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Cytotoxic and anti-inflammatory cembranoids from the Vietnamese soft coral *Lobophytum laevigatum*

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ABSTRACT

Four new cembranoids, namely laevigatol A–D (**1**–**4**), and six known metabolites (**5**–**10**), were isolated from the Vietnamese soft coral *Lobophytum laevigatum*. The structures of these compounds were elucidated by extensive spectroscopic analyses, and the absolute stereochemistry of **1** was determined using the modified Mosher's method. Compounds **5**, and **7**–**10** exhibited cytotoxic activity against selected human cancer cell lines. Compounds **1**, **2**, **8**, and **9** showed dose-dependent inhibitory effects on the TNFα-induced NF-κB transcriptional activity in Hep-G2 cells. Moreover, compounds **1**, **2**, **8**, and **9** significantly inhibited the induction of COX-2 and iNOS mRNA dose-dependently, indicating that these compounds attenuated the synthesis of these transcripts at the transcriptional level.

1. Introduction

Soft corals of the genus *Lobophytum* have been proven to be a rich source of cembranoids and their cyclized derivatives.[1–10] Some of these metabolites have been reported to possess cytotoxicity against various cancer cell lines,[6–9] and anti-inflammatory activity.[9,10] However, the chemical constituents as well as biological activities of the soft coral *Lobophytum laevigatum* have been little known to date. This prompted us to investigate the secondary metabolites of *L. laevigatum*, and their potential cytotoxic and anti-inflammatory activities. From a methanol extract of *L. laevigatum*, four new cembranoids with unusual tetrahydrofuran functionalities, namely laevigatol A–D (**1**–**4**), and six previously characterized metabolites (**5**–**10**) were isolated. Structures of these compounds were elucidated by extensive spectroscopic analyses and comparison with data reported in the literature. The absolute configuration of **1** was determined by the modified Mosher's method. The cytotoxic activity of compounds **1**–**10** was evaluated against human cancer cell lines, including leukemia (HL-60), lung (A549), colon (HCT-116), and breast (MCF-7). Moreover, the effects of compounds **1**–**10** on the tumor necrosis factor α (TNFα)-induced NF-κB transcriptional activity in Hep-G2 cells were determined. To confirm the inhibitory effects of the compounds on NF-κB transcriptional activity, we examined the effects of the compounds on the up-regulation of proinflammatory proteins inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in TNFα-stimulated Hep-G2 cells.

2. Results and discussion

The HR-FTICR-MS of laevigatol A (**1**) exhibited a pseudo molecular ion peak at *m*/*z* 335.22232 $[M+H]^+$, consistent with the molecular formula $C_{20}H_{30}O_4$, and appropriate for six degrees of unsaturation. The ^{13}C NMR spectrum of **1** (Table 1) displayed 20 carbon signals, and a DEPT experiment showed the presence of four methyls, six methylenes, five methines, and five quaternary carbons. The IR spectrum of **1** revealed the presence of a hydroxyl group (ν_{max} 3441 cm^{-1}), which was further confirmed by the observation of a methine proton at δ_H 5.28 (1H, d, J = 2.4 Hz) attached to a carbon at δ_C 99.2 (CH), indicating by HMQC spectrum. The NMR spectroscopic data (Tables 1 and 2) suggested that **1** possessed a cembrane skeleton with two epoxy groups and two macrocylic double bonds by comparison with data of a similar metabolite.[11] The two epoxy functionalities were determined by

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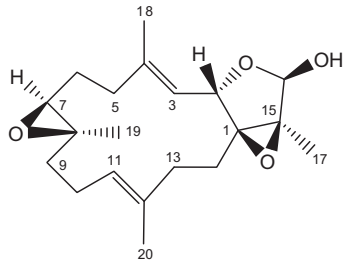

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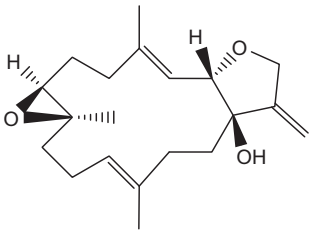

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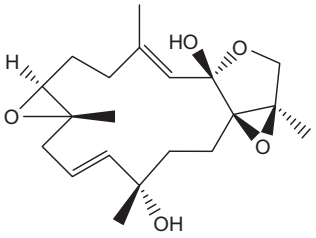

