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Steroid and cembranoids from the Dongsha atoll soft coral *Lobophytum sarcophytoides*

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ABSTRACT

A novel sterol, sarcophytosterol (**1**), and four new cembranoids, sarcophytolins A–D (**2**–**5**), have been isolated from the soft coral *Lobophytum sarcophytoides* along with four known metabolites **6**–**9**. The relative structures of new metabolites were elucidated on the basis of extensive spectroscopic analyses and X-ray diffraction analysis of **1**, and the absolute configurations of **1** and **5** were determined by Mosher's method and CD spectrum, respectively. Compound **1** is a steroid possessing an unusual (20*R*,23*R*,24*R*)-23,24-dimethyl-20-hydroxy side chain. Compounds **3**, **6**, **8**, and **9** were shown to exhibit cytotoxicity toward a limited panel of cancer cell lines. Furthermore, while **1** could effectively enhance the expression of the pro-inflammatory iNOS protein in LPS-stimulated RAW264.7 macrophage cells, compounds **2**, **3**, **5**–**7** and **9** were found to effectively inhibit the accumulation of the pro-inflammatory of iNOS.

1. Introduction

Worldwide chemical investigations on marine invertebrates have demonstrated structural diversity in the terpenoid and steroid constituents of octocorals.[1] In the course of our chemical studies on the octocorals, metabolites with interesting bioactivity from Formosan soft corals, including sesquiterpenoids,[2,3] diterpenoids,[4–7] and steroids,[8–10] have been isolated. Recently, Taiwanese soft corals of the *Lobophytum* genus have been shown to be the important sources of the bioactive terpenoids,[11–14] and steroids. Our investigation of natural products from the Dongsha atoll soft coral, *Lobophytum sarcophytoides* (Moser, 1919, family Alcyoniidae), has also led to the isolation of five new compounds, sarcophytosterol (**1**) and sarcophytolins A–D (**2**–**5**), along with four known metabolites **6**–**9**.[15–17] The relative structures of new compounds were established, and that of **1** was further confirmed by X-ray analysis. The absolute configuration of **1** was determined by Mosher's method while that of **5** was established by CD spectrum. Some of metabolites **1**–**9** were found to exhibit cytotoxicity toward a limited panel of human tumor cell lines, including breast adenocarcinoma (MCF-7), colon adenocarcinoma (WiDr), laryngeal adenocarcinoma (HEp 2), medulloblastoma (Daoy), T-cell acute lymphoblastic leukemia (CCRF-CEM), and colon adenocarcinoma (DLD-1). The ability of **1**–**9** to inhibit the up-regulation of pro-inflammatory iNOS (inducible nitric oxide synthase) and COX-2 (cyclooxygenase-2) proteins in LPS (lipopolysaccharide)-stimulated RAW264.7 macrophage cells was also evaluated. The results revealed that compounds **3**, **6**, **8**, and **9** showed some extent of cytotoxicity toward the above cancer cell lines. Compounds **2**, **3**, **5**–**7**, and **9** were found to effectively inhibit the expression of the pro-inflammatory iNOS protein. On the contrary, **1** could significantly enhance the accumulation of iNOS.

2. Results and discussion

Sarcophytosterol (**1**), $[\alpha]_D^{25}$ −77 (*c* 0.28, $CHCl_3$), was obtained as colorless crystal, mp 121–125 °C, and had the molecular formula $C_{29}H_{48}O_2$ as determined by HRESIMS (*m*/*z* calcd 451.3552, found 451.3549 $[M+Na]^+$), requiring six degrees of unsaturation. The infrared spectrum of **1** showed absorption of hydroxy group (ν_{max} 3392 cm^{-1}). The ^{13}C NMR spectroscopic data of **1** (Table 1) revealed the presence of 29 carbon signals, which were identified by the assistance of DEPT spectrum into seven methyls, eight sp^3 methylenes, seven sp^3 methines (including an oxygenated carbon resonating at δ 71.8), two sp^2 methines, five sp^3 quaternary carbons

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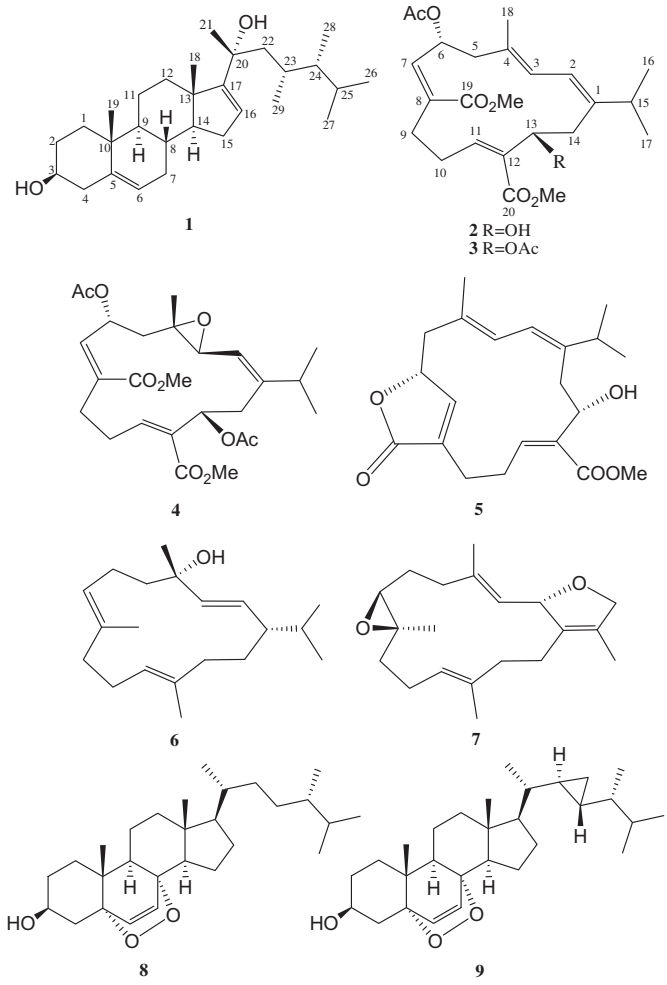


