

Article

New 9 α -Hydroxy-5 α ,6 α -epoxysterols from the Vietnamese Marine Sponge *Ircinia echinata*

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Abstract: Chemical investigation of the methanol extract of the Vietnamese marine sponge *Ircinia echinata* led to the isolation of six new 9 α -hydroxy-5 α ,6 α -epoxysterols: 5 α ,6 α -epoxycholesta-7,22(*E*)-dien-3 β ,9 α -diol (**1**), 5 α ,6 α -epoxycholesta-7,24(28)-dien-3 β ,9 α -diol (**2**), (24*R*)-5 α ,6 α -epoxy-24-ethyl-cholesta-7-en-3 β ,9 α -diol (**3**), 5 α ,6 α -epoxycholesta-7-en-3 β ,9 α -diol (**4**), (24*S*)-5 α ,6 α -epoxyergosta-7,22-dien-3 β ,9 α -diol (**5**), and (24*R*)-5 α ,6 α -epoxy-24-methyl-cholesta-7-en-3 β ,9 α -diol (**6**) along with the known 5 α ,6 α -epoxysterols: 5 α ,6 α -epoxystigmasta-7-en-3 β -ol (**7**), 5 α ,6 α -epoxystigmasta-7,22-dien-3 β -ol (**8**), and 5 α ,6 α -epoxyergosta-7-en-3 β -ol (**9**). Their structures and their configurations were established on the basis of high resolution mass spectra and extensive 1D and 2D NMR spectroscopic data and by comparison with the literature. Their cytotoxic activity, evaluated against three human cancer cell lines, MCF-7, Hep-G2 and LU-1, revealed that only compounds **3** and **4** exhibited significant antiproliferative activity and compound **3** showed a selective inhibition towards the MCF-7 human breast cancer cells.

Keywords: marine sponge; *Ircinia echinata*; 9 α -hydroxy-5 α ,6 α -epoxysterols; cytotoxicity

1. Introduction

Marine sponges of the genus *Ircinia* are known as a rich source of varied bioactive natural products, including fatty acids [1], steroids [2,3], sesqui- [4] and sester-terpenes [5–7], peptides [8], and macrolides [9,10]. Previous reports revealed that steroids from marine sponges of the genus *Ircinia* exhibited a wide range of biological activities, as illustrated with the antileishmanial epidioxysterols from the Colombian marine sponge *Ircinia campana* [11], the cytotoxic 5,6-epoxysterols from the Chinese sponge *Ircinia aruensis* [12], the cytotoxic pentacyclic steroids possessing a cis C/D ring junction from the Okinawan marine sponge *Ircinia* sp. [13], the antibacterial 9,11-secosterol [14], or the derivative linked with a 1,4-quinone from a Korean marine sponge *Ircinia* sp. [15].

The East Sea in Vietnam covers an area of approximately three million km², and has about 3000 km of coastline. The marine biodiversity of the East Sea is considered to be among the most extensive in the world, but remains poorly understood and explored. Recently, Kiem *et al.* reported the isolation of several furanosesterterpenes and six known sterols from the sponge *Ircinia echinata* collected at Co To,

Quang Ninh, Vietnam [16,17]. In this paper, we describe the isolation and structural characterization of six new 9α -hydroxy- $5\alpha,6\alpha$ -epoxysterols (**1–6**), together with three known $5\alpha,6\alpha$ -epoxysterols from *I. echinata* (Order Dictyoceratida, Family Irciniidae) collected at Bai Tu Long, Vietnam. The cytotoxic activity of the nine $5\alpha,6\alpha$ -epoxysterols was evaluated against different human tumor cell lines.

2. Results and Discussion

Dried materials of *I. echinata* (0.5 kg) were extracted with MeOH at room temperature. The MeOH-soluble product was purified by a flash chromatography silica column followed by silica gel, Sephadex LH-20, and repeated high performance liquid chromatography (HPLC) columns to yield compounds **1–9** (Figure 1).