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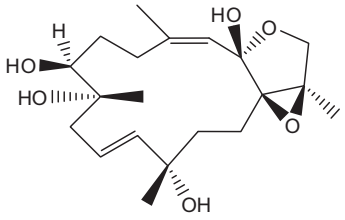

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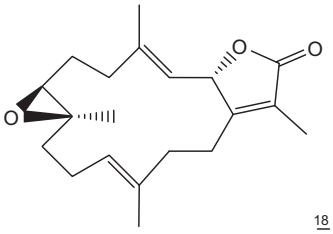

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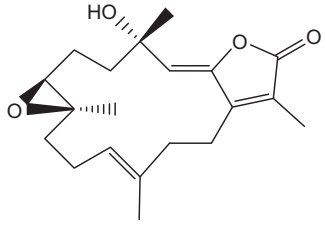

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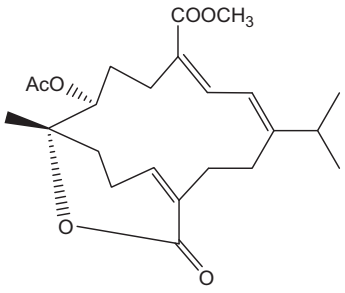

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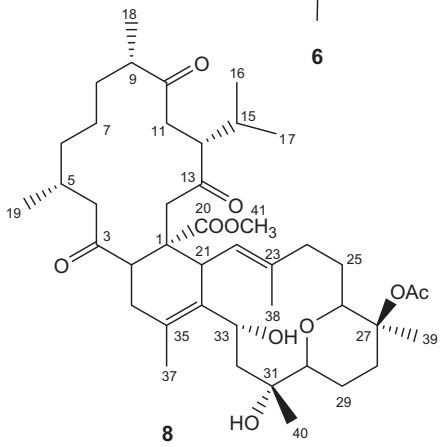

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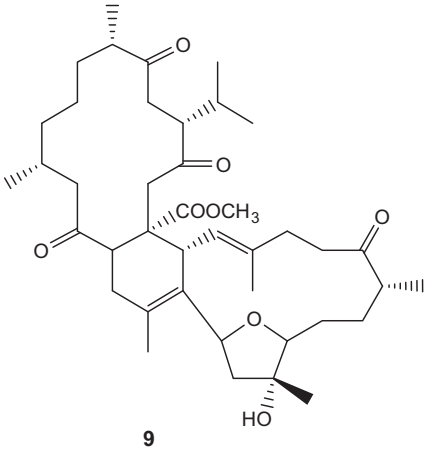

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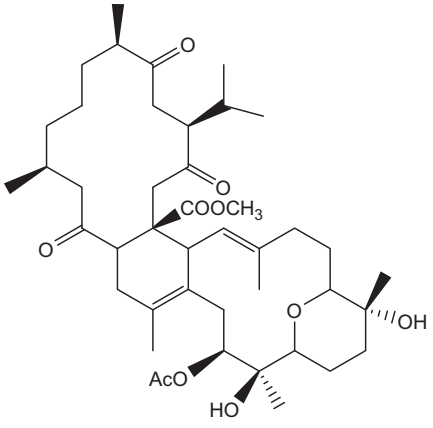

10

the NMR signals at δ_H 2.64 (1H, t, J = 4.2) and at δ_C 71.3 (qC), 69.0 (qC), 62.6 (CH), and 60.5 (qC). The positions of the two epoxy groups were confirmed by the HMBC correlations of H_3-17 to C-1 and C-15, and of H_3-19 to C-7 and C-8. Moreover, the HMBC correlations from H-16 to C-1 and C-2 indicated that the hydroxyl group was located at C-16. The NMR signals of the two trisubstituted double bonds were observed at δ_H 5.40 (1H, d, J = 11.4 Hz) and 5.10 (1H, dd, J = 10.2, 4.8 Hz), and at δ_C 140.7 (qC), 136.7 (qC), 125.2 (CH), and 124.6 (CH). The HMBC interactions between H_3-18/C-3, H_3-18/C-4, H_3-18/C-5, H_3-20/C-11, H_3-20/C-12, and H_3-20/C-13 led to the assignment of two double bonds at C-3 and C-11, respectively. The geometry of the two trisubstituted olefins was identified as *E*, due to the ^{13}C NMR chemical shift values of the olefinic methyls C-18 (δ_C 16.2) and C-20 (δ_C 15.5) (less than 20 ppm).[12] On the basis of the above analysis, the planar structure of **1** was established unambiguously.

