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PAPER

Hirsutosterols A–G, polyoxygenated steroids from a Formosan soft coral *Cladiella hirsuta*†

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Seven new polyoxygenated steroids, hirsutosterols A–G (**1–7**), were isolated from the Formosan soft coral *Cladiella hirsuta*. Their structures were elucidated by spectroscopic methods, particularly in 1D and 2D NMR experiments. The absolute configurations of **1** and **5** were determined by Mosher's method. Sterols **4–6** possess hydroxy groups at C-9 and C-11 and might be oxidatively cleaved to the corresponding 9,11-secosterols. Hirsutosterol A (**1**) was found to exhibit a stronger cytotoxicity against a limited panel of cancer cell lines.

Introduction

In the course of our chemical study on the octocorals, metabolites with promising anti-inflammatory and/or cytotoxic activities, including sesquiterpenoids,[1,2] cembranoids,[3–5] eunicellins[6–8] and steroids,[9–10] have been discovered. As many secondary metabolites have been isolated from soft corals of the genus *Cladiella*,[11–25] we investigated the chemical constituents of a Formosan soft coral *Cladiella hirsuta* which has not been chemically studied before with the aim of discovering bioactive natural products. The above study has led to the isolation of a series of eunicellin-type metabolites.[26] In this paper, we report the isolation, structure determination and biological activity of seven new polyoxygenated steroids, hirsutosterols A–G (**1–7**, Scheme 1), from further investigation of the soft coral *C. hirsuta*. The structures of **1–7** were established by extensive spectroscopic analysis, including 2D NMR (^{1}H–^{1}H COSY, HSQC, HMBC, and NOESY) spectroscopy. Cytotoxicity of metabolites **1–7** against a limited panel of human tumor cell lines including human liver carcinoma (Hep G2 and Hep G3B), human breast carcinoma (MDA-MB-231 and MCF-7) human lung carcinoma (A-549), and human oral cancer cells (Ca9-22) was investigated, and the ability of **1–7** to inhibit up-regulation of the pro-inflammatory iNOS (inducible nitric oxide synthase) and COX-2 (cyclooxygenase-2) proteins in LPS (lipopolysaccharide)-stimulated RAW264.7 macrophage cells was also evaluated. Herein, we report the isolation, structure elucidation and bioactivities of their natural products.

Results and discussion

The soft coral was extracted with acetone. The organic extract was concentrated to an aqueous suspension and was further partitioned between EtOAc and water. The combined EtOAc-soluble fraction was concentrated under reduced pressure and the residue was repeatedly purified by chromatography to yield metabolites **1–7**.

Hirsutosterol A (**1**) was isolated as a white power. Its HRESIMS exhibited a $[M + Na]^+$ peak at m/z 513.3552 and established a molecular formula $C_{30}H_{50}O_5$, implying six degrees of unsaturation. The IR spectrum of **1** revealed the presence of hydroxy, ester, and conjugated enone functionalities from absorptions of 3317, 1737, and 1669 cm^{-1}. The ^{13}C NMR spectroscopic data of **1** exhibited thirty carbon signals (Table 1), which were assigned by the assistance of DEPT spectrum to seven methyls, nine methylenes (including one oxymethylene), nine sp^3 methines (including two oxymethines), and three sp^2 and two sp^3 quaternary carbons. The carbonyl and olefinic resonances in the ^{13}C NMR spectrum data of **1** (Table 1) appeared at δ 205.1 (qC), 147.8 (CH) and 136.6 (qC), respectively, revealing the presence of a conjugated enone. The 3H singlet appearing at δ 2.04 in the ^{1}H NMR spectrum (Table 3) and the carbonyl signal at δ 170.5 in the ^{13}C NMR spectrum were ascribable to an acetoxy group. Therefore, the remaining three degrees of unsaturation identified compound **1** as a tricyclic compound. Detailed analysis of the ^{1}H–^{1}H COSY and HMBC correlations (Fig. 1) further established the molecular skeleton of **1** as a 9,11-secosterol derivative bearing two hydroxy groups at C-6 and C-11, one acetoxy group at C-3, and a conjugated enone at olefinic carbons C-7 and C-8, and carbonyl carbon C-9. From the above results, the structure of compound **1** was shown to be very similar to