Table 1
^{1}H and ^{13}C NMR data of **1**

C/H	**1**	
	^{1}H[a]	^{13}C[b]
1	1.09 m; 1.86 m	37.1 (CH_2)[d]
2	1.51 m; 1.84 m	31.6 (CH_2)
3	3.53 m	71.8 (CH)
4	2.24 m; 2.30 m	42.3 (CH_2)
5		141.0 (qC)
6	5.37 d (4.0)[c]	121.5 (CH)
7	1.62 m; 2.01 m	31.5 (CH_2)
8	1.69 m	30.4 (CH)
9	1.02 m	50.3 (CH)
10		36.6 (qC)
11	1.60 m	20.9 (CH_2)
12	1.59 m; 2.10 m	36.2 (CH_2)
13		47.4 (qC)
14	1.41 m	57.9 (CH)
15	1.87 m; 2.06 m	31.0 (CH_2)
16	5.50 d (1.5)	123.8 (CH)
17		160.9 (qC)
18	1.00 s	18.1 (CH_3)
19	1.04 s	19.3 (CH_3)
20		75.9 (qC)
21	1.37 s	29.6 (CH_3)
22	1.50 m; 1.57 m	49.0 (CH_2)
23	1.83 m	29.6 (CH)
24	1.07 m	45.4 (CH)
25	1.42 m	30.8 (CH)
26	0.86 d (7.0)	21.0 (CH_3)
27	0.89 d (7.0)	21.4 (CH_3)
28	0.76 d (7.0)	11.6 (CH_3)
29	0.78 d (7.0)	15.7 (CH_3)

[a] Spectra recorded at 500 MHz in $CDCl_3$.
[b] Spectra recorded at 125 MHz in $CDCl_3$.
[c] The *J* values are in hertz in parentheses.
[d] Attached protons were determined by DEPT experiments.

(including one oxygenated carbon resonating at δ 75.9, and two olefinic ones resonating at δ_C 141.0 and δ_C 160.9). From the ^{1}H NMR spectrum of **1**, the signals of two olefinic protons (δ_H 5.37, d, J=4.0 Hz and δ_H 5.50, d, J=1.5 Hz), one hydroxymethine group (δ_H 3.53, m), and three singlet methyls (δ_H 1.00, s, δ_H 1.04, s, and δ_H 1.37, s) were also observed. On the basis of the above analyses, it was suggested that **1** is a steroidal compound.

The COSY and HMBC (Fig. 1) correlations were further used to establish the molecular skeleton of **1**. From the $^{1}H-^{1}H$ COSY spectrum of **1**, it was possible to establish four proton sequences (a–d). The HMBC experiment used to connect the above substructures was found to show the following key correlations: H_3-19 to C-1, C-5, C-9, and C-10; H_2-4 to C-5 and C-6; H-6 to C-10; H_3-18 to C-12, C-13, C-14, and C-17; H-16 to C-13, C-17, and C-20; H_3-21 to C-17, C-20, and C-22; and both H_3-28 and H_3-29 to C-23 and C-24. Thus, **1** was found to possess two double bonds at C-5/C-6 and C-16/C-17 positions, two methyl substituents at C-23 and C-24, and a hydroxy group at C-20. The structure of **1** could be determined unambiguously from a single-crystal X-ray diffraction analysis (Fig. 2). Furthermore, the absolute configuration of **1** was finally determined using Mosher's method.[18] The (*S*)- and (*R*)-MTPA esters of **1** (**1a** and **1b**, respectively) were prepared using the corresponding (*R*)-(−) and (*S*)-(+)-MTPA chloride, respectively. The determination of $\Delta\delta$ values ($\delta_S-\delta_R$) for protons neighboring C-3 led to the assignment of the *S* configuration at C-3 in **1** (Fig. 3), and **1** was found to be the sterol with an unusual (20*R*,23*R*,24*R*)-23,24-dimethyl-20-hydroxy side chain.

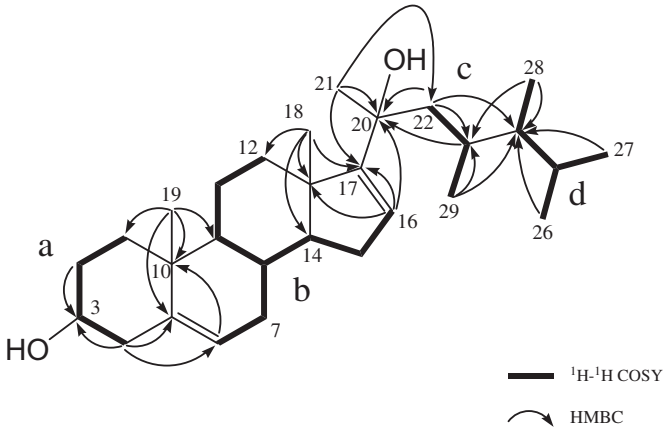


Figure 1. $^{1}H-^{1}H$ COSY and HMBC correlations for **1**.

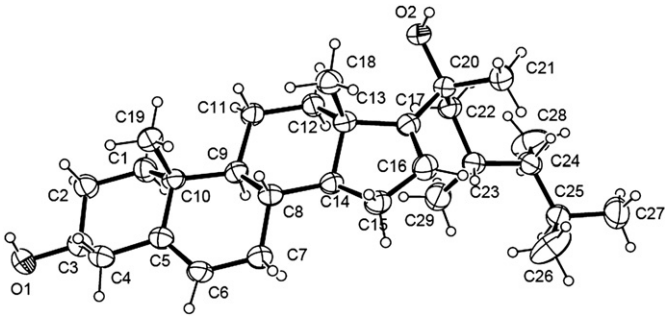
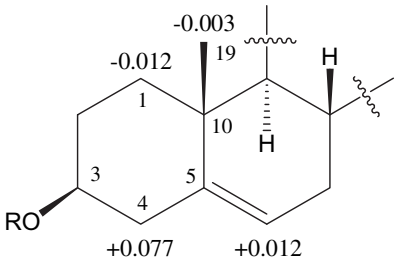


Figure 2. X-ray crystal structure for **1**.



