (ppm) = $\delta_S - \delta_R$

by preparative TLC, compound **1** afforded a desulfated derivative **1a**. The ^{1}H and ^{13}C NMR spectra of **1a** were identical to those of **1**, except for the upfield shifted chemical 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 $C_{26}H_{44}O_5$ by a pseudomolecular ion *m/z* 481.3181 $[M + COOH]^-$ (481.3165 required, Δ –1.6 mmu) in the HR-ESITOFMS. Thus, the structure of **1a** was identified as (22*E*,24ξ)-24-methyl-26,27-bisnor-5α-cholest-22-en-3β,5,6β,15α,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 $C_{29}H_{51}O_{10}S^-$ 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

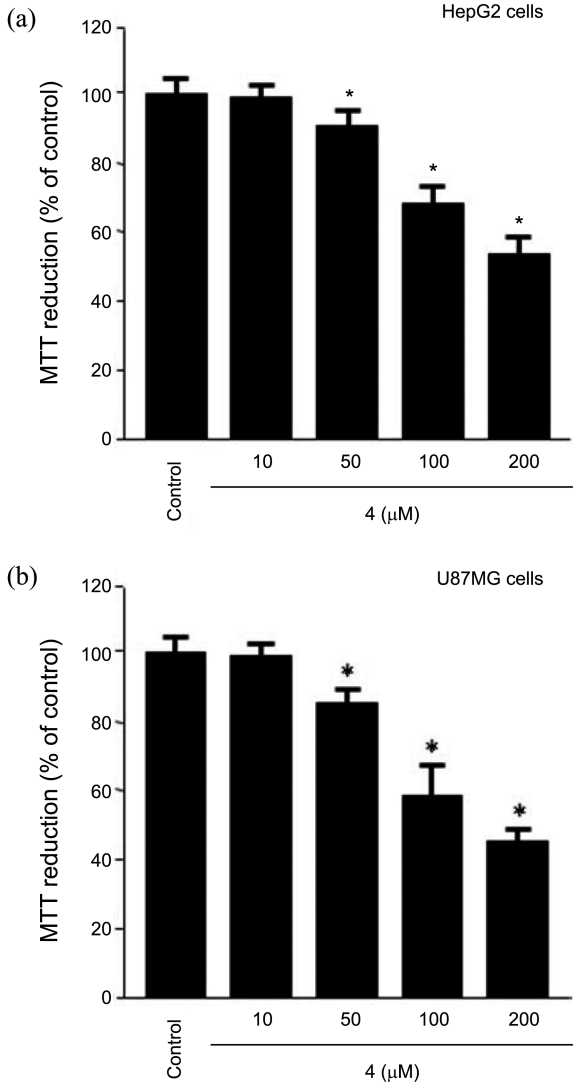

Figure 2. Effects of compound **4** on cell viability. HepG2 and U87MG cells were incubated for 24 h with various concentrations of compound **4** (10–200 μM). Cell viability was determined by MTT assay. Bar represents the mean ± S.D. of three independent experiments. $^*P < 0.05$ *vs*. control.