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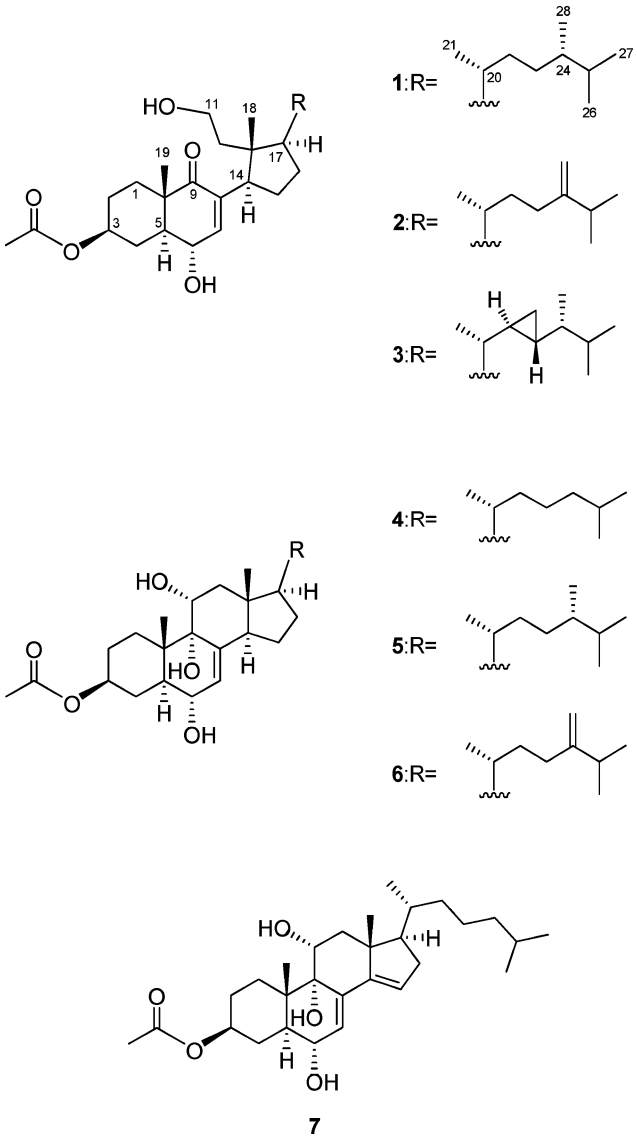

Scheme 1

that of a known compound, 3β,6α,11-trihydroxy-9,11-seco-5α-cholest-7-ene-9-one.[27] The relative configuration of **1** was mostly confirmed to be the same as that of 3β,6α,11-trihydroxy-9,11-seco-5α-cholest-7-ene-9-one by comparison of the chemical shifts and coupling constants for the protons of both compounds and was further confirmed by NOE correlations (Fig. 2). Furthermore, the 24*S* configuration of **1** was determined by comparison of the NMR data with those of yonarasterol B which was isolated from the soft coral *Clavularia viridis*.[28] The proton shift of H_3-28, δ_H = 0.78 ppm, was found to be identical with that of yonarasterol B. Also, the carbon shifts of C-21–C-28 are in excellent agreement with those of yonarasterol B and (24*S*)-methylcholestanol (*vs* those of (24*R*)-methylcholestanol).[29] Thus, the structure of compound **1** was fully established. In order to resolve the absolute structure of **1**, we determined the configuration at C-6 using Mosher's method.[30,31] The *S*- and *R*-α-methoxy-α-trifluoromethylphenylacetic (MTPA) esters of **1** (**1a** and **1b**, respectively) were prepared by using the corresponding *R*-(−)- and *S*-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chlorides, respectively. The values

Table 1 ^{13}C NMR data for compounds **1–4**

Position	**1**[a]	**2**[b]	**3**[c]	**4**[b]
1	31.6, CH_2[d]	31.6, CH_2	31.6, CH_2	31.8, CH_2
2	26.4, CH_2	26.4, CH_2	26.7, CH_2	27.2, CH_2
3	72.2, CH	72.0, CH	72.0, CH	72.6, CH
4	29.0, CH_2	29.0, CH_2	29.0, CH_2	30.2, CH_2
5	48.3, CH	48.4, CH	48.5, CH	42.7, CH
6	69.0, CH	69.4, CH	69.4, CH	69.9, CH
7	147.8, CH	147.2, CH	147.2, CH	127.9, CH
8	136.6, qC	136.9, qC	137.1, qC	140.0, qC
9	205.1, qC	204.9, qC	204.9, qC	74.3, qC
10	44.7, qC	44.8, qC	44.8, qC	40.5, qC
11	59.2, CH_2	59.4, CH_2	59.4, CH_2	69.4, CH_2
12	40.8, CH_2	40.8, CH_2	40.8, CH_2	46.8, CH_2
13	46.0, qC	46.1, qC	46.2, qC	42.9, qC
14	42.5, CH	42.5, CH	42.3, CH	50.6, CH
15	26.5, CH_2	26.6, CH_2	27.5, CH_2	22.9, CH_2
16	26.2, CH_2	26.2, CH_2	26.6, CH_2	27.9, CH_2
17	49.5, CH	49.4, CH	50.5, CH	55.8, CH
18	17.3, CH_3	17.3, CH_3	17.2, CH_3	12.2, CH_3
19	16.0, CH_3	16.1, CH_3	15.7, CH_3	15.5, CH_3
20	35.3, CH	34.9, CH	39.2, CH	36.0, CH
21	18.8, CH_3	18.6, CH_3	18.3, CH_3	18.7, CH_3
22	33.0, CH_2	34.0, CH_2	24.0, CH_2	35.9, CH_2
23	31.2, CH_2	31.5, CH_2	24.6, CH_2	23.8, CH_2
24	39.0, CH	156.6, qC	44.8, qC	39.4, CH
25	31.4, CH	33.7, CH	32.8, CH	28.0, CH
26	17.5, CH_3	21.8, CH_3	18.5, CH_3	22.5, CH_3
27	20.4, CH_3	21.9, CH_3	20.7, CH_3	22.8, CH_3
28	15.4, CH_3	106.1, CH_2	16.7, CH_3	
29			10.5, CH_2	
3-OAc	170.5, qC	170.5, qC	170.5, qC	170.6, qC
	21.2, CH_3	21.3, CH_3	21.3, CH_3	21.4, CH_3

[a] 75 MHz in $CDCl_3$. [b] 100 MHz in $CDCl_3$. [c] 125 MHz in $CDCl_3$. [d] Multiplicities deduced by DEPT.