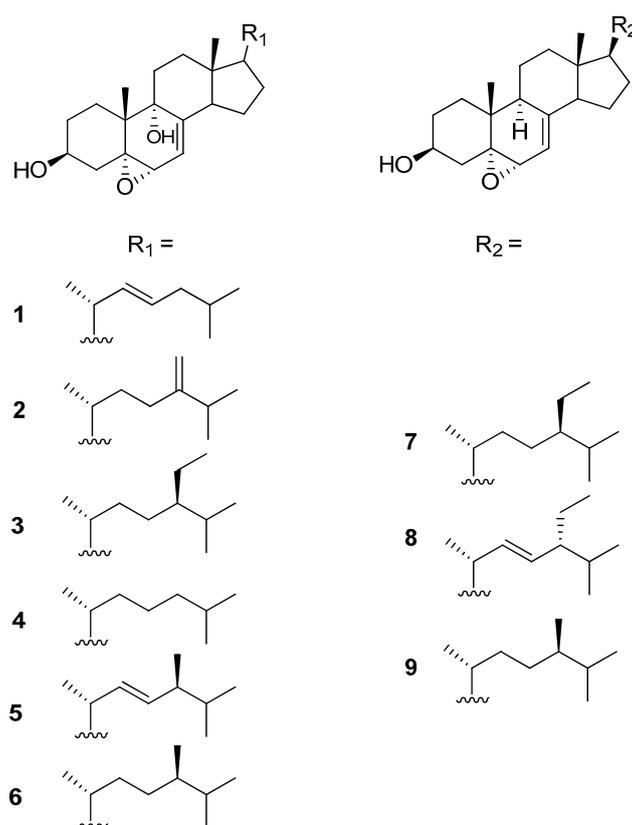


Figure 1. Structure of $5\alpha,6\alpha$ -epoxysterols **1–9** isolated from *Ircinia echinata*.

Compound **1** was isolated as white microcrystals. The HRESIMS exhibited a fragment ion peak at m/z 379.2953 $[M-2H_2O + H]^+$ (calculated 379.3001 for $C_{27}H_{39}O$), corresponding for compound **1** to a molecular formula of $C_{27}H_{42}O_3$. Seven degrees of unsaturation was thus deduced for **1**. The presence of a cholestanol skeleton was suggested by the 1H nuclear magnetic resonance (NMR) spectrum (Table 1), which exhibited two singlet methyls at δ_H 0.66 (CH₃-18) and 1.12 (CH₃-19), three doublet methyls at δ_H 1.03 ($J = 6.5$ Hz, CH₃-21) and 0.89 ($J = 6.7$ Hz, CH₃-26 and CH₃-27), and one oxygenated methine proton at δ_H 4.0 (m, H-3). Additionally, a set of complex protons was noted for the aliphatic region, including two olefinic protons at δ_H 5.32 (m, H-23) and 5.24 (dd, $J = 8.0$ and 15.0 Hz, H-22). The ^{13}C NMR spectrum of compound **1** confirmed the presence of 27 carbons, including five methyls, eight methylenes, six sp^3 methines, four sp^3 quaternary carbons, and one sp^2 quaternary carbon (Table 2). The chemical shifts of carbons at δ_C 68.2 (C-3), 78.9 (C-5), 73.7 (C-6), and 76.1 (C-9) suggested their linkage to oxygen. Analysis of the homonuclear correlated spectroscopy (COSY) spectrum of **1** revealed the structural elements, as presented in Figure 2. The connections of these spin–spin coupling systems were also shown from heteronuclear multiple bond correlations (HMBC) that confirmed the

cholestanol skeleton for **1**. Therefore, correlations from the methyl protons at δ_{H} 1.12 (CH₃-19) with the carbons at δ_{C} 28.2 (C-1), 78.9 (C-5), 76.1 (C-9), and 41.4 (C-10), and from the multiplet proton at δ_{H} 4.0 (H-3) with the carbons at δ_{C} 78.9 (C-5) and 28.2 (C-1) assigned the presence of the A-ring. Similarly, the HMBC correlations from the proton at δ_{H} 3.65 (H-6) with the carbons at δ_{C} 78.9 (C-5) and 40.7 (C-4) as well as the correlations from the methyl protons at δ_{H} 1.12 (CH₃-19) with the carbons at δ_{C} 78.9 (C-5), 76.1 (C-9), 41.4 (C-10), and 28.2 (C-1) established the B-ring. Finally, the C/D ring system was determined by HMBC correlations from the methylene protons at δ_{H} 1.60–1.91 (CH₂-11), with the carbons at δ_{C} 76.1 (C-9) and 36.5 (C-12), and from the methyl protons at δ_{H} 0.66 (CH₃-18), with the carbons at δ_{C} 36.5 (C-12), 44.8 (C-13), 51.9 (C-14), and 57.3 (C-17). HMBC correlations from the methylenic proton at δ_{H} 5.33 (H-7), with the carbons at δ_{C} 143.8 (C-8), 76.1 (C-9), and 51.9 (C-14), allowed the linking of rings B and C. Taking into account the molecular formula and the seven degrees of unsaturation previously established, as well as the presence of one unsaturation in the alkyl chain, the epoxy ring was suggested to be at the C-5/C-6 position for compound **1**. Furthermore, correlations from the methyl protons at δ_{H} 1.03 (CH₃-21), with carbons at δ_{C} 57.3 (C-17), 41.8 (C-20), and 139.1 (C-22), and from the methyl protons at 0.89 (CH₃-26 and 27), with carbons at δ_{C} 43.1 (C-24), and 29.8 (C-25), allowed determining the planar structure of **1** (Figure 2).