The relative configuration of **1** was determined by analysis of NOE correlations and with the aid of molecular modeling using

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

Position	1	2	3	4
1	71.3	81.6	69.2	70.8
2	78.1	84.8	104.2	103.8
3	125.2	121.9	125.3	122.9
4	140.7	139.7	138.5	145.3
5	38.6	38.0	39.0	35.8
6	26.1	25.9	24.0	31.2
7	62.6	62.5	64.3	71.3
8	60.5	60.2	62.7	74.7
9	40.8	40.5	41.7	43.9
10	24.4	24.1	121.3	119.7
11	124.6	123.8	140.6	140.6
12	136.7	136.6	75.8	76.0
13	36.0	34.9	33.8	33.0
14	28.0	33.1	21.1	20.6
15	69.0	152.8	65.7	66.4
16	99.2	69.4	69.9	71.3
17	11.8	106.6	12.2	12.3
18	16.2	16.7	18.0	20.6
19	17.4	17.0	17.8	28.9
20	15.5	15.5	31.3	31.8

Spectra were recorded at 150 MHz in $CDCl_3$.

MM2 force field calculations (Fig. 2). The NOE interactions of H_3-18 to H-2 and H-6b (δ_H 1.62, m), but not to H-3, and of H_3-20 to H-10a (δ_H 2.26, m), but not to H-11, confirmed the *E* geometry of the two olefins and the β-orientation of H-2.[13] The NOE correlations between H-3/H-5a (δ_H 2.35, m), H-3/H-7, H-3/H-14a (δ_H 1.76, m), and H-3/H_3-19 indicated that H-7 and H_3-19 are oriented towards α-side of **1**. Additionally, the NOE cross peaks between H-7/H-9b (δ_H 0.91, ddd, *J* = 13.2, 13.2, 3.0 Hz), H-9b/H_3-20, H_3-20/H_3-17, and H_3-17/H-16 clearly revealed that H-9b, H-16 and H_3-17 are α-oriented, and the β-orientation of the epoxy group at C-1/C-15.[11] On the basis of the above analysis and detailed examinations of other NOE correlations (Fig. 2), the relative stereochemistry of **1** was established. Finally, the absolute configuration of **1** was determined using a modified Mosher's method.[14] The (*S*)- and (*R*)-MTPA esters of **1** (**1a** and **1b**, respectively) were prepared using the corresponding (*R*)- and (*S*)-MTPA chlorides, respectively. The determination of $\Delta\delta$ values (δ_S–δ_R) for protons neighboring C-16 led to the assignment of the *R* configuration at C-16 in **1** (Fig. 1). Therefore, the absolute stereochemistry of **1** was identified as 3*E*,11*E*,1*S*,2*S*,7*S*,8*S*,15*S*, and *16R*.

Laevigatol B (**2**) was found to possess the molecular formula $C_{20}H_{30}O_3$ from the interpretation of its HR-FTICR-MS (*m*/*z* 319.22712 $[M+H]^+$) and NMR spectroscopic data (Tables 1 and 2), corresponding to six degrees of unsaturation. The NMR spectroscopic data of **2** were found to be quite similar to those of (7*R*,8*R*)-epoxycembranolide,[15] except for the lack of a carbonyl group, and the presence of hydroxyl and oxymethylene groups in **2**. The absorption band at ν_{max} 3454 cm^{-1} in the IR spectrum, and a signal of an oxygenated quaternary carbon at δ_C 81.6 in the ^{13}C NMR spectrum of **2** revealed the presence of a hydroxyl group. Furthermore, the NMR signals at δ_H 4.63 and 4.44 (each 1H, d, *J* = 13.8 Hz), and at δ_C 69.4 (CH_2) indicated the presence of the oxymethylene group. The HMBC cross peaks from H-2 to C-1, C-4, and C-16, and from H_2-17 to C-1, C-15, and C-16 allowed the assignment of the hydroxyl and oxymethylene groups at C-1 and C-16, respectively. In the NOESY spectrum of **2** (Fig. 2), the NOE interactions between H-2/H_3-18, H_3-18/H-6b (δ_H 1.63, m), H-3/H-7, and H-3/H_3-19 indicated the β- and α-orientations of H-2 and H-7, respectively.[13] Further analyses of other NOE interactions (Fig. 2) revealed that **2** possessed the same relative configurations at C-1, C-2, C-7, and C-8 as those of **1**.