1a: R= (*S*)-MTPA
1b: R= (*R*)-MTPA

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

Sarcophytolin A (**2**) exhibited a pseudomolecular ion peak in the HRESIMS at *m*/*z* 457.2206 $[M+Na]^{+}$, establishing the molecular formula $C_{24}H_{34}O_7$. Thus, eight degrees of unsaturation were determined for **2**. The IR spectrum of **2** revealed the presence of hydroxy (ν_{max} 3444 cm^{-1}) and carbonyl (ν_{max} 1714 cm^{-1}) group. The ^{13}C NMR and DEPT (Table 2) spectral data of **2** showed signals of six methyls, four methylenes, seven methines (including two oxymethines), three carbonyls, and four sp^2 quaternary carbons. The ^{1}H and ^{13}C NMR spectra (Table 2) displayed resonances for one acetoxy moiety including one methyl (δ_H 2.01, 3H, s; δ_C 21.1) and one oxygenated quaternary carbon (δ_c 170.1, qC), one isopropyl moiety including two sp^2 methyls (δ_H 1.01, 3H, d, *J*=7.0 Hz; δ_C 21.2 and δ_H 1.02, 3H, d, *J*=7.0 Hz; δ_C 22.6), two methyl ester moieties including two methyls (δ_H 3.75, s; δ_C 51.6, and δ_H 3.70, s; δ_C 51.3) and two carbonyls (δ_C 168.2 and 167.0, each qC), four trisubstituted double bonds including four sp^2 methines (δ_H 5.98, 1H, d, *J*=11.5 Hz, δ_C 119.8; δ_H 5.92, 1H, d, *J*=11.5 Hz, δ_C 124.9; δ_H 5.64, 1H, d, *J*=9.5 Hz, δ_C 137.1; δ_H 6.03, 1H, t, *J*=7.5 Hz, δ_C 140.0), and four sp^2 quaternary carbons (δ_C 142.6, 131.3, 134.7, and 135.1). The remaining one degree of unsaturation identified **2** as a monocyclic diterpenoid. The gross structure of **2** was further established by 2D NMR experiments. From the ^{1}H–^{1}H COSY spectrum (Fig. 4), it was possible to establish the proton sequences from H-2 to H_3-18 through H-3, H_2-5 to H-7, H-9 to H-11, H-13 to H-14, and H_3-16 to H_3-17 through H_2-15. The key HMBC correlations of H-2, H_2-14, H_3-16, and H_3-17 to C-1; H-2 to C-3; H_3-18 to C-4 and C-5; H-7 to C-9 and C-19; H-9 to C-8 and C-19; H-11 to C-12 and C-13; and H-13 to C-12 and C-20, permitted the connection of the carbon skeleton. In addition, one acetoxy group positioned at C-6 was confirmed from the HMBC correlations of H-6 (δ_H 6.09, td, *J*=9.5, 2.0 Hz) and protons of an acetate methyl (δ_H 2.01, s) to the carbonyl (δ_C 170.1). As both C-19 and C-20 resonated at δ 167.0 and 168.2, respectively, and protons of two methoxyls

Table 2
^{1}H and ^{13}C NMR data of **2**–**5**

C/H	**2**		**3**		**4**		**5**	
	^{1}H[a]	^{13}C[b]	^{1}H[c]	^{13}C[d]	^{1}H[a]	^{13}C[b]	^{1}H[a]	^{13}C[b]
1		142.6 (qC)[f]		141.5 (qC)		150.3 (qC)		143.5 (qC)
2	5.98 d (11.5)[e]	119.8 (CH)	6.01 d (11.6)	120.1 (CH)	5.07 d (9.0)	118.8 (CH)	6.00 d (11.5)	119.5 (CH)
3	5.92 d (11.5)	124.9 (CH)	5.97 d (11.6)	124.8 (CH)	3.42 d (9.0)	59.3 (CH)	5.85 d (11.5)	126.4 (CH)
4		131.3 (qC)		131.7 (qC)		59.8 (qC)		130.4 (qC)
5	2.27 t (12.0)							
	2.47 dd (12.0, 3.5)	45.3 (CH_2)	2.29 t (11.6)	45.4 (CH_2)	1.49 t (12.5)	44.8 (CH_2)	2.07 dd (12.5, 10.0)	45.6 (CH_2)
			2.47 td (12.0, 3.6)		2.45 dd (12.5, 3.0)		2.84 dd (12.5, 6.0)	
6	6.09 ddd (12.0,9.5, 3.5)	69.3 (CH)	6.10 m	69.4 (CH)	5.98 ddd (12.5,9.5, 3.0)	67.7 (CH)	5.10 ddd (10.0, 6.0, 2.0)	79.7 (CH)
7	5.64 d (9.5)	137.1 (CH)	5.61 d (9.6)	137.0 (CH)	5.58 d (9.5)	134.8 (CH)	7.54 d (2.0)	151.3 (CH)
8		134.7 (qC)		135.0 (C)		136.9 (qC)		132.1 (qC)
9	2.19 m	33.5 (CH_2)	2.16 m	34.0 (CH_2)	2.27 m	33.2 (CH_2)	2.50 m	24.3 (CH_2)
	2.79 dd (14.5, 7.5)		2.87 m		2.79 ddt (15.0, 7.0, 2.0)			
10	2.48 m	27.2 (CH_2)	2.58 m	27.4 (CH_2)	2.60 m	26.4 (CH_2)	2.46 m	26.1 (CH_2)
	2.61 m				2.70 dtd (16.5, 7.0, 2.0)		3.12 m	
11	6.03 t (7.5)	140.0 (CH)	6.06 m	141.5 (CH)	6.00 t (7.0)	141.9 (CH)	6.15 dd (7.0, 4.0)	139.5 (CH)
12		135.1 (qC)		132.0 (qC)		131.4 (qC)		136.9 (qC)
13	4.57 dd (10.0, 3.5)	71.2 (CH)	5.82 dd (11.2, 4.0)	71.6 (CH)	5.79 dd (11.0, 4.0)	71.2 (CH)	4.81 td (9.5, 5.0)	71.1 (CH)
14	2.42 dd (13.0, 3.5)	36.9 (CH_2)	2.42 m 2.94 m	34.2 (CH_2)	2.52 dd (14.0, 4.0)	34.3 (CH_2)	2.52 dd (13.0, 5.0)	38.7 (CH_2)
	2.86 dd (13.0, 10.0)				3.05 dd (14.0, 11.0)		2.65 dd (13.0, 9.5)	
15	2.29 sept (7.0)	33.5 (CH)	2.41 sept (6.8)	32.6 (CH)	2.34 sept (6.5)	32.3 (CH)	2.41 sept (7.0)	33.8 (CH)
16	1.01 d (7.0)	21.2 (CH_3)	1.03 d (6.4)	20.9 (CH_3)	1.00 d (6.5)	21.2 (CH_3)	1.06 d (7.0)	21.3 (CH_3)
17	1.02 d (7.0)	22.6 (CH_3)	1.04 d (6.8)	22.9 (CH_3)	1.07 d (6.5)	22.3 (CH_3)	1.06 d (7.0)	22.5 (CH_3)
18	1.81 s	17.0 (CH_3)	1.84 s	17.0 (CH_3)	1.47 s	17.3 (CH_3)	1.81 s	17.4 (CH_3)
19		167.0 (qC)		166.8 (qC)		167.0 (qC)		173.8 (qC)
20		168.2 (qC)		166.8 (qC)		166.7 (qC)		167.7 (qC)
6-OAc	2.01 s	170.1 (qC)	2.07 s	169.8 (qC)	2.01 s	169.9 (qC)		
		21.2 (CH_3)		21.3 (CH_3)		21.0 (CH_3)		
13-OAc			2.02 s	170.1 (qC)	2.06 s	169.9 (qC)		
				21.0 (CH_3)		21.2 (CH_3)		
19-OMe	3.75 s	51.6 (CH_3)	3.76 s	51.6 (CH_3)	3.79 s	51.9 (CH_3)		
20-OMe	3.70 s	51.3 (CH_3)	3.67 s	51.4 (CH_3)	3.75 s	51.7 (CH_3)	3.70 s	51.4 (CH_3)