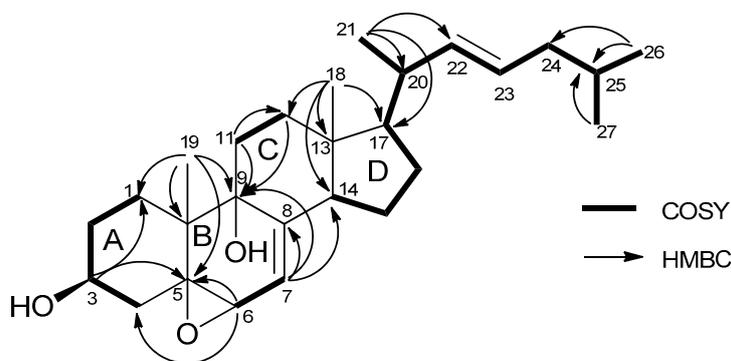


Figure 2. Selected HMBC and COSY correlations in compound **1**.

The relative configuration of compound **1** was suggested by proton coupling constants and nuclear Overhauser effect spectroscopy (NOESY) spectrum analyses (Figure 3). Since the signal at δ_{H} 4.0 (H-3) appeared as a multiplet in the ¹H NMR spectrum, its coupling constants could not be directly determined. However, the proton axial at δ_{H} 2.11 (CH₂-4) showed two anti-coupling constants ($J = 11.4$ and 13.8 Hz). These data indicated an axial orientation for the proton H-3. In the NOESY spectrum, the signal at δ_{H} 2.11 (H-4_{ax}) showed correlations with the proton at δ_{H} 3.65 (H-6) and the methyl at δ_{H} 1.12 (CH₃-19), suggesting their co-facial dispositions. Furthermore, NOESY correlations of H-11_{ax} with both methyls at δ_{H} 0.66 (CH₃-18) and at δ_{H} 1.12 (CH₃-19) were observed. Therefore, the α -configuration was deduced for the hydroxyl group at C-9 and at the 5-6-epoxy function. The transfused junction for the C/D rings was established by NOESY correlations from the signal at δ_{H} 2.49 (H-14) with the proton at δ_{H} 1.61 (H-12_{ax}), and between the signals at δ_{H} 1.91 (H-11_{ax}) and δ_{H} 0.66 (CH₃-18). Additionally, protons at δ_{H} 1.37 (H-17) and at δ_{H} 2.49 (H-14) were determined to be co-facial, as indicated by their correlation in the NOESY spectrum. In addition, NOESY correlations between CH₃-18, CH₃-19, and CH-20 indicated that they are oriented on the same side of the molecule. The 20*R* absolute configuration was supported by the chemical shift at δ_{H} 1.03 of CH₃-21 [18–20]. Furthermore, a strong coupling constant between H-22/H-23 ($J = 15.0$ Hz) assigned the *E*-configuration for the C-22/C-23 double bond. Therefore, according to this intensive analysis of 2D NMR spectra, the structure of the new compound **1** was identified as 5 α ,6 α -epoxycholesta-7,22(*E*)-dien-3 β ,9 α -diol.

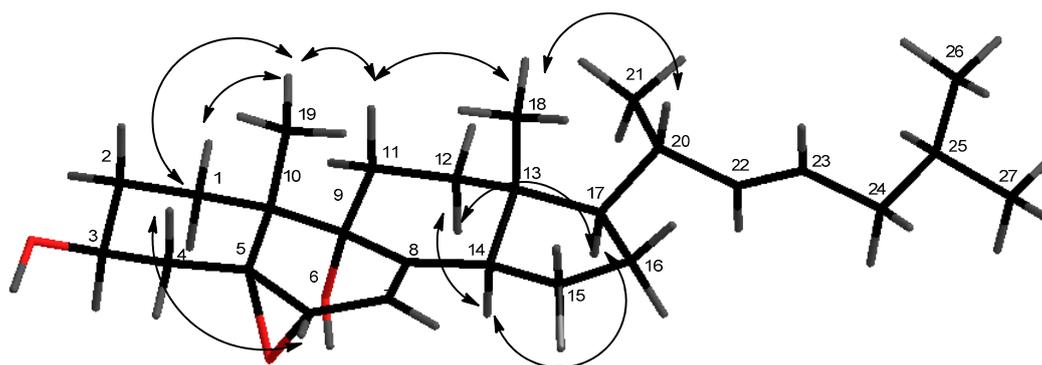


Figure 3. Key NOESY correlations of compound 1.