The HR-FTICR-MS (*m*/*z* 333.20641 $[M+H-H_2O]^+$) and NMR spectroscopic data (Tables 1 and 2) of laevigatol C (**3**) gave a molecular formula of $C_{20}H_{30}O_5$, implying six degrees of unsaturation. The ^{13}C NMR (Table 1) and DEPT spectra exhibited signals of four methyls, six methylenes, four methines and six quaternary carbons. The ^{1}H and ^{13}C NMR spectroscopic data of **3** were found to be quite similar to those of **1**, except for the migration of the double bond at C-11 in **1** to C-10 [δ_H 5.39 (1H, m) and 5.52 (1H, d, *J* = 16.2 Hz),

Table 2
^{1}H NMR data for compounds **1**–**4**

Position	1	2	3	4
1				
2	4.91, d (11.4)	4.78, d (10.2)		
3	5.40, d (11.4)	5.15, d (10.2)	5.35, s	5.44, s
4				
5	2.35, m	2.30, m	2.33, m	2.25, m
	2.31, m	2.27, m	2.21, m	2.04, m
6	1.91, m	1.81, m	1.98, m	1.97, m
	1.62, m	1.63, m	1.53, m	1.81, m
7	2.64, t (4.2)	2.66, t (4.2)	2.70, dd (10.2, 1.8)	4.01, d (10.2)
8				
9	2.12, m	2.10, m	2.56, dd (12.0, 6.0)	2.42, t (12.0)
	0.91, ddd (13.2, 13.2, 3.0)	0.93, ddd (13.2, 13.2, 3.6)	1.70, m	2.35, m
10	2.26, m	2.24, m	5.39, m	5.10, m
	1.88, m	1.90, m		
11	5.10, dd (10.2, 4.8)	5.03, t (8.4)	5.52, d (16.2)	5.40, d (16.2)
12				
13	2.20, m	2.29, m	2.25, m	2.22, m
	1.93, m	1.79, m	1.67, m	1.68, m
14	1.76, m	2.15, m	2.36, m	2.18, m
	1.69, m	1.22, m	1.55, m	1.57, m
15				
16	5.28, d (2.4)	4.63, d (13.8)	3.95, d (10.2)	4.01, d (10.2)
		4.44, d (13.8)	3.83, d (10.2)	3.90, d (10.2)
17	1.47, s	5.20, t (2.4)	1.44, s	1.46, s
		5.08, t (2.4)		
18	1.81, s	1.81, s	1.79, s	1.98, s
19	1.25, s	1.24, s	1.28, s	1.44, s
20	1.55, s	1.58, s	1.20, s	1.22, s

Spectra were recorded at 600 MHz in $CDCl_3$. Coupling constants (*J*) are in Hz.

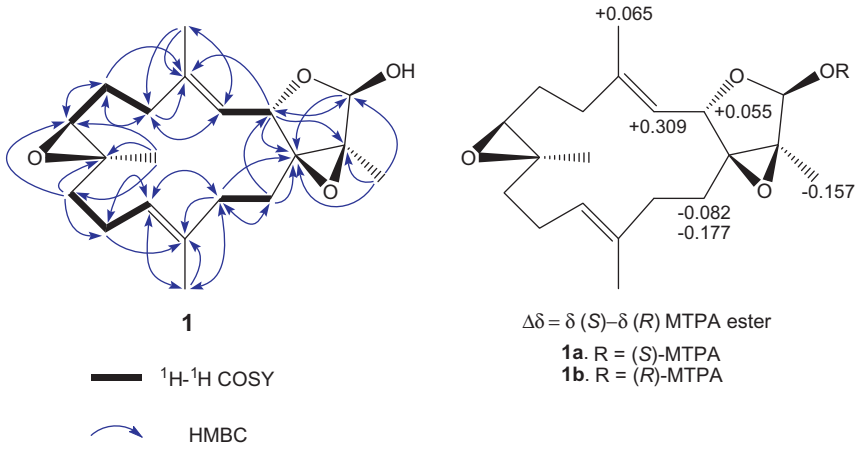

Figure 1. Selective ^{1}H–^{1}H COSY and HMBC correlations and ^{1}H NMR chemical shift differences of MTPA esters of **1**.