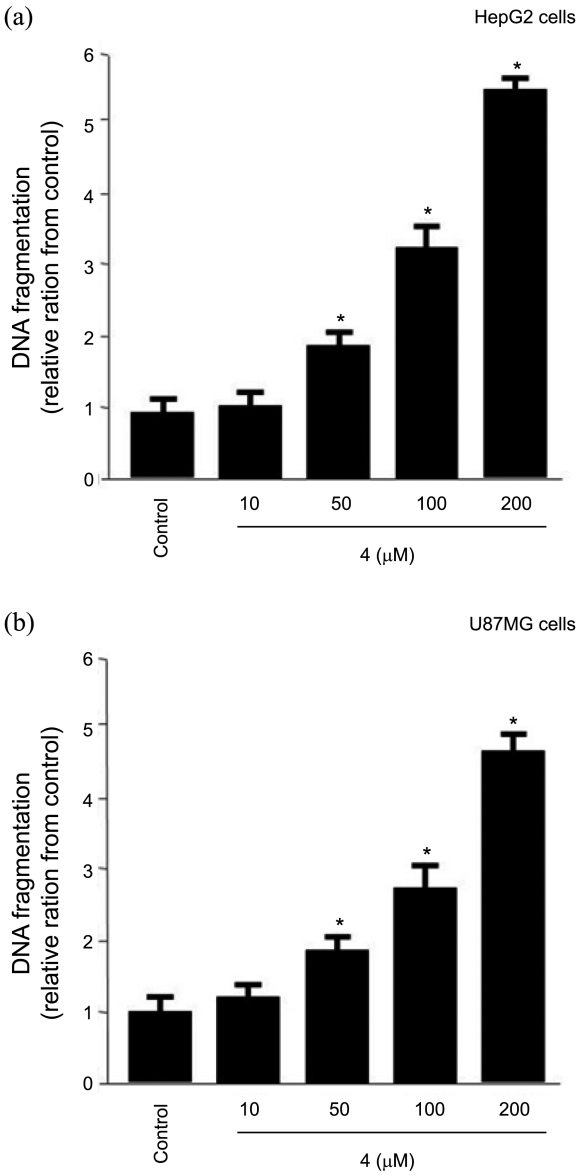

Figure 3. Effects of compound **4** on DNA fragmentation in HepG2 and U87MG cells. Cells were incubated for 24 h with various concentrations of compound **4** (10–200 μM). DNA fragmentation was evaluated using the Cellular DNA fragmentation ELISA kit. Bar represents the mean ± S.D. of three independent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