Compound 2 was isolated as white microcrystals. The HRESIMS of compound 2 exhibited a fragment ion peak at m/z 393.3070 $[M-2H_2O + H]^+$ (calculated 393.3157 for $C_{28}H_{41}O$), corresponding for compound 2 to a molecular formula of $C_{28}H_{44}O_3$ with seven degrees of unsaturation. The 1H and ^{13}C NMR data showed that compound 2 shares the same Δ^7 sterol nucleus as 1, with the presence of an epoxy group at C-5/C-6 and a hydroxyl group at C-9 (Table 1). However, significant differences were noted in the 1H NMR spectrum for signals of the side chain. In particular, the presence of gem olefinic protons at δ_H 4.67 and 4.73 was observed in the 1H NMR spectrum for compound 2. The HMBC correlations from these olefinic protons with the carbons at δ_C 32.1 (C-23) and 34.9 (C-25) located the double bond at position C-24/C-28. Careful analysis of 2D NMR spectra revealed the structure of compound 2 as 5 α ,6 α -epoxycholesta-7,24(28)-dien-3 β ,9 α -diol (Figure 1).

Compound 3 was obtained as white microcrystals. The HRESIMS of 3 exhibited a fragment ion peak at m/z 427.3597 $[M-H_2O + H]^+$ (calculated 427.3576 for $C_{29}H_{47}O_2$), corresponding for compound 3 to a molecular formula of $C_{29}H_{48}O_3$ with six degrees of unsaturation. Examination of the NMR data indicated that compound 3 also shared the same Δ^7 -5,6-epoxyhydroxy sterol nucleus as compounds 1 and 2, but possesses a different side chain (Figure 1). Comparison of the NMR data of compound 3 with that of 2 revealed the presence of a methine and an ethyl group in 3 instead of the C-24/C-28 double bond resonance in the structure of 2. The ethyl group (CH_2 -28- CH_3 -29) was determined to be located at C-24 from the COSY correlations between the methyl at δ_H 0.88 (CH_3 -29) and the methylene at δ_H 1.35–1.18 (CH_2 -28), which in turn correlated with the methine at δ_H 0.96 (CH -24). Although only one diastereomer was isolated, the difference between C-26 and C-27 chemical shifts, which is 0.70 ppm, suggests the 24 *R* configuration for compound 3 (instead of 0.55 ppm in the case of *S*-configuration) [21,22]. The new sterol 3 was thus deduced to be (24*R*)-5 α ,6 α -epoxy-24-ethyl-cholesta-7-en-3 β ,9 α -diol (Figure 1).

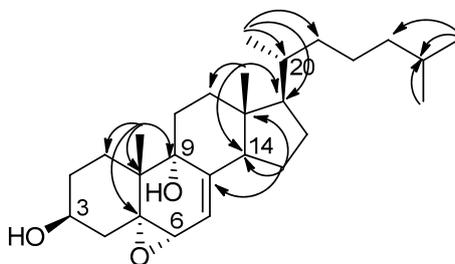
Compound 4 was isolated as white microcrystals. The HRESIMS of compound 4 exhibited a fragment ion peak at m/z 399.3259 $[M-H_2O + H]^+$ (calculated 399.3263 for $C_{27}H_{43}O_2$), corresponding for compound 4 to a molecular formula of $C_{27}H_{44}O_3$ with six degrees of unsaturation. The 1H NMR signals of 4 were close to those of 1, except for the presence of two methylenes instead of the C-22/C-23 double bond. Analysis of 2D NMR spectra, in particular of HMBC correlations (Figure 4), established the structure of compound 4 as 5 α ,6 α -epoxycholesta-7-en-3 β ,9 α -diol (Figure 1). Compound 4 was previously prepared from the free radical chain oxidation process of 7-dehydrocholesterol, but this is its first report as a natural product [23].

Table 1. ¹H NMR data of compounds 1–6 recorded in CD₃OD (δ_{H} , multiplicity, *J* in Hz).