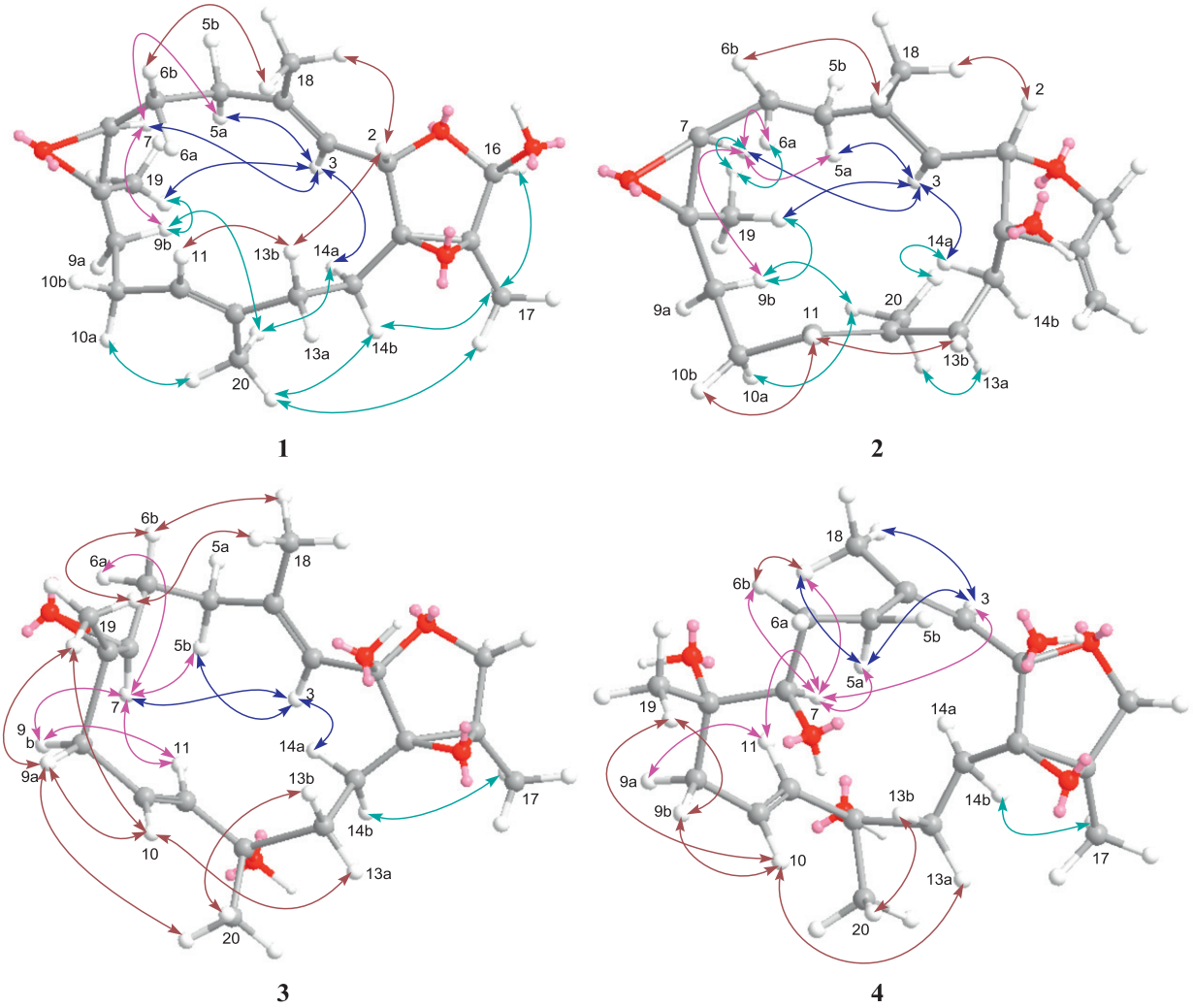

Figure 2. Key NOE correlations and computer-generated perspective model using MM2 force field calculations for **1**–**4**.