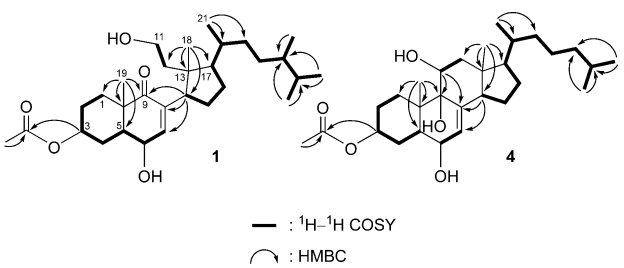

Fig. 1 Key ^{1}H–^{1}H COSY and HMBC correlations of **1** and **4**.

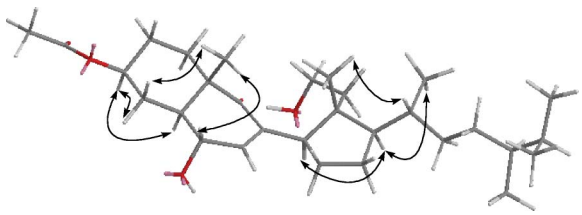

Fig. 2 Key NOESY correlations of **1**.

of $\Delta\delta$ [δ(*S*-MTPA ester) – δ(*R*-MTPA ester)] for H-7 and H-14 were negative, while the values of $\Delta\delta$ for H-3, H-5 and 3-OAc were positive, revealing the *S*-configuration at C-6 (Fig. 3). It was found that the 3-*O*-deacetyl derivative of **1** has been isolated from a soft coral.[32]

The molecular formula of hirsutosterol B (**2**) was assigned as $C_{30}H_{48}O_5$ from the HRESIMS and NMR data (Tables 1 and 2).

Table 2 ^{13}C NMR data for compounds **5–7**

Position	**5**[a]	**6**[b]	**7**[c]
1	31.8, CH_2[d]	31.8, CH_2	31.7, CH_2
2	27.2, CH_2	27.2, CH_2	27.1, CH_2
3	72.8, CH	72.6, CH	72.5, CH
4	30.3, CH_2	30.2, CH_2	30.2, CH_2
5	42.6, CH	42.7, CH	42.8, CH
6	69.8, CH	69.9, CH	69.8, CH
7	127.8, CH	127.9, CH	127.7, CH
8	139.9, qC	140.0, qC	139.8, qC
9	74.4, qC	74.3, qC	74.9, qC
10	40.5, qC	40.5, qC	40.6, qC
11	69.4, CH_2	69.4, CH_2	69.7, CH_2
12	46.7, CH_2	46.8, CH_2	43.4, CH_2
13	42.9, qC	42.9, qC	43.8, qC
14	50.6, CH	50.6, CH	149.1, qC
15	23.0, CH_2	22.9, CH_2	118.4, CH
16	27.8, CH_2	27.8, CH_2	26.4, CH_2
17	55.7, CH	55.6, CH	49.1, CH
18	12.2, CH_3	12.2, CH_3	19.2, CH_3
19	15.5, CH_3	15.5, CH_3	15.8, CH_3
20	36.4, CH	36.0, CH	31.9, CH
21	18.9, CH_3	18.7, CH_3	15.3, CH_3
22	33.5, CH_2	34.3, CH_2	23.2, CH_2
23	30.6, CH_2	30.9, CH_2	26.2, CH_2
24	39.0, CH	156.6, qC	33.9, CH
25	31.5, CH	33.8, CH	38.1, CH
26	17.6, CH_3	21.8, CH_3	18.0, CH_3
27	20.5, CH_3	22.0, CH_3	20.2, CH_3
28	15.4, CH_3	106.1, CH_2	
29			
3-OAc	170.7, qC	170.6, qC	170.6, qC
	21.4, CH_3	21.4, CH_3	21.4, CH_3

[a] 75 MHz in $CDCl_3$. [b] 100 MHz in $CDCl_3$. [c] 125 MHz in $CDCl_3$. [d] Multiplicities deduced by DEPT.

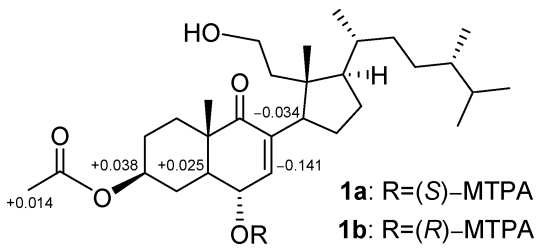

Fig. 3 ^{1}H NMR chemical shift differences $\Delta\delta$ ($\delta_S - \delta_R$) in ppm for the MTPA esters of **1**.