N ^o	1	2	3	4	5	6
1	2.20 dt (4.3, 13.5)	2.20 dt (4.3, 13.5)	2.20 dt (4.3, 13.8)	2.20 dt (4.2, 13.8)	2.20 dt (4.2, 13.8)	2.20 dt (4.2, 13.8)
	1.35 m	1.35 m	1.36 m	1.35 m	1.36 m	1.36 m
2	1.86 m	1.86 m	1.88 m	1.85 m	1.86 m	1.86 m
	1.51 m	1.50 m	1.52 m	1.52 m	1.51 m	1.51 m
3	4.00 m	4.00 m	4.00 m	4.00 m	4.0 m	4.0 m
4	1.66 m	1.66 m	1.66 m	1.67 m	1.66 m	1.67 m
	2.11 dd (11.4, 13.8)	2.12 dd (11.2, 13.5)	2.12 dd (11.2, 13.8)	2.12 dd (11.4, 13.2)	2.12 dd (11.4, 13.2)	2.12 dd (11.4, 13.8)
6	3.65 m	3.64 m	3.66 m	3.66 m	3.65 m	3.65 m
7	5.33 m	5.33 m	5.34 dd (1.8, 4.8)	5.34 dd (2.4, 4.8)	5.34 dd (2.4, 4.8)	5.33 dd (1.8, 4.8)
11	1.60 m	1.97 m	1.97 m	1.97 m	1.60 m	1.60 m
	1.91 m	1.59 m	1.60 m	1.60 m	1.90 m	1.97 m
12	1.61 m	1.60 m	1.59 m	1.59 m	1.61 m	1.60 m
	1.83 m	1.86 m	1.86 m	1.86 m	1.83 m	1.86 m
14	2.49 m	2.50 m	2.49 m	2.50 m	2.50 m	2.49 m
15	1.55 m	1.61 m	1.61 m	1.61 m	1.56 m	1.61 m
	1.48 m	1.51 m	1.53 m	1.53 m	1.48 m	1.52 m
16	1.34 m	1.36 m	1.35 m	1.34 m	1.34 m	1.34 m
	1.80 m	1.90 m	1.91 m	1.90 m	1.80 m	1.91 m
17	1.37 m	1.36 m	1.36 m	1.34 m	1.37 m	1.34 m
18	0.66 s	0.65 s	0.65 s	0.65 s	0.66 s	0.65 s
19	1.12 s					
20	2.05 m	1.45 m	1.41 m	1.41 m	2.04 m	1.41 m
21	1.03 d (6.5)	0.99 d (6.8)	0.97 d (6.5)	0.96 d (6.6)	1.03 d (6.6)	0.96 d (6.6)
22	5.24 dd (8.0, 15.0)	1.18 m, 1.60 m	1.60 m, 1.07 m	1.41 m, 1.14 m	5.20 m	1.11 m, 1.40 m
23	5.32 m	1.92 m, 2.13 m	1.25 m	1.40 m, 1.19 m	5.21 m	1.28 m, 1.53 m
24	1.84 m		0.96 m	1.16 m	1.85 m	1.23 m
25	1.58 m	2.24 m	1.71 m	1.53 m	1.47 m	1.55 m
26	0.89 d (6.7)	1.03 d (6.7)	0.86 d (6.8)	0.89 d (6.6)	0.88 d (7.2)	0.88 d (7.2)
27	0.89 d (6.7)	1.04 d (6.7)	0.84 d (6.8)	0.89 d (6.6)	0.86 d (7.2)	0.83 d (7.2)
28		4.67 brs, 4.73 brs	1.35 m, 1.18 m		0.94 d (7.2)	0.81 d (7.2)
29			0.88 m			

Table 2. ^{13}C NMR data for compounds 1–6 recorded in CD_3OD .

N ^o	1	2	3	4	5	6
1	28.2 t	28.2 t	28.3 t	28.2 t	28.3 t	28.3 t
2	31.6 t					
3	68.2 d					
4	40.7 t	40.7 t	40.8 t	40.7 t	40.7 t	40.7 t
5	78.9 s					
6	73.7 d					
7	121.0 d	121.1 d	121.0 d	121.0 d	121.0 d	121.0 d
8	143.8 s					
9	76.1 s	76.0 s	76.1 s	76.0 s	76.1 s	76.1 s
10	41.4 s					
11	29.1 t	28.9 t	29.0 t	28.9 t	29.2 t	29.0 t
12	36.5 t	36.6 t	36.6 t	36.6 t	36.5 t	36.5 t
13	44.8 s	44.9 s	44.9 s	44.9 s	44.8 s	44.8 s
14	51.9 d	51.8 d	51.8 d	51.8 d	51.9 d	51.8 d
15	24.0 t	24.1 t	24.1 t	24.1 t	24.0 t	24.0 t
16	29.3 t	29.1 t	29.2 t	29.1 t	29.6 t	29.1 t
17	57.3 d	57.4 d	57.4 d	57.5 d	57.4 d	57.5 d
18	12.2 q	12.0 q	12.2 q	12.0 q	12.2 q	12.0 q
19	22.2 q					
20	41.8 d	37.3 d	37.9 d	37.5 d	41.9 d	37.5 d
21	21.5 q	19.3 q	19.4 q	19.3 q	21.5 q	19.3 q
22	139.1 d	35.8 t	35.0 t	37.2 t	137.2 d	35.0 t
23	127.8 d	32.1 t	27.2 t	24.9 t	133.4 d	31.4 t
24	43.1 t	157.3 s	47.4 d	40.6 t	44.6 d	40.3 d
25	29.8 d	34.9 d	30.2 d	29.2 d	34.5 d	33.7 d
26	22.7 q	22.3 q	20.1 q	22.9 q	20.7 q	20.5 q
27	22.7 q	22.4 q	19.4 q	23.2 q	20.1 q	18.5 q
28		106.1 t	24.1 t		18.6 q	15.8 q
29			12.7 q			