δ_C 121.3 and 140.6], and the hydroxylation of C-2 (δ_C 104.2) and C-12 (δ_C 75.8) in **3**. The geometry of the double bond at C-10 was defined as *E* from the coupling constant of 16.2 Hz between H-10 and H-11. Furthermore, the oxymethine group at C-16 in **1** was replaced by an oxymethylene group in **3** with NMR signals at δ_H 3.83, 3.95 (each 1H, d, J = 10.2 Hz) and δ_C 69.9 (CH_2). The observation of a ^{13}C NMR signal at δ_C 104.2 (qC) indicated that the oxymethine group at C-2 in **1** was changed to a hemiketal carbon in **3**. Based on analyses of the HMBC and ^{1}H–^{1}H COSY correlations (Fig. 3), the overall planar structure of **3** was established.

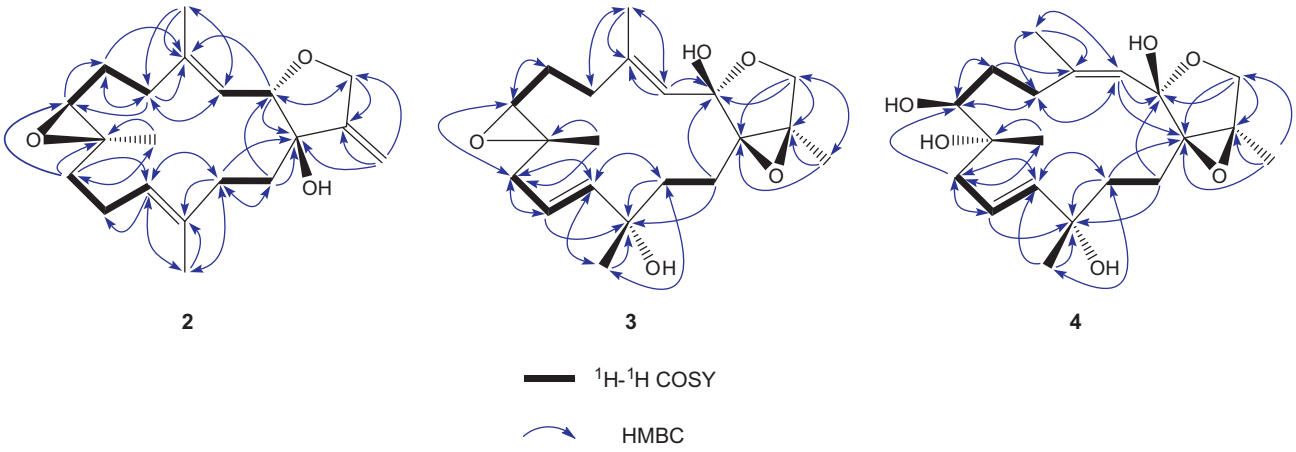

Figure 3. Selective ^{1}H–^{1}H COSY and HMBC correlations of **2**–**4**.

The relative configuration of **3** was identified by assistance of the NOESY experiment. In the NOESY spectrum (Fig. 2), H_3-18 showed a NOE interaction with H-6b (δ_H 1.53, m), but not with H-3, together with the ^{13}C NMR chemical shift value (δ_C 18.0) for the olefinic methyl C-18, indicating *E* geometry of the C-3/C-4-double bond. The relative stereochemistry of the hydroxyl group at C-12 was determined by careful comparison of the NMR spectroscopic data of **3** with those of crassumolide D.[9] The remarkable differences in the ^{13}C NMR chemical shift values of C-12 and C-20 between **3** and crassumolide D clearly revealed the α-orientation of the hydroxyl group at C-12. The NOE cross peaks from H_3-20 to H-9a (δ_H 2.56, dd, *J* = 12.0, 6.0 Hz), from H-9a to H-10 and H_3-19, and from H_3-19 to H_3-18 allowed to determine that H-9a and H_3-19 are both β-oriented. Furthermore, the NOE correlations between H-9b (δ_H 1.70, m)/H-7, and between H-7/H-3 indicated the α-orientation of H-7. H_3-17 exhibited NOE interaction with H-14b (δ_H 1.55, m) but not with H_3-20, revealing the α- and the β-orientations of H_3-17 and the epoxy group at C-1/C-15, respectively.[11]