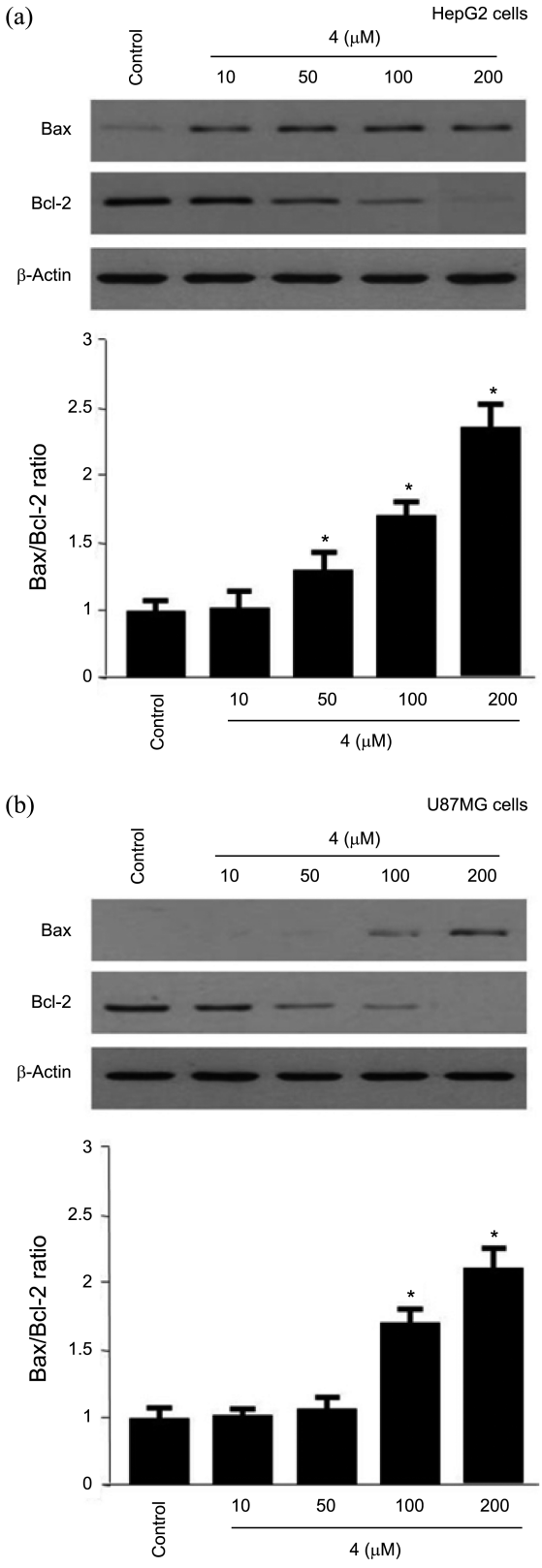

Figure 4. Effects of compound **4** on the expression of Bcl-2 family proteins in HepG2 and U87MG cells. Cells were incubated for 24 h with various concentrations of compound **4** (10–200 μM). Western blot analyses for proteins expression were performed as described in Experimental. Representative blots 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-5α-cholesta-3β,5,6β,8,15α,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 ^{1}H 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 Δδ values ($\delta_S - \delta_R$) for protons neighboring C-28 led to the assignment of

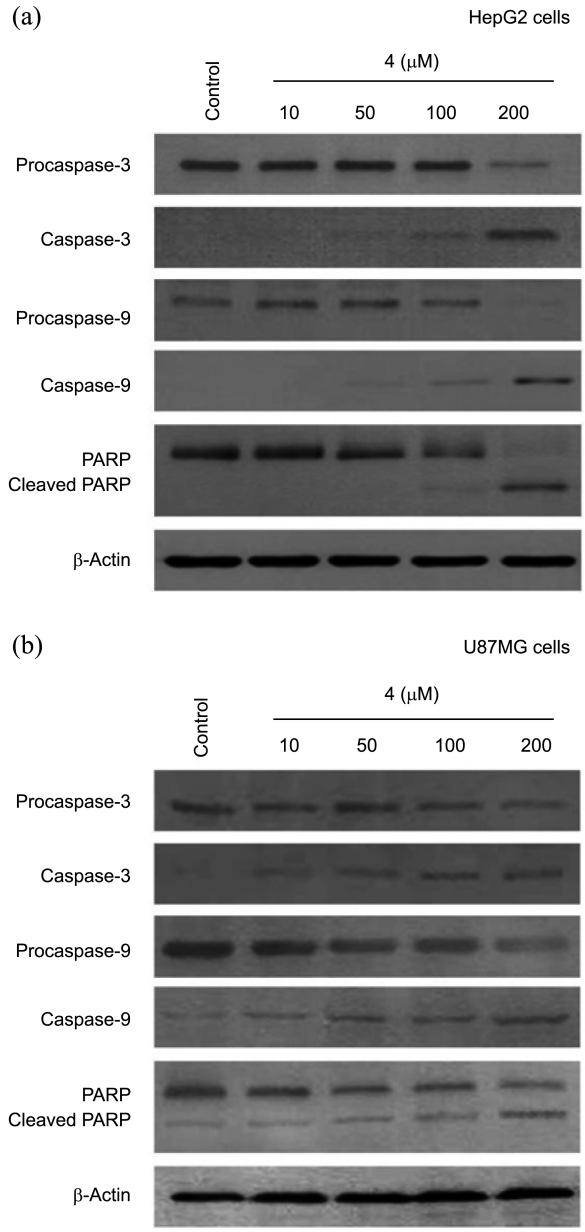

Figure 5. Effects of compound **4** on caspase-3, caspase-9, and PARP activation in HepG2 and U87MG cells. Cells were incubated for 24 h with various concentrations of compound **4** (10–200 μM). Western blot analyses for proteins expression were performed as described in Experimental. Representative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

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

Three remaining sterols were determined to be (22*E*,24*R*, 25*R*)-24-methyl-5α-cholest-22-en-3β,5,6β,15α,25,26-hexol 26-*O*-sulfate (**2**),[6] (25*S*)-5α-cholestane-3β,5,6β,15α,16β,26-hexaol (**4**),[10] and Δ7-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 physiological 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 μM) 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)