**Figure 4.** Selected HMBC correlations in compound 4.

Compound 5 was isolated as white microcrystals. The HRESIMS of compound 5 exhibited a fragment ion peak at m/z 411.3284 $[\text{M}-\text{H}_2\text{O} + \text{H}]^+$ (calculated 411.3263 for $\text{C}_{28}\text{H}_{43}\text{O}_2$), corresponding for compound 5 to a molecular formula of $\text{C}_{28}\text{H}_{44}\text{O}_3$ with seven degrees of unsaturation. A comparison of the ^1H and ^{13}C NMR data of compound 5 with that of sterol 1 revealed the presence of an additional methyl group at δ_{H} 0.94 (d, $J = 7.2$ Hz) and its corresponding resonance at δ_{C} 18.6, suggesting the replacement of a methylene at C-24 in compound 1 by a methine linked to a methyl group for compound 5. The location of the additional methyl group in C-24 was confirmed by HMBC correlations from the methyl at δ_{H} 0.94 (CH_3 -28), with the carbons at δ_{C} 133.4 (C-23) and 34.5 (C-25). The configuration at C-24 of compound 5 was determined by comparison of the ^{13}C NMR chemical shifts with those of the related structures [21,22]. Accordingly, due to the chemical difference shift of 0.6 ppm between the C-26 and C-27 carbon atoms and the chemical shift of C-28 at δ_{C} 18.6 ppm, the *S*-configuration at C-24 was suggested for compound 5. The new sterol 5 was thus deduced to be (24*S*)-5 α ,6 α -epoxyergosta-7,22-dien-3 β ,9 α -diol (Figure 1).

Compound **6** was isolated as white microcrystals. The HRESIMS of compound **6** exhibited a fragment ion peak at m/z 413.3414 $[M-H_2O + H]^+$ (calculated 413.3420 for $C_{28}H_{45}O_2$), corresponding to a molecular formula of $C_{28}H_{46}O_3$ with six degrees of unsaturation. A detailed examination of 1H and ^{13}C NMR data (Tables 1 and 2) showed that compound **6** also belongs to the 9α -hydroxy- $5\alpha,6\alpha$ -epoxysterols. Comparison with compound **4** revealed the presence of an additional methyl group in C-24. The configuration of C-24 in compound **6** was determined to be *R*, since the chemical shift difference between C-26 and C-27 carbon atoms is 2.0 ppm, whereas in the *S* configuration the expected value for a saturated 24-methyl side chain should be 3.0 ppm [21,22]. The new sterol **6** was thus deduced to be (24*R*)- $5\alpha,6\alpha$ -epoxy-24-methyl-cholesta-7-en- $3\beta,9\alpha$ -diol (**6**).

Three known compounds $5\alpha,6\alpha$ -epoxystigmasta-7-en- 3β -ol (**7**), $5\alpha,6\alpha$ -epoxystigmasta-7,22-dien- 3β -ol (**8**), and $5\alpha,6\alpha$ -epoxyergosta-7-en- 3β -ol (**9**) were also isolated and characterized from the MeOH extract of *I. echinata*. Their structures were determined by spectral data and comparison with those reported in the literature from the marine sponge *Ircinia aruensis* [12].

Evaluation of all steroids isolated from *I. echinata* was performed against three cancer cell lines: MCF-7 (human breast cancer cells), HepG-2 (human liver hepatocellular carcinoma cells), and Lu-1 (human lung adenocarcinoma cells). All compounds were inactive until $32 \mu\text{g}\cdot\text{mL}^{-1}$, except the two new steroids (24*R*)- $5\alpha,6\alpha$ -epoxy-24-ethyl-cholesta-7-en- $3\beta,9\alpha$ -diol (**3**) and $5\alpha,6\alpha$ -epoxycholesta-7-en- $3\beta,9\alpha$ -diol (**4**) (Table 3). Compound **3** exhibited a selective inhibition towards the MCF-7 cells with an IC_{50} value of $15.88 \pm 1.36 \mu\text{g}\cdot\text{mL}^{-1}$, whereas the steroid **4** showed a similar range of cytotoxic activity against the three cancer cell lines (Table 3 and Figure S43). Comparison of the active sterol **3** with the inactive non-hydroxylated sterol **7** could show, in this case, the importance of hydroxylation in position C-9. Furthermore, an additional ethyl group on the alkyl chain could exert a selective activity against MCF-7.

Table 3. Cytotoxic evaluation of the active $5\alpha,6\alpha$ -epoxysterols **3–4** against human cancer cell lines (IC_{50} values are expressed in $\mu\text{g}\cdot\text{mL}^{-1}$).

Compound	Human Cancer Cell Lines		
	MCF-7	HepG-2	Lu-1
3	15.88 ± 1.36	>32	>32
4	15.88 ± 0.09	15.95 ± 0.20	22.92 ± 0.09
Ellipticine	0.34 ± 0.01	0.38 ± 0.05	0.41 ± 0.04

Ellipticine was used as positive control. Values presented as the mean \pm (SEM) ($n = 3$).