[a] Spectra recorded at 500 MHz in $CDCl_3$.
[b] Spectra recorded at 125 MHz in $CDCl_3$.
[c] Spectra recorded at 400 MHz in $CDCl_3$.
[d] Spectra recorded at 100 MHz in $CDCl_3$.
[e] The *J* values are in hertz in parentheses.
[f] Attached protons were determined by DEPT experiments.

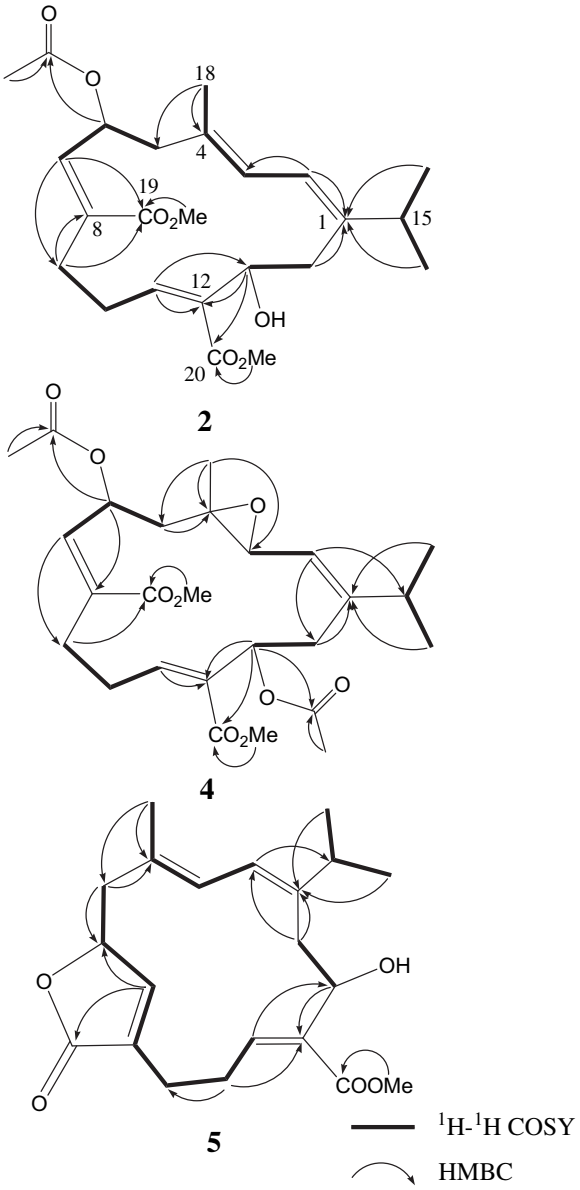


Figure 4. $^{1}H-^{1}H$ COSY and HMBC correlations for **2**, **4**, and **5**.

gave HMBC correlations to these carbonyl carbons, thus the positions of two methyl esters at C-8 and C-12 were confirmed.

The relative stereochemistry of **2** was further confirmed by NOE correlations (Fig. 5). The *E* geometries of two trisubstituted double bonds at C-1/C-2 and C-3/C-4 in **2** were assigned from the NOE interactions between H-2 (δ 5.98, d, J=11.5 Hz) with the protons of the isopropyl methyls (δ 1.01, d, J=7.0 Hz and 1.02, d, J=7.5 Hz) and H_3-18 (δ 1.81, s). The strong NOE correlations between H-3 and one proton of H_2-5, which was assumed to be H-5α (δ_H 2.27 t, J=12.0 Hz), and H-14 (δ_H 2.86, dd, J=13.0, 3.5 Hz), were observed therefore, this H-14 was characterized as H-14α. Furthermore, H-5β (δ_H 2.47, dd, J=12.0, 3.5 Hz) and H-14β (δ_H 2.42, dd, J=13.0, 10.0 Hz) exhibited NOE interactions with H-6 (δ_H 6.09, ddd, J=12.0, 9.5, 3.5 Hz) and H-13 (δ_H 4.57, dd, J=10.0, 3.5 Hz), respectively, revealing the α-orientation of the acetoxy group at C-6 and the hydroxyl group at C-13. Furthermore, the chemical shifts of H-7 and H-11 at δ 5.64 and δ 6.03, respectively, were in favor of *Z* configurations for both C-7/C-8 and C-11/C-12 double bonds,[19,20] as also confirmed by NOE correlations of both H-7 and H-11 with one proton of H_2-9. On