Compound **2** is the 24,28-dehydrogenated derivative of **1**, as shown by the two exocyclic methylene protons resonating at δ 4.72 (s) and 4.66 (s). This was further confirmed by the HMBC correlations from H_3-20 to C-17, C-20, C-22; H_2-23 to C-24; H_3-26 and H_3-27 to C-24, C-25; and H_2-28 to C-23, C-25. Thus, the structure of compound **2** was established. The 3-*O*-deacetyl derivative of **2** has been discovered from marine invertebrates.[33–35]

Hirsutosterol C (**3**) was obtained as a white power that gave a pseudomolecular ion peak at *m/z* 525.3559 [M + Na]$^+$ in the HRESIMS, consistent with the molecular formula $C_{31}H_{50}O_5$ and implying seven degrees of unsaturation. The ^{1}H and ^{13}C NMR spectral data of the A–D rings in **3** were nearly identical with those of **1** and **2**. Furthermore, a cyclopropyl group was found to be present in the side chain [δ_H 0.54 (1H, m), 0.35 (1H, m), 0.15 (2H, m); δ_C 24.6 (CH), 24.0 (CH), 10.5 (CH_2)] of **3**. A methyl substitution at C-24 was revealed by ^{1}H NMR (δ 0.86) and ^{13}C NMR (δ 16.7) data. By comparison of the proton shifts of H_3-21, H_3-26, H_3-27, and H_3-28 with those of the four synthetic demethylgorgosterol isomers,[36] it was suggested that the stereochemistry of the side chain in **3** should be assigned as 22*R*, 23*R*, and 24*R* (Fig. 4). Thus, the structure of compound **3** was established.

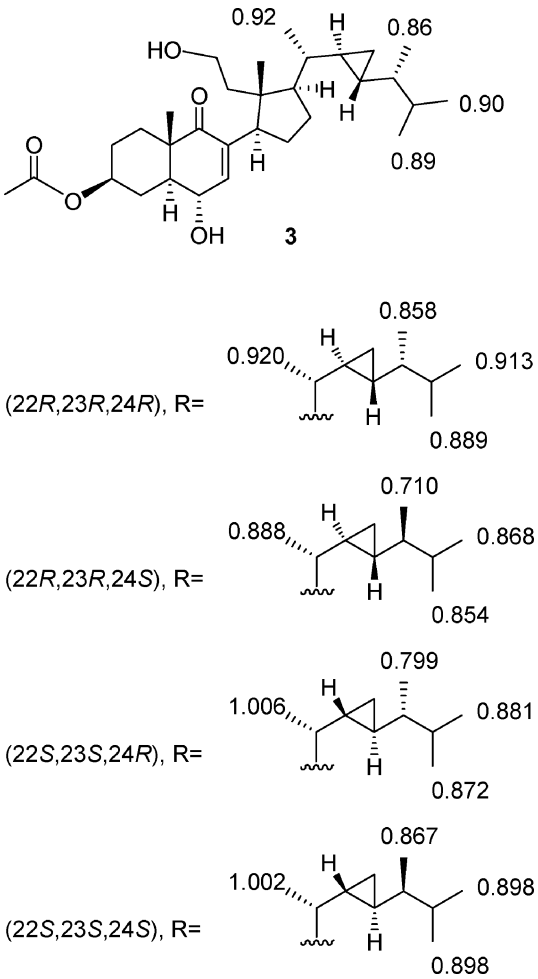

Fig. 4 ^{1}H NMR chemical shifts of the side-chain methyl groups of **3** and synthetic isomers of demethylgorgosterols.[36]

Hirsutosterol D (**4**) was isolated as a white power. Its molecular formula, $C_{29}H_{48}O_5$, was established by the HRESIMS spectrum (*m/z* 499.3397 [M + Na]$^+$) and ^{13}C NMR data, implying six degrees of unsaturation. The IR absorptions were observed at 3329 and 1733 cm^{-1}, suggesting the presence of a hydroxy and carbonyl group. The structure of this compound was deduced from its ^{13}C NMR and DEPT spectra, which showed that the compound has 29 carbons, including six methyls, nine sp^3 methylenes, one sp^2 methine, eight sp^3 methines (including three oxymethines), and two sp^2 and three sp^3 quaternary carbons. From the ^{1}H and ^{13}C NMR spectra (Tables 1 and 3), **4** was found to possess one acetoxy group [δ_H 2.03, s; δ_C 170.6 (qC), 21.4 (CH_3)], in addition to one trisubstituted olefin [δ_H 5.43, (br s), δ_C 140.0 (qC), 127.9 (CH)]. Detailed analysis of the ^{1}H–^{1}H COSY and HMBC correlations (Fig. 1) further established the planar structure of **4** as a cholesterol derivative bearing three hydroxy groups at C-6, C-9 and C-11, one acetoxyl group at C-3, and one 7,8-trisubstituted double bond.