3. Materials and Methods

3.1. General Experimental Procedures

Flash chromatography was carried out on a Buchi C-615 pump system (Rungis, France). Analytical and semi-preparative reverse-phase (Luna C18 or biphenyl Kinetex, Phenomenex, Le Pecq, France) columns were performed with an Alliance HPLC apparatus (model 2695, Waters, Saint-Quentin-en-Yvelines, France), equipped with a photodiode array detector (model 2998, Waters), an evaporative light-scattering detector (model Sedex 80, Sedere, Alfortville, France), and the Empower software. Mass spectra were recorded on an API Q-STAR PULSAR I (Applied Biosystem, Concord, ON, Canada) and on a Maxis II-ETD (Bruker, Wissenbourg, France). 1D and 2D NMR (COSY, HSQC, HMBC, NOESY) spectra were recorded on a Bruker AVANCE 600 (Bruker, Wissenbourg, France).

3.2. Biological Materials

Specimens of *I. echinata* (Keller, 1889) (Order Dictyoceratida, Family Irciniidae) were collected at Bai Tu Long (Quang Ninh, Vietnam) in August 2014. The sponge sample was identified by Professor

Do Cong Thung of the Institute of Marine Environment and Resources, and a voucher specimen (HM01-39 α) has been deposited at the Institute of Marine Biochemistry (VAST, Vietnam).

3.3. Isolation of 5 α ,6 α -Epoxysterols from the Vietnamese Marine Sponge *Ircinia echinata*

The air-dried sponge of *I. echinata* (0.5 kg) was extracted with MeOH at room temperature (3 times \times 2 L). The solvent was removed under reduced pressure to give 15 g of the MeOH crude extract. An aliquot of 5 g was subjected to flash chromatography on silica gel (from 0% to 100% MeOH in CH₂Cl₂) to yield 12 fractions. Fraction F10 (150 mg) and fraction F12 (380 mg) were each chromatographed on a silica gel column (n-hexane/acetone gradient), and then on a Sephadex LH-20 column, using MeOH/CH₂Cl₂ (9/1) as eluent to afford subfraction F10.2.2 (9 mg) and F12.2.1 (9 mg), respectively. Subfraction F10.2.2 was purified by a semi-preparative reverse phase HPLC (Luna 5 μ C18 Phenomenex, 250 \times 10 mm, flow rate 3 mL.min⁻¹) eluting with a gradient solvent system MeOH/H₂O/HCOOH from 92/8/0.1 to 95/5/0.1 for 20 min to give compounds **7** (0.8 mg), **8** (0.5 mg), and **9** (0.8 mg). Similarly, subfraction F12.2.1 was then purified by HPLC on a semi-preparative reverse phase column, using the gradient solvent system MeOH/H₂O/HCOOH from 85/15/0.1 to 95/5/0.1 for 20 min to yield the new pure compounds **1** (2.0 mg), **2** (1.5 mg), and **3** (2.0 mg), as well as impure sub-fractions A and B. Sub-fraction A was purified using reverse phase HPLC (Kinetex 5 μ biphenyl Phenomenex, 250 \times 4.6 mm, flow rate 1 mL.min⁻¹) with MeOH/H₂O (75/25) as eluent, to furnish the new compounds **4** (0.8 mg) and **5** (0.5 mg). Sub-fraction B was purified using reverse phase HPLC (Kinetex 5 μ biphenyl Phenomenex, 250 \times 4.6 mm, flow rate 1 mL.min⁻¹) with MeOH/H₂O (80/20) as eluent, to yield the new compound **6** (0.4 mg).

3.3.1. 5 α ,6 α -Epoxycholesta-7,22(*E*)-dien-3 β ,9 α -diol (**1**)

Compound **1**: white microcrystals; m.p. 150–151 °C; $[\alpha]_D^{25}$ –15.0 (*c* 0.1, MeOH). The HRESIMS results showed $[M-2H_2O + H]^+$ found at *m/z* 379.2953 (calculated 379.3001 for C₂₇H₃₉O), and $[M-3H_2O + H]^+$ found at *m/z* 361.2913 (calculated 361.2895 for C₂₇H₃₇). For ¹H and ¹³C NMR, see Tables 1 and 2.

3.3.2. 5 α ,6 α -Epoxycholesta-7,24(28)-dien-3 β ,9 α -diol (**2**)

Compound **2**: white microcrystals; m.p. 181–182 °C; $[\alpha]_D^{25}$ –11.0 (*c* 0.1, MeOH). The HRESIMS results showed $[M-2H_2O + H]^+$ found at *m/z* 393.3070 (calculated 393.3157 for C₂₈H₄₁O), and $[M-3H_2O + H]^+$ found at *m/z* 375.3035 (calculated 375.3052 for C₂₈H₃₉). For ¹H and ¹³C NMR, see Tables 1 and 2.