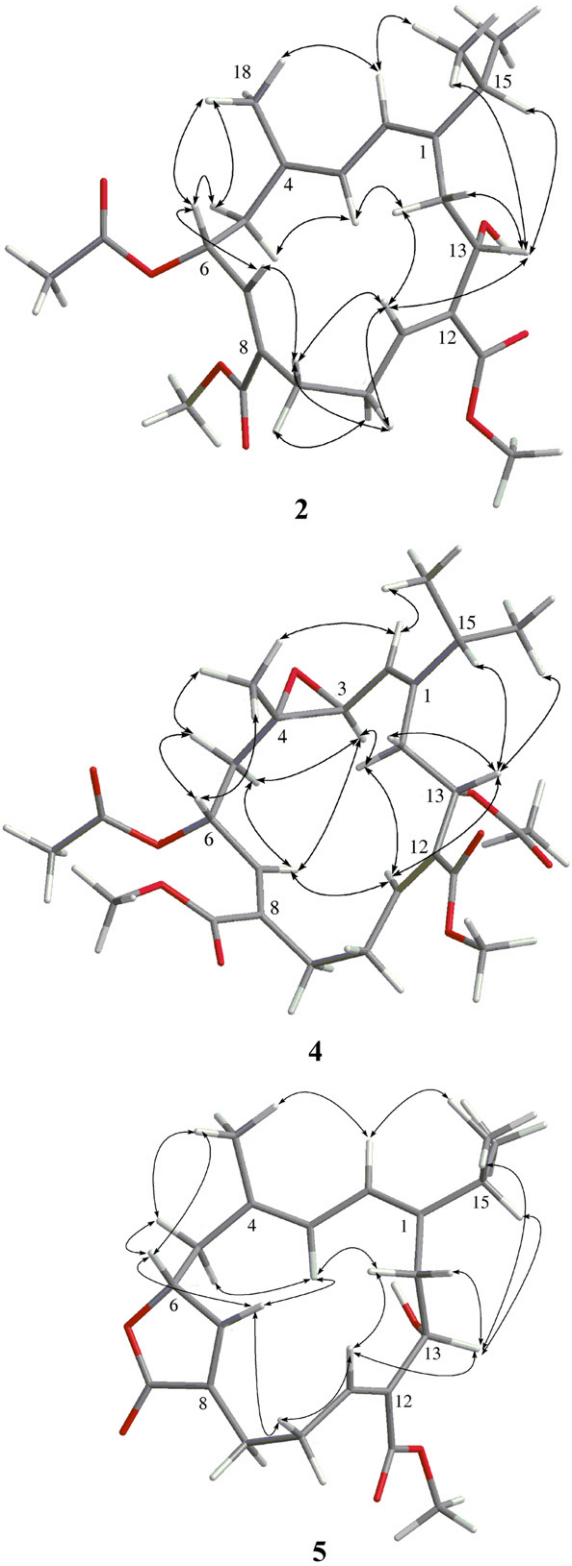


Figure 5. Selected NOE correlations for **2**, **4**, and **5**.

the basis of the above findings and other detailed NOE correlations (Fig. 5), the relative structure of sarcophytolin A (**2**) was deduced. Further investigation on the absolute configuration of **2** by Mosher's method[18] did not succeed, as although the (*R*)-MTPA ester of **2** could be easily prepared by reaction of **2** with (*S*)-(+)-MTPA chloride, however, **2** could not be converted into its (*R*)-MTPA ester

by repeated reactions with (*R*)-(−) -MTPA chloride, a phenomenon still awaits explanation.

Sarcophytolin B (**3**) was found to possess the molecular formula $C_{26}H_{36}O_8$ as revealed by the HRESIMS spectrum (*m*/*z* 499.2305 $[M+Na]^+$) and NMR data (Table 2). The IR absorption band at 1737 cm^{-1} indicated the presence of ester carbonyl group in **3**. The ^1H NMR spectrum of **3**, measured in $CDCl_3$ (Table 2), displayed seven methyl groups including one olefinic methyl (δ_H 1.84, 3H, s), two secondary methyls (δ_H 1.03 and 1.04, 6H, d, *J*=6.4 and 6.8 Hz, respectively), two acetate methyls (δ_H 2.07 and 2.02, 6H, s) and two ester methyls (δ_H 3.76 and 3.67, 6H, s). In the lower field, the four protons, which showed overlapped signals at δ_H 5.9–6.2, measured in $CDCl_3$, were well resolved by measuring the ^1H NMR spectrum in C_6D_6, which showed signals of H-2, H-3, H-6, and H-11 at δ_H 6.29 (d, *J*=11.2 Hz), 6.17 (d, *J*=11.6 Hz), 6.61 (ddd, *J*=11.6, 3.6, 2.0 Hz), and 6.22 (t, *J*=6.8 Hz), respectively. Comparison of the NMR data of **3** with those of **2** (Table 2) revealed that the hydroxy group in **2** was replaced by signals of an acetoxy group, including one carbonyl (δ_C 170.1) and one acetate methyl (δ_H 2.02, 3H, s). Thus, **3** is the acetyl derivative of **2**, as confirmed by the interpretations of the $^1H-^1H$ COSY and HMBC correlations (Fig. 4). We observed further that acetylation of **2** gave a product, which was found to be identical with **3** by comparison of the physical and spectroscopic data. Thus, the structure of compound **3** was then determined.

Sarcophytolin C (**4**) showed the pseudomolecular ion peak $[M+Na]^+$ at *m*/*z* 515.2253 in the HRESIMS, suggesting the molecular formula $C_{26}H_{36}O_9$ and nine degrees of unsaturation. The IR spectrum suggested the presence of ester carbonyl group in **4** (ν_{max} 1747 cm^{-1}). The ^{13}C NMR spectroscopic data (Table 2) of **4** were found to be very similar to those of **3**, except for the replacement of a trisubstituted double bond in the butadiene system of **3** by an epoxide moiety in **4** (δ_c 59.3, CH, C-3 and 59.8, qC, C-4). It was also found that the chemical shifts and *J* values for H-2 (δ_H 5.16, d, *J*=8.0 Hz) and H-3 (δ_H 3.42, d, *J*=9.0 Hz) of a known 3,4-epoxycembranoid[21] are very similar to those of **4**. Thus, **4** might also possess 3,4-epoxy group in cembrene. The HMBC correlations displayed from H_3-18 (δ_H 1.47, s) to the epoxide carbons C-3 and C-4, coupled with $^1H-^1H$ COSY correlations found between the epoxy proton and the olefinic proton at δ_H 5.07 (d, *J*=9.0 Hz), confirmed the C-3/C-4 and C-1/C-2 positions of the epoxy group and a trisubstituted double bond, respectively. Further investigations on the $^1H-^1H$ COSY and HMBC correlations (Fig. 4) positioned two trisubstituted double bonds, two methyl ester groups, and two acetoxy groups at C-7/C-8, C-11/C-12, C-8, C-12, C-6 and C-13, respectively.