Table 3 ^{1}H NMR data for compounds **1–7**

Position	**1**[a]	**2**[b]	**3**[c]	**4**[b]	**5**[a]	**6**[b]	**7**[c]
1	1.94, m	1.93, m	1.97, m	2.02, m	2.02, m	2.02, m	2.02, m
	1.53, m	1.53, m	1.55, m	1.76, m	1.76, m	1.76, m	1.78, m
2	1.86, m	1.86, m	1.95, m	1.86, m	1.86, m	1.84, m	1.86, m
	1.50, m	1.50, m	1.52, m	1.50, m	1.50, m	1.47, m	1.49, m
3	4.64, m	4.66, m	4.66, m	4.68, m	4.65, m	4.66, m	4.68, m
4	2.35 br d (11.9)[d]	2.36 br d (12.0)	2.35 br d (12.5)	2.36, m	2.36, m	2.35, m	2.38, m
	1.49, m	1.54, m	1.54, m	1.37, m	1.37, m	1.33, m	1.37, m
5	1.84, m	1.84, m	1.85, m	1.76, m	1.76, m	1.74, m	1.78, m
6	4.24, d (9.6)	4.26, d (9.8)	4.26, d (8.5)	3.70, d (9.6)	3.68, d (8.8)	3.70, d (9.6)	3.71, d (9.5)
7	6.60, br s	6.59, br s	6.59, br s	5.43, br s	5.41, br s	5.43, br s	5.49, br s
11	3.84, m	3.89, m	3.90, m	4.11, dd (10.8, 5.2)	4.09, m	4.11, dd (10.4, 4.8)	4.18, t (5.0)
	3.65, m	3.67, m	3.68, m				
12	1.60, m	1.60, m	1.65, m	2.15, m	2.15, m	2.15, m	2.01, m
	1.03, m	1.04, m	1.04, m	1.52, m	1.52, m	1.50, m	1.53, m
14	3.38, t (9.7)	3.43, t (9.2)	3.44, t (9.0)	2.34, m	2.34, m	2.34, m	
15	1.91, m	1.95, m	2.11, m	1.56, m	1.56, m	1.58, m	5.02, t (7.0)
	1.56, m	1.52, m	1.60, m	1.38, m	1.38, m	1.35, m	
16	1.58, m	1.58, m	1.64, m	1.95, m	1.95, m	1.95, m	2.36, m
	1.40, m	1.42, m	1.64, m	1.28, m	1.28, m	1.32, m	1.94, m
17	1.70, m	1.75, m	1.84, m	1.30, m	1.29, m	1.36, m	2.33, m
18	0.63, s	0.64, s	0.62, s	0.59, s	0.58, s	0.59, s	0.66, s
19	1.14, s	1.15, s	1.15, s	1.03, s	1.02, s	1.03, s	1.04, s
20	1.35, m	1.44, m	0.93, m	1.34, m	1.32, m	1.37, m	1.56, m
21	0.95, d (6.3)	0.99, d (6.8)	0.92, d (6.0)	0.94, d (6.0)	0.95, d (6.5)	0.97, d (6.8)	0.79, d (7.0)
22	1.42, m	1.55, m	0.35, m	1.36, m	1.42, m	1.54, m	1.75, m
	0.94, m	1.15, m		0.97, m	0.94, m	1.15, m	1.54, m
23	1.38, m	2.10, m	0.54, m	1.32, m	1.38, m	2.07, m	2.36, m
	0.93, m	1.86, m		1.15, m	0.93, m	1.87, m	1.88, m
24	1.21, m		0.54, m	1.35, m	1.21, m		1.35, m
				1.12, m			1.12, m
25	1.55, m	2.22, m	1.68, m	1.52, m	1.55, m	2.22, m	1.28, m
26	0.78, d (6.7)	1.02, d (6.8)	0.89, d (7.0)	0.86, d (6.8)	0.78, d (6.5)	1.02, d (6.8)	0.80, d (7.0)
27	0.85, d (6.7)	1.03, d (6.8)	0.90, d (7.0)	0.88, d (6.8)	0.85, d (6.5)	1.03, d (6.8)	0.85, d (7.0)
28	0.78, d (6.7)	4.72, s	0.86, d (7.0)		0.78, d (6.5)	4.72, s	
		4.66, s				4.66, s	
29			0.15, m				
3-OAc	2.04, s	2.05, s	2.05, s	2.03, s			

[a] 300 MHz in $CDCl_3$. [b] 400 MHz in $CDCl_3$. [c] 500 MHz in $CDCl_3$. [d] J values (in Hz) in parentheses.

In the NOESY spectrum of **4** (Fig. 5), observation of the NOE correlations between H_3-18 with both H-20 and H-11, and H_3-19 with H-11, and H-6 suggested that H-20, H_3-19, H_3-18, H-11, and H-6 are β-oriented. Also, correlations between H-3 with both H-5 and H-4α (δ 2.36); and H-17 with both H_3-21 and H-14 suggested that H_3-21, H-17, H-14, H-5, and H-3 are all α-oriented. Sterol compounds with a similar oxidation pattern have been previously synthesised from $\Delta^{7,9(11)}$-sterols.