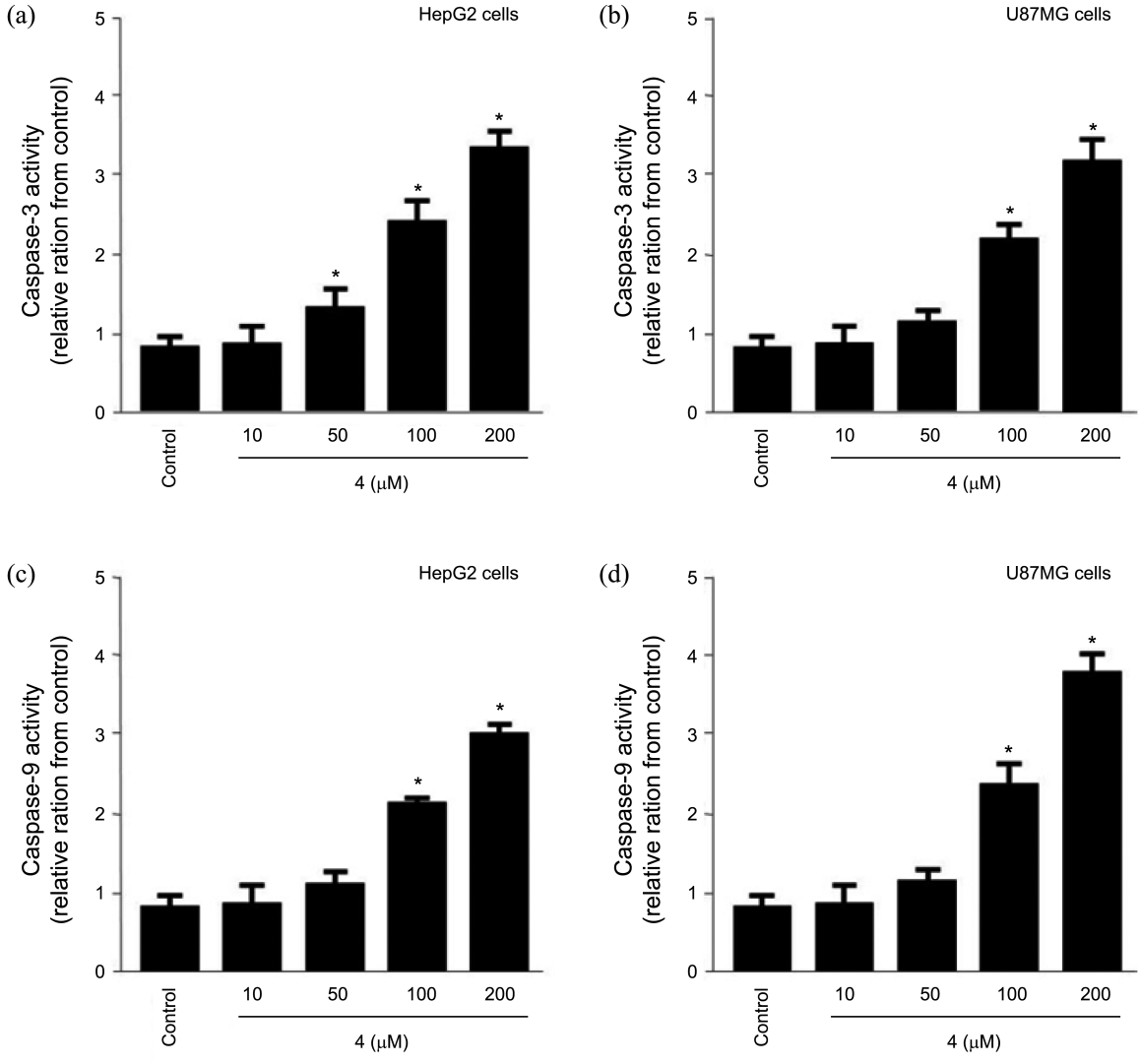

Figure 6. Effects of compound **4** on caspase-3 and caspase-9 activity in HepG2 and U87MG cells. Cells were incubated for 24 h with various concentrations of compound **4** (10–200 μM). Caspase-3 and caspase-9 activities were measured by a colorimetric assay. Bar represents the mean ± S.D. of three 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 is 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 μM) 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 antitumoric 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*,24ξ)-24-methyl-26,27-bisnor-5α-cholest-22-en-3β,5,6β,15α,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. ^{1}H and ^{13}C 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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