The relative stereochemistry of **4** was resolved by analysis of NOE correlations (Fig. 5). Assuming the α-orientation of H-3, and from NOE correlations between H-3 and both H-5α (δ_H 1.49, t, *J*=12.5 Hz) and H-14α (δ_H 3.05, dd, *J*=14.0, 11.0 Hz), and the correlations between H-5β (δ_H 2.45, dd, *J*=12.5, 3.0 Hz) and H-6 (δ_H 5.98, ddd, *J*=12.5, 9.5, 3.0 Hz), and H-14β (δ_H 2.52, dd, *J*=14.0, 4.0 Hz) and H-13 (δ_H 5.79, dd, *J*=11.0, 4.0 Hz), the α-orientations of two acetoxy groups at C-6 and C-13 were shown as in Figure 5. On the basis of the above findings and other detailed NOE correlations (Fig. 5), the structure of compound **4**, a 3,4-epoxy derivative of **3**, was determined.

Sarcophytolin D (**5**) was found to have the molecular formula $C_{21}H_{28}O_5$, as revealed from the HRESIMS (*m*/*z* 383.1832 $[M+Na]^+$) and NMR data (Table 2), implying eight degrees of unsaturation. The NMR data (Table 2) revealed the presence of four trisubstituted double bonds and the absence of one carbomethoxy methyl in comparison with those of **1**. Also, the IR absorptions at 3479, 1747, and 1714 cm^{-1} indicated the presence of hydroxy, ester carbonyl, and α,β-unsaturated ester groups. Similar to **2** and **3**, two of these double bonds represented a conjugated diene moiety, as shown in structure of **5** (δ_H 6.00 and 5.85, each 1H, d, *J*=11.5 Hz, for both protons, δ_C 119.5 and 126.4, CH; 143.5 and 130.4, qC). Moreover, it was found that **5** differed from **2**–**4** by the presence of an α,β-unsaturated-γ-lactone ring (δ_H 7.54 d, *J*=2.0 Hz and 5.10 ddd, *J*=10.0, 6.0, 2.0 Hz, δ_C 151.3 and 79.7, CH; 173.8 and 132.1, qC). The interpretation of $^1H-^1H$ COSY and HMBC correlations (Fig. 4) were successively used to determine the positions of hydroxy group, double bonds, methyl ester group, and α,β-unsaturated-γ-lactone ring and resulted in the establishment of the planar structure of **5**. Compound **5** is found to be a 12-carbomethoxy-13-hydroxy-cembra-1,3,7,11- tetraen-6,19-olide.

The absolute stereochemistry of **5** was further confirmed by NOE correlations (Fig. 5) and CD spectrum. The *E* geometries for both C-1/C-2 and C-3/C-4 double bonds and the *Z* geometries for both C-7/C-8 and C-11/C-12 double bonds were determined by the same procedure as for the identification of compound **2**, and the β-orientations of H-6 and H-13 were established as well. The CD spectrum of **5** revealed that the negative Cotton effect at 206 nm and positive Cotton effect at 231 and 254 nm were observed, suggesting the *R* configuration at C-6.[22] Thus, the structure of compound **5** was determined.

It is worth noting that metabolite **1** is a sterol possessing a rarely found (20*R*,23*R*,24*R*)-23,24-dimethyl-20-hydroxy side chain. The cytotoxic activity of **1**–**9** against six human cancer cell lines, breast adenocarcinoma (MCF-7), colon adenocarcinoma (WiDr), laryngeal adenocarcinoma (HEp 2), medulloblastoma (Daoy), T-cell acute lymphoblastic leukemia (CCRF-CEM), and colon adenocarcinoma (DLD-1) was also assayed. The results showed that in compounds **1**–**9**, compound **8** exhibited more stronger cytotoxicity against MCF-7, WiDr, HEp 2, and DLD-1 cancer cell lines with ED_{50} values of 4.8, 11.8, 14.1, and 3.2 μg/mL, respectively, and **6** was found to be more cytotoxic than other isolates against Daoy and CCRF-CEM cancer cell lines with ED_{50} values of 11.4 and 7.4 μg/mL, respectively, and have the same ED_{50} value of 3.2 μg/mL toward DLD-1 cell line, as that of **8**. Steroid **9** has been shown to exhibit moderate cytotoxicity toward MCF-7 cells (ED_{50} 7.2 μg/mL) and cembranoid **3** showed weak cytotoxicity against MCF-7 and Daoy cell lines (Table 3). Furthermore, the in vitro anti-inflammatory effect of cembranoids **1**–**9** was tested. In this assay, the up-regulation of the pro-inflammatory iNOS and COX-2 proteins of LPS-stimulated RAW264.7 macrophage cells was evaluated using immunoblot analysis. At a concentration of 10 μM, compounds **2**, **3**, **5**–**7**, and **9** were found to effectively reduce the levels of iNOS to 39.0±14.1%, 24.4±11.3%, 38.4±14.9%, 38.6±24.4%, 49.6±3.9%, and 54.2±4.4%, respectively, while **1** significant enhanced the accumulation of iNOS to 218.2±28.6%, relative to the control cells stimulated with LPS only. It was shown that no significant reduction of COX-2 protein expression could be found for these compounds. Thus, compounds **2**, **3**, **5**–**7**, and **9** might be the useful anti-inflammatory agents to inhibit the expression of iNOS protein (Fig. 6).

Table 3
Cytotoxicity (ED_{50} μg/mL) of compounds **1**–**9**

Compound	MCF-7	WiDr	HEp 2	Daoy	CCRF-CEMM	DLD-1
1	(—)[a]	(—)	(—)	(—)	(—)	(—)
2	22.4	(—)	(—)	(—)	(—)	(—)
3	14.4	33.7	24.8	14.4	(—)	(—)
4	31.1	(—)	(—)	35.4	(—)	(—)
5	(—)	(—)	(—)	(—)	(—)	(—)
6	15.3	16.1	14.4	11.4	7.4	3.2
7	(—)	(—)	(—)	(—)	(—)	(—)
8	4.8	11.8	14.1	16.4	(—)	3.2
9	7.2	22.5	21.0	19.5	36.3	(—)

[a] (—): Compound is considered inactive when ED_{50}>40 μg/mL.