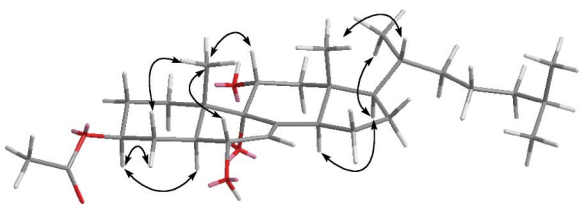

Fig. 5 Key NOESY correlations of **4**.

The HRESIMS spectrum of hirsutosterol (**5**) exhibited a pseudomolecular ion peak at m/z 513.3558 $[M + Na]^+$, consistent with a molecular formula of $C_{30}H_{50}O_5$. A comparison of the NMR data of **5** (Tables 2 and 3) with those of **4** and **1** showed that **5** has the same A–D rings as that of **4** and the identical side chain (C-20–C-28) as that of **1**. The absolute configuration of **5** was also determined by the use of a Mosher's method. The (*S*)- and (*R*)-MTPA esters of **5** (**5a** and **5b**, respectively) were prepared using the corresponding *R*-(–)- and *S*-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chlorides, respectively. The determination of the chemical shift differences ($\delta_S - \delta_R$) for the protons neighboring C-6 led to the assignment of an *S* configuration at C-6 of **5** (Fig. 6), respectively. Thus, the absolute structure of sterol **5** was established. Similarly, the HRESIMS of

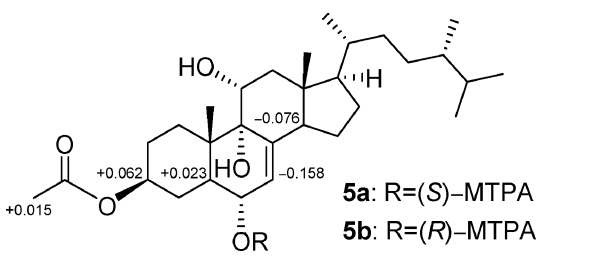

Fig. 6 ^{1}H NMR chemical shift differences $\Delta\delta$ ($\delta_S - \delta_R$) in ppm for the MTPA esters of **5**.

Table 4 Cytotoxicity data for compounds **1–7**

Cell lines IC_{50} (μM)						
Compound	Hep G2	Hep G3B	Ca9-22	A549	MCF-7	MDA-MB-231
1	16.9	9.4	8.2	18.4	17.8	16.1
2	[a]—	8.6	16.0	—	—	—
3	30.1	13.9	11.6	31.3	39.4	30.1
4	30.9	22.5	20.2	31.7	33.4	31.3
5	—	—	—	18.4	—	—
6	32.0	15.2	17.6	—	34.6	26.8
7	35.0	28.1	26.6	38.4	29.7	42.0
Doxorubicin	0.4	1.3	0.2	2.6	2.9	2.0

[a] $IC_{50} > 50$ μM.

hirsutosterol F (**6**) exhibited a $[M + Na]^+$ peak at m/z 511.3396 and established a molecular formula of $C_{30}H_{48}O_5$. The ^1H and ^{13}C NMR signals of **6** showed that **6** has identical A–D rings as those of **4** and **5**, and the same side chain as that of **2**. Thus, the structure of compound **6** was established.

Hirsutosterol G (**7**), was further isolated as a white solid. It's molecular formula, $C_{29}H_{46}O_5$, was established by HRESIMS. The spectroscopic data of **7** (IR, ^1H and ^{13}C NMR) were similar to those of **4**, except that a 14,15-carbon, carbon single bond in **4** [δ_C 50.6, CH, C-14 and 22.9, CH_2, C-15] were replaced by signals of a trisubstituted double bond [δ_C 149.1, CH, C-14 and 118.4, CH_2, C-15] in **7**. This was further confirmed by HMBC correlations observed from H_3-18 (δ 0.66, s) to C-14 (δ 149.1). The structure of compound **7** was thus established.

The cytotoxicity of compounds **1–7** against the proliferation of a limited panel of cancer cell lines, including human liver (Hep G2 and Hep G3B), breast (MDA-MB-23) and gingival (Ca9-22) carcinoma cells, was evaluated (Table 4). The results showed that secosterol **1**, the more potent one of compounds **1–7**, exhibited cytotoxicity towards Hep G2, Hep G3B, MDA-MB-23, and Ca9-22 cancer cell lines with IC_{50}s of 16.9, 9.4, 8.2, 18.4, 17.8 and 16.1 μM, respectively. Secosterols **2** and **3** were found to exhibit cytotoxicity towards Hep G3B and Ca9-22 cancer cells. The *in vitro* anti-inflammatory effects of compounds **1–7** were also tested, however, all of **1–7** did not show significant activity in inhibiting the expression of the pro-inflammatory iNOS and COX-2 proteins in LPS-stimulated RAW264.7 macrophage cells.