3.3.3. (24*R*)-5 α ,6 α -Epoxy-24-ethyl-cholesta-7-en-3 β ,9 α -diol (**3**)

Compound **3**: white microcrystals; m.p. 172–173 °C; $[\alpha]_D^{25}$ –17.0 (*c* 0.08, MeOH). The HRESIMS results showed $[M-H_2O + H]^+$ found at *m/z* 427.3597 (calculated 427.3576 for C₂₉H₄₇O₂), and $[M-2H_2O + H]^+$ found at *m/z* 409.3499 (calculated 409.3470 for C₂₉H₄₅O), and $[M-3H_2O + H]^+$ found at *m/z* 391.3371 (calculated 391.3365 for C₂₉H₄₃). For ¹H and ¹³C NMR, see Tables 1 and 2.

3.3.4. 5 α ,6 α -Epoxycholesta-7-en-3 β ,9 α -diol (**4**)

Compound **4**: white microcrystals; m.p. 158–159 °C; $[\alpha]_D^{25}$ –46.0 (*c* 0.08, MeOH). The HRESIMS results showed $[M-H_2O + H]^+$ found at *m/z* 399.3259 (calculated 399.3263 for C₂₇H₄₃O₂), $[M-2H_2O + H]^+$ found at *m/z* 381.3161 (calculated 381.3157 for C₂₇H₄₁O), and $[M-3H_2O + H]^+$ found at *m/z* 363.3053 (calculated 363.3052 for C₂₇H₃₉). For ¹H and ¹³C NMR, see Tables 1 and 2.

3.3.5. (24*S*)-5 α ,6 α -Epoxyergosta-7,22-dien-3 β ,9 α -diol (**5**)

Compound **5**: white microcrystals; m.p. 190–192 °C; $[\alpha]_D^{25}$ –32.0 (*c* 0.08, MeOH). The HRESIMS results showed $[M-H_2O + H]^+$ found at *m/z* 411.3284 (calculated 411.3263 for C₂₈H₄₃O₂), $[M-2H_2O + H]^+$ found at *m/z* 393.3128 (calculated 393.3157 for C₂₈H₄₁O), and $[M-3H_2O + H]^+$ found at *m/z* 375.3044 (calculated 375.3052 for C₂₈H₃₉). For ¹H and ¹³C NMR, see Tables 1 and 2.

3.3.6. (24R)-5 α ,6 α -Epoxy-24-methyl-cholesta-7-en-3 β ,9 α -diol (6)

Compound **6**: white microcrystals; m.p. 207–208 °C; $[\alpha]_D^{25} -15.0$ (c 0.05, MeOH). The HRESIMS showed $[M-H_2O + H]^+$ found at m/z 413.3414 (calculated 413.3420 for C₂₈H₄₅O₂), $[M-2H_2O + H]^+$ found at m/z 395.3306 (calculated 395.3314 for C₂₈H₄₃O), and $[M-3H_2O + H]^+$ found at m/z 377.3202 (calculated 377.3208 for C₂₈H₄₁). For ¹H and ¹³C NMR, see Tables 1 and 2.

3.4. Evaluation of Cytotoxic Activity

Cytotoxicity assays were carried out in triplicate in a 96-well microtiter plates against HepG-2, Lu-1, and MCF-7, using a modification of the published method [24]. Cells were maintained in Dulbecco's D-MEM medium, supplemented with 10% fetal calf serum, L-glutamine (2 mM), penicillin G (100 U.L⁻¹), streptomycin (100 μ g.mL⁻¹), and gentamicin (10 μ g.mL⁻¹). Stock solutions of the compounds were prepared in DMSO/H₂O (1/9), and the cytotoxicity assays were carried out against cancer cells (3 \times 10³ cells.mL⁻¹). After 72 h of incubation at 37 °C in air/CO₂ (95:5), with or without the test compounds, cell growth was estimated by colorimetric measurement of stained living cells using neutral red. Optical density was determined at 540 nm with a Titertek Multiscan photometer. The IC₅₀ value was defined as the concentration of the sample necessary to inhibit cell growth to 50% of the control. Ellipticine was used as a reference compound.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1660-3397/16/11/424/s1>, HRESIMS as well as 1D and 2D NMR spectra of compounds 1–6 along with growth inhibition curves of active compounds 3 and 4 against three human cancer cell lines.

Author Contributions: T.T.V.T., B.N.T., and A.L. performed the purification of the compounds. A.D. performed NMR spectra, V.C.P. and M.-L.B.-K. designed the study. All of the authors contributed to the data analysis and structure elucidation of the compounds, as well as to edit the manuscript.

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