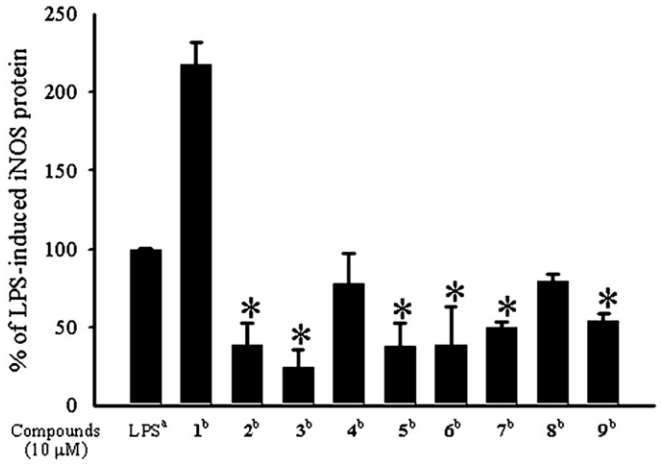


Figure 6. Effect of compounds **1**–**9** on iNOS protein expression of RAW264.7 macrophage cells by immunoblot analysis. The values are mean±SEM (n=6). Relative intensity of the LPS alone stimulated group was taken as 100%. Under the same experimental condition CAPE (caffeic acid phenylethyl ester, 10 μM) reduced the levels of the iNOS to 2.5±3.7%, respectively.*Significantly different from LPS alone stimulated group (*P<0.05). [a]Stimulated with LPS, [b]stimulated with LPS in the presence of **1**–**9** (10 μM).

3. Experimental section

3.1. General experimental procedures

Melting points were determined using a Fisher-Johns melting point apparatus. Optical rotations were measured on a JASCO P-1020 polarimeter. Ultraviolet spectra were recorded on a JASCO V-650 spectrophotometer. IR spectra were recorded on a JASCO FT/IR-4100 infrared spectrophotometer. CD spectrum was measured on a JASCO J-815 spectrophotometer. IR spectra were recorded on a Varian 400MR FT-NMR (or Varian Unity INOVA500 FT-NMR) instrument at 400 MHz (or 500 MHz) for ^{1}H and 100 MHz (or 125 MHz) for ^{13}C in $CDCl_3$. LRMS and HRMS were obtained by ESI on a Bruker APEX II mass spectrometer. 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 Merck Hibar Si-60 column (250×21 mm, 7 μm) and on a Hitachi L-2455 HPLC apparatus with a Inertsil ODS-3 column (250×20 mm, 5 μm).

3.2. Organism

L. sarcophytoides was collected by hand using scuba off the coast of Dongsha, Taiwan, in April 2007, at a depth of 5–10 m and stored in a freezer until extraction. A voucher sample was deposited at the Department of Marine Biotechnology and Resources, National Sun Yat-sen University.

3.3. Extraction and separation

The frozen bodies of *L. sarcophytoides* (1.2 kg, wet wt) were minced and exhaustively extracted with EtOAc (1 L×5). The EtOAc extract (1.20 g) was chromatographed over silica gel by column chromatography and eluted with EtOAc in *n*-hexane (0–100%, stepwise) then with MeOH in EtOAc (50–100%, stepwise) to yield thirteen fractions. Fraction 5 eluted with *n*-hexane–EtOAc (20:1) was purified over silica gel using *n*-hexane–EtOAc (10:1) to afford six subfractions (A1–A6). Subfraction A2 was further purified by reverse-phase HPLC using MeOH–H_2O (12:1) to afford **6** (3.8 mg). Fraction 8 eluted with *n*-hexane–EtOAc (4:1) was further separated over silica gel using *n*-hexane–EtOAc (5:1) to afford six subfractions (B1–B6). Subfraction B3 was further purified by reverse-phase HPLC using MeOH–H_2O (10:1) to afford **8** (2.6 mg) and **9** (6.9 mg). Subfraction B5 was purified by normal-phase HPLC using gradient solvent *n*-hexane–EtOAc (5:1–3:1) to afford **2** (7.1 mg), **3** (4.3 mg), and **5** (6.5 mg). Fraction 9 eluted with *n*-hexane–EtOAc (2:1) was purified over silica gel using *n*-hexane–EtOAc (4:1) to afford four subfractions (C1–C4). Subfraction C3 was further purified by reverse-phase HPLC using acetonitrile–H_2O (1:1) to afford **1** (15.5 mg). Fraction 10 eluted with *n*-hexane–EtOAc (1:1) was purified over silica gel using *n*-hexane–EtOAc (1:1) to afford four subfractions (D1-D4). Subfraction D2 was separated by reverse-phase HPLC using MeOH–H_2O (2.5:1) to afford **4** (3.3 mg).

*3.3.1. Sarcophytosterol (**1**).* Colorless needles; mp 121–125 °C; $[\alpha]_D^{25}$ −77 (*c* 0.28, $CHCl_3$); IR (neat) ν_{max} 3392, 2965, 2931, 1457, and 1375 cm^{-1}; ^{13}C and ^{1}H NMR data, see Table 1; ESIMS *m*/*z* 451 $[M+Na]^+$; HRESIMS *m*/*z* 451.3549 $[M+Na]^+$ (calcd for $C_{29}H_{48}O_2Na$, 451.3552).

*3.3.2. Sarcophytolin A (**2**).* Colorless oil; $[\alpha]_D^{25}$ −71 (*c* 0.44, $CHCl_3$); IR (neat) ν_{max} 3444, 2955, 2871, 1714, 1654, 1435, and 1243 cm^{-1}; UV (MeOH) λ_{max} 248 and 214 nm (log ϵ=3.7, 3.7); ^{13}C and ^{1}H NMR data, see Table 2; ESIMS *m*/*z* 457 $[M+Na]^+$; HRESIMS *m*/*z* 457.2206 $[M+Na]^+$ (calcd for $C_{24}H_{34}O_7Na$, 457.2202).

*3.3.3. Sarcophytolin B (**3**).* Colorless oil; $[\alpha]_D^{25}$ −92 (*c* 0.33, $CHCl_3$); IR (neat) ν_{max} 2955, 1737, 1371, and 1237 cm^{-1}; UV (MeOH) λ_{max} 247 and 211 nm (log ϵ=3.6, 3.6); ^{13}C and ^{1}H NMR data, see Table 2; ESIMS *m*/*z* 499 $[M+Na]^+$; HRESIMS *m*/*z* 499.2305 $[M+Na]^+$ (calcd for $C_{26}H_{36}O_8Na$, 499.2308).