Compounds **4–7** were isolated from the same organism, thus from a biosynthetic consideration they should possess identical absolute configurations at the chiral centers C-3, C-5, C-6, C-10, C-14, and C-17, in the A–D rings of the steroids.

Conclusion

Steroids **4–6** possess hydroxy groups at both vicinal carbons, C-9 and C-11, thus, compounds of this type might be oxidatively cleaved to form 9,11-secosterols, such as **1–3**. Cholest-7-ene-3β,6α,9α,11α-tetraols (**4–6**) and cholesta-7,14-diene-3β,6α,9α,11α-tetraol (**7**) have unique oxidation patterns which were discovered for the first time. The cytotoxicity data suggest that the 9,11-secosterols **1–3**, in particular **1**, are worthy of further anti-tumor study.

Experimental

General experimental procedures

Optical rotations were measured on a JASCO P-1020 polarimeter. IR spectra were recorded on a JASCO FT/IR-4100 infrared spectrophotometer. ESIMS were obtained with a Bruker APEX II mass spectrometer. The NMR spectra were recorded on a Varian Unity INOVA 500 FT-NMR at 500 MHz for ^1H and 125 MHz for ^{13}C or on a Varian 400 MR FT-NMR at 400 MHz for ^1H and 100 MHz for ^{13}C, or on a Bruker AVANCE-DPX 300 FT-NMR at 300 MHz for ^1H and 75 MHz for ^{13}C, respectively. Silica gel (Merck, 230–400 mesh) was used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60 F-254, 0.2 mm) were used for analytical TLC. High-performance liquid chromatography was performed on a Hitachi L-7100 HPLC apparatus with a C-18 column (250 ×21.2 mm, 5 μm).

Extraction and isolation

The frozen bodies of *C. hirsuta* (3.1 kg, wet wt.) were sliced and exhaustively extracted with acetone (3 × 10 L). The organic extract was concentrated to an aqueous suspension and was further partitioned between EtOAc and H_2O. The EtOAc layer was dried with anhydrous Na_2SO_4. After removal of the solvent *in vacuo*, the residue (32.8 g) was subjected to column chromatography on silica gel and eluted with EtOAc in *n*-hexane (0–100% of EtOAc, gradient) and further with MeOH in EtOAc of increasing polarity to yield 25 fractions. Fraction 17, eluted with *n*-hexane–EtOAc (2 : 1), was rechromatographed over a Sephadex LH-20 column, using acetone as the mobile phase to afford four subfractions (A1–A4). Subfraction A2 was separated by reverse-phase HPLC (MeOH–H_2O, 5 : 1 to 3 : 1) to afford compounds **1** (57.8 mg), **2** (15.6 mg), **3** (1.1 mg), **4** (4.5 mg), **5** (10.2 mg), **6** (4.2 mg), and **7** (1.4 mg), respectively. For ^1H, ^{13}C NMR, and ESIMS spectra, see ESI.†

Hirsutosterol A (1): white power; $[\alpha]_D^{25}$ +26 (*c* 5.78, $CHCl_3$); IR (neat) ν_{max} 3317, 1737 and 1669 cm^{-1}; ^{13}C and ^1H NMR data (300 MHz; $CHCl_3$), see Tables 1 and 3; ESIMS m/z 513 $[M + Na]^+$; HRESIMS m/z 513.3552 $[M + Na]^+$ (calcd for $C_{30}H_{50}O_5Na$, 513.3556).

Hirsutosterol B (2): white power; $[\alpha]_D^{25}$ +32 (*c* 1.56, $CHCl_3$); IR (neat) ν_{max} 3343, 1737 and 1673 cm^{-1}; ^{13}C and ^1H NMR data (400 MHz; $CHCl_3$), see Tables 1 and 3; ESIMS m/z 511 $[M + Na]^+$; HRESIMS m/z 511.3396 $[M + Na]^+$ (calcd for $C_{30}H_{48}O_5Na$, 511.3399).

Hirsutosterol C (3): white power; $[\alpha]_D^{25}$ +33 (*c* 0.11, $CHCl_3$); IR (neat) ν_{max} 3384, 1735, and 1671 cm^{-1}; ^{13}C and ^1H NMR data (500 MHz; $CDCl_3$), see Tables 1 and 3; ESIMS m/z 525 $[M + Na]^+$; HRESIMS m/z 525.3559 $[M + Na]^+$ (calcd for $C_{31}H_{50}O_5Na$, 525.3556).

Hirsutosterol D (4): white power; $[\alpha]_D^{25}$ −44 (*c* 0.45, $CHCl_3$); IR (neat) ν_{max} 3329 and 1733 cm^{-1}; ^{13}C and ^1H NMR data (400 MHz; $CDCl_3$), see Tables 1 and 3; ESIMS m/z 499 $[M + Na]^+$; HRESIMS m/z 499.3397 $[M + Na]^+$ (calcd for $C_{29}H_{48}O_5Na$, 499.3399).