The molecular formula of laevigatol D (**4**) was analyzed to be $C_{20}H_{32}O_6$ according to its HR-FTICR-MS (*m*/*z* 369.22764 $[M+H]^+$) and NMR spectroscopic data (Tables 1 and 2), and appropriate for five degrees of unsaturation. Comparison of the NMR data and the IR spectrum of **4** with those of **3** indicated that the structure of **4** was nearly identical to that of **3**, except for the conversion of the epoxide ring at C-7/C-8 in **3** to two hydroxyl groups in **4** [(δ_H 4.01 (1H, d, *J* = 10.2 Hz), δ_C 71.3 and 74.7], and the remarkable differences in chemical shifts of the trisubstituted olefin at C-3 [δ_H 5.44 (1H, m), δ_C 122.9 and 145.3] and the olefinic methyl C-18 [δ_H 1.98 (3H, s) and δ_C 20.6]. The geometry of the C-3/C-4-double bond was assigned as *Z*, based on the ^{13}C NMR chemical shift of the olefinic methyl C-18 (δ_C 20.6, over than 20 ppm)[12] and the NOE correlation between H-3 and H_3-18. The configuration of the hydroxyl group at C-8 was determined as α by comparison of the NMR spectroscopic data of **4** with those of both 7α,8β-dihydroxydeepoxysarcophine and 7β,8α -dihydroxydeepoxysarcophine.[16] The ^{1}H and ^{13}C NMR chemical shifts (δ_H 1.44 and δ_C 28.9) for the methyl group C-19 sensitive to the stereochemistry at C-8 were in good agreement with the values reported for the 8α-hydroxyl isomer.[16] The NMR chemical shift values for C-12 (δ_C 76.0) and C-20 (δ_H 1.22 and δ_C 31.8) of **4** were identical with those of **3**, revealing that the hydroxyl group at C-12 and H_3-20 are β- and α-oriented, respectively. H_3-19 showed NOE interaction with H-9b (δ_H 2.35, m) and H-10 but not with H-7, while H-7 exhibited NOE cross peaks with H-3, H-5a (δ_H 2.25, m), and H-11, clearly indicating that H-7 is oriented towards the opposite side of H_3-19. This allowed to identify the relative stereochemistry of the hydroxyl group at C-7 as β. Moreover, H_3-17 showed NOE correlation with H-14b (δ_H 1.57, m) but not with H_3-20, suggesting that H_3-17 and the C1/C15-epoxy group are α- and β-oriented, respectively.[11]

The known metabolites (+)-sarcophine (**5**),[17] 7β,8β-epoxy-4α-hydroxycembra-1(15),2,11-trien-16,2-olide (**6**),[16] emblide (**7**),[18] ximaolide F (**8**),[19] methyl tortuoate B (**9**),[20] and nyalolide (**10**),[21] were identified by comparison of their spectroscopic data with those reported in the literature.

The evaluation of cytotoxicity of compounds **1–10** was carried out in selected cancer cell lines, including HL-60, A549, HCT-116, and MCF-7 carcinoma cells (Table 3). The results showed that compounds **8–10** exhibited cytotoxicity against HL-60, A549, and HCT-116 cancer cell lines with IC_{50} values ranging from 9.0 to 28.7 μM, while compounds **5** and **7** displayed cytotoxicity against only HL-60 cells with IC_{50} values of 34.7 and 38.8 μM, respectively. Only compound **10** was cytotoxic towards MCF-7 cells with IC_{50} values of 35.5 μM. Comparison of the cytotoxic data and structures of compounds **1–10** revealed that biscembranoids (**8–10**) showed stronger and broader cytotoxicity than others against the cancer cell lines tested.

The anti-inflammatory activity of compounds **1–10** was evaluated through the inhibition of TNFα-induced NF-κB luciferase reporter, and by attenuation of TNFα-induced pro-inflammatory protein (iNOS and COX-2) expression in Hep-G2 cells.

The NF-κB luciferase assay is designed to monitor the activity of NF-κB-regulated signal transduction pathways in cultured cells. The NF-κB-responsive luciferase construct encodes the firefly luciferase reporter gene under the control of a minimal CMV promoter and tandem repeats of the NF-κB transcriptional response element. Using this assay, the inhibitory activity of compounds **1–10** on NF-κB activation was readily monitored. It is known that

Table 3
Cytotoxicity data for compounds **1–10**

Compound	Cell lines IC_{50} (μM)			
	HL-60	A549	HCT-116	MCF-7
5	34.7 ± 0.6	>40[a]	>40	>40
7	38.8 ± 3.8	>40	>40	>40
8	9.0 ± 0.8	28.4 ± 1.9	16.4 ± 0.2	>40
9	25.8 ± 0.2	24.4 ± 0.3	19.7 ± 0.6	>40
10	28.1 ± 0.2	28.7 ± 0.2	17.5 ± 4.0	35.5 ± 3.6
Mitoxantrone[b]	7.9 ± 0.3	7.8 ± 0.1	7.2 ± 0.3	7.1 ± 0.5

Compounds **1–4**, and **6** were inactive for all cell lines.
[a] A compound is considered inactive with IC_{50} >40 μM.
[b] Positive control.