*3.3.4. Sarcophytolin C (**4**).* Colorless oil; $[\alpha]_D^{25}$ −10 (*c* 0.42, $CHCl_3$); IR (neat) ν_{max} 2960, 2927, 1730, 1653, and 1436 cm^{-1}; ^{13}C and ^{1}H NMR data, see Table 2; ESIMS *m*/*z* 515 $[M+Na]^+$; HRESIMS *m*/*z* 515.2253 $[M+Na]^+$ (calcd for $C_{26}H_{36}O_9Na$, 515.2257).

*3.3.5. Sarcophytolin D (**5**).* Colorless oil; $[\alpha]_D^{25}$ +74 (*c* 0.10, $CHCl_3$); IR (neat) ν_{max} 3479, 2958, 2927, 2873, 1747, 1714, 1651, and 1435 cm^{-1}; UV (MeOH) λ_{max} 247 and 209 nm (log ϵ=3.5, 3.6); CD (*c* 0.1, MeOH) (mdeg) 206 (−6.5), 231 (39.3), 254 (20.5) nm; ^{13}C and ^{1}H NMR data, see Table 2; ESIMS *m*/*z* 383 $[M+Na]^+$; HRESIMS *m*/*z* 383.1834 $[M+Na]^+$ (calcd for $C_{21}H_{28}O_5Na$, 383.1832).

*3.3.6. Acetylation of **2**.* A solution of **2** (1.5 mg) in pyridine (0.1 mL) was mixed with Ac_2O (0.1 mL), and the mixture was stirred at room temperature for 24 h. After evaporation of excess reagent, the residue was subjected to column chromatograph over Si gel using *n*-hexane–EtOAc (1:3) to yield compound **3** (0.5 mg, 30%).

3.4. Preparation of (*S*)- and (*R*)-MTPA esters of 1

To a solution of **1** (1.0 mg) in pyridine (100 μL) was added (*R*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (10 μL), and the solution was then allowed to stand overnight at room temperature. The reaction mixture was added to 1.0 mL of H_2O, followed by extraction with EtOAc (1.0 mL×3). The EtOAc-soluble layers were combined, dried over anhydrous $MgSO_4$, and evaporated. The residue was purified by a short silica gel column using EtOAc–*n*-hexane (1:5) to yield the (*S*)-MTPA ester **1a** (0.4 mg, 27%). The same procedure was applied to obtain the (*R*)-MTPA ester **1b** (0.3 mg, 20%) from the reaction of (*S*)-(+)-α-methoxy-α-(trifluoromethyl) phenylacetyl chloride with **1** in pyridine. Selective ^{1}H NMR ($CDCl_3$, 400 MHz) data of **1a**: δ 1.172 (1H, t, *J*=7.2 Hz, H-1a), 2.435 (2H, m, H-4), 5.436 (1H, br s, H-6), 0.995 (3H, s, H-18), 1.038 (3H, s, H-19), 1.375 (3H, s, H-21), 0.859 (3H, d, *J*=7.0 Hz, H-26), 0.890 (3H, d, *J*=7.0 Hz, H-27), 0.758 (3H, d, *J*=7.0 Hz, H-28), 0.786 (3H, d, *J*=7.0 Hz, H-29); selective ^{1}H NMR ($CDCl_3$, 400 MHz) data of **1b**:

δ 1.184 (1H, t, J=7.2 Hz, H-1a), 2.358 (2H, m, H-4), 5.424 (1H, br s, H-6), 0.995 (3H, s, H-18), 1.041 (3H, s, H-19), 1.375 (3H, s, H-21), 0.873 (3H, d, J=7.0 Hz, H-26), 0.893 (3H, d, J=7.0 Hz, H-27), 0.753 (3H, d, J=7.0 Hz, H-28), 0.789 (3H, d, J=7.0 Hz, H-29).

3.5. X-ray diffraction analysis of sarcophytosterol (1)[23]

A suitable colorless crystal ($0.8\times0.6\times0.3$ mm^3) of **1** was grown by slow evaporation of the EtOAc solution. Diffraction intensity data were acquired with a Rigaku AFC7S single-crystal X-ray diffractometer with graphite-monochromated Mo Kα radiation (λ=0.71073 Å). Crystal data for **1**: $C_{29}H_{49}O_3$ (formula weight 445.68), approximate crystal size, $0.8\times0.6\times0.3$ mm^3, monoclinic, space group, $P2_1$ (# 4), T=298(2) K, a=6.641(5) Å, α=90°, b=17.573(13) Å, β=90°, c=23.399 (11), γ=90°, V=2731(3) Å^3, D_c=1.084 Mg/m^3, Z=4, F(000)=988, $\mu_{(Mo\ K\alpha)}$=0.068 mm^{-1}. A total of 5415 reflections were collected in the range $2.09°<\theta<26.05°$, with 4939 independent reflections [R(int)= 0.0516], completeness to θ_{max} was 99.7%; psi-scan absorption correction applied; full-matrix least-squares refinement on F^2, the number of data/restraints/parameters were 4939/0/308; goodness-of-fit on F^2=0.979; final R indices [$I>2\sigma(I)$], R_1=0.0645, wR_2=0.1621; R indices (all data), R_1=0.1462, wR_2=0.1986, largest difference peak and hole, 0.156 and −0.224 e/Å^{-3}.

3.6. Cytotoxicity testing

Cell lines were purchased from the American Type Culture Collection (ATCC). Cytotoxicity assays of compounds **1**–**9** were performed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 -diphenyltetrazolium bromide] colorimetric method.[24,25]

3.7. In vitro anti-inflammatory assay

Macrophage (RAW264.7) cell line was purchased from ATCC. In vitro anti-inflammatory activity of compounds **1**–**9** were measured by examining the inhibition of lipopolysaccharide (LPS) induced upregulation of iNOS (inducible nitric oxide synthetase) and COX-2 (cyclooxygenase-2) proteins in macrophages cells using western blotting analysis.[26,27]

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Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tet.2010.06.094.

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23. Crystallographic data for compound **1** has been deposited with the Cambridge Crystallographic Data Centre (deposition number CCDC 775719). Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 1223 336033 or e-mail: deposit@ccdc.cam.ac.uk).
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