Hirsutosterol E (5): white power; $[\alpha]_D^{25}$ −22 (*c* 1.02, $CHCl_3$); IR (neat) ν_{max} 3336 and 1735 cm^{-1}; ^{13}C and ^1H NMR data (300 MHz; $CDCl_3$), see Tables 2 and 3; ESIMS m/z 513 $[M + Na]^+$; HRESIMS m/z 513.3558 $[M + Na]^+$ (calcd for $C_{30}H_{50}O_5Na$, 513.3556).

Hirsutosterol F (6): white power; $[\alpha]_D^{25}$ −13 (*c* 0.42, $CHCl_3$); IR (neat) ν_{max} 3328 and 1733 cm^{-1}; ^{13}C and ^1H NMR data (400 MHz;

$CDCl_3$), see Tables 2 and 3; ESIMS *m/z* 511 $[M + Na]^+$; HRESIMS *m/z* 511.3396 $[M + Na]^+$ (calcd for $C_{30}H_{48}O_5Na$, 511.3399).

Hirsutosterol G (7): white power; $[\alpha]_D^{25}$ −16 (*c* 0.14, $CHCl_3$); IR (neat) ν_{max} 3355 and 1733 cm^{-1}; ^{13}C and ^1H NMR data (500 MHz; $CDCl_3$), see Tables 2 and 3; ESIMS *m/z* 497 $[M + Na]^+$; HRESIMS *m/z* 497.3246 $[M + Na]^+$ (calcd for $C_{29}H_{46}O_5Na$, 497.3243).

Preparation of (*S*)-and (*R*)-MTPA Esters of 1

To a solution of **1** (0.5 mg) in pyridine (0.4 mL) was added *R*-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl (MTPA) chloride (25 μL), and the mixture was allowed to stand for 24 h at room temperature. The reaction was quenched by addition of 1.0 mL of water, and the mixture was subsequently extracted with EtOAc (3 × 1.0 mL). The EtOAc-soluble layers were combined, dried over anhydrous $MgSO_4$ and evaporated. The residue was subjected to column chromatography over silica gel using *n*-hexane–EtOAc (6 : 1) to yield the (*S*)-MTPA ester, **1a** (0.7 mg, 74%). The same procedure was used to prepare the (*R*)-MTPA ester, **1b** (0.6 mg, 63%) from the reaction of (*S*)-MTPA chloride with **1** in pyridine. Selective ^1H NMR ($CDCl_3$, 400 MHz) of **1a**: 6.204 (1H, s, H-7), 5.623 (1H, d, *J* = 10.0, H-6), 4.554 (1H, m, H-3), 3.270 (1H, m, H-14), 2.123 (1H, m, H-5), 2.033 (3H, s, 3-OAc). Selective ^1H NMR ($CDCl_3$, 400 MHz) of **1b**: δ 6.345 (1H, s, H-7), 5.597 (1H, d, *J* = 10.0, H-6), 4.363 (1H, m, H-3), 3.304 (1H, m, H-14), 2.019 (1H, m, H-5), 2.019 (1H, s, 3-OAc).

Preparation of (*S*)-and (*R*)-MTPA esters of 5

To a solution of **5** (0.5 mg) in pyridine (0.4 mL) was added *R*-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl (MTPA) chloride (25 μL), and the mixture was allowed to stand for 24 h at room temperature. The reaction was quenched by addition of 1.0 mL of water, and the mixture was subsequently extracted with EtOAc (3 × 1.0 mL). The EtOAc-soluble layers were combined, dried over anhydrous $MgSO_4$ and evaporated. The residue was subjected to column chromatography over silica gel using *n*-hexane–EtOAc (6 : 1) to yield the (*S*)-MTPA ester, **5a** (0.6 mg, 83%). The same procedure was used to prepare the (*R*)-MTPA ester, **5b** (0.6 mg, 83%) from the reaction of (*S*)-MTPA chloride with **5** in pyridine. Selective ^1H NMR ($CDCl_3$, 400 MHz) of **5a**: 5.190 (1H, d, *J* = 10.4, H-6), 5.178 (1H, s, H-7), 4.584 (1H, m, H-3), 2.293 (1H, m, H-14), 2.122 (1H, m, H-5), 2.025 (3H, s, 3-OAc). Selective ^1H NMR ($CDCl_3$, 400 MHz) of **5b**: δ 5.336 (1H, s, H-7), 5.174 (1H, d, *J* = 10.4, H-6), 4.522 (1H, m, H-3), 2.369 (1H, m, H-14), 2.099 (1H, m, H-5), 2.010 (3H, s, 3-OAc).

Cytotoxicity testing

Cell lines were purchased from the American Type Culture Collection (ATCC). Cytotoxicity assays were performed using the MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method.[37,38]

In vitro anti-inflammatory assay

Macrophage (RAW264.7) cell line was purchased from ATCC. The *in vitro* anti-inflammatory activity of compounds **1–7** were measured by examining the inhibition of lipopolysaccharide (LPS) induced upregulation of iNOS (inducible nitric oxide synthetase) and COX-2 (cyclo-oxygenase-2) proteins in macrophage cells using Western blotting analysis.[39,40]

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