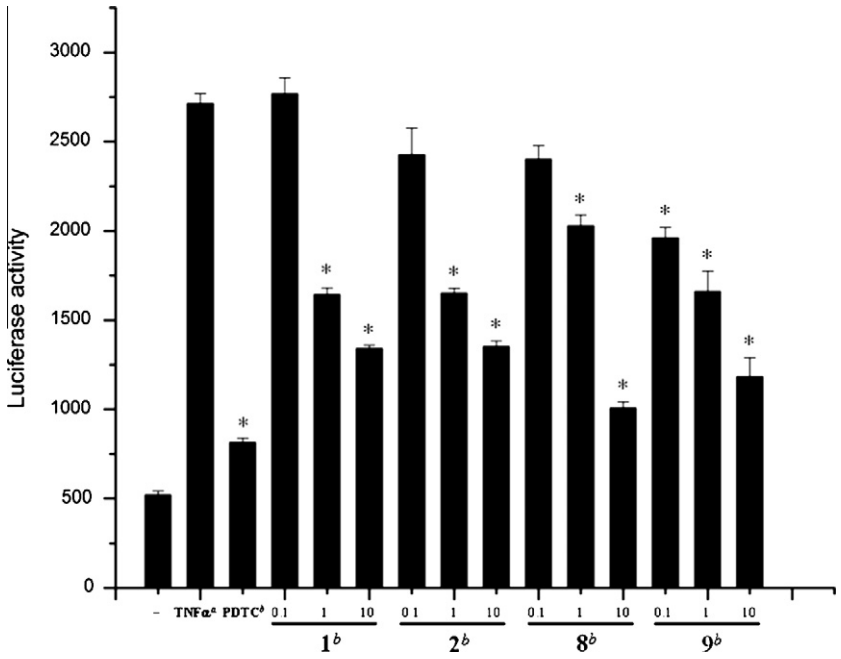

Figure 4. Effect of compounds **1**, **2**, **8**, and **9** on the TNFα-induced NF-κB luciferase reporter activity in Hep-G2 cells. The values are mean ± SD (n = 6). [a]Stimulated with TNFα. [b]Stimulated with TNFα in the presence of **1**, **2**, **8**, **9** (0.1, 1, and 10 μM), and PDTC. PDTC: pyrrolidine dithiocarbamate, positive control (10 μM). *Significantly different from TNFα-stimulated group (P <0.05).

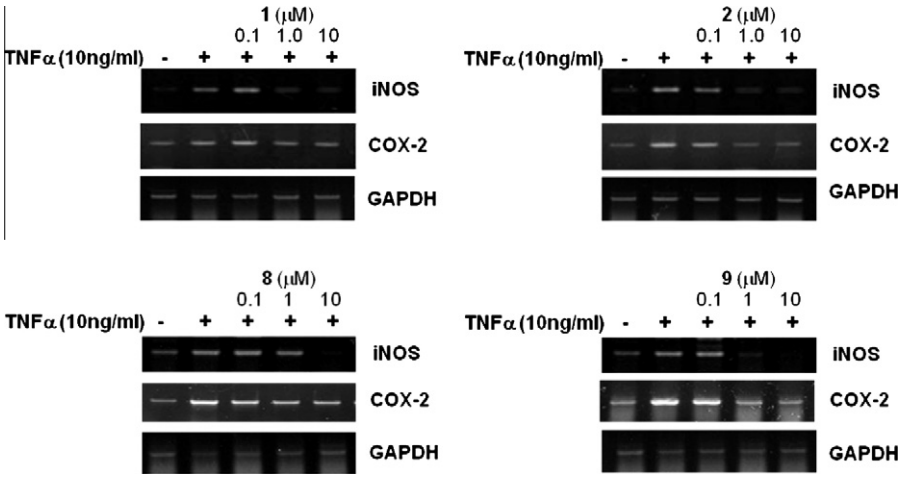

Figure 5. Effects of compounds **1**, **2**, **8**, and **9** on iNOS and COX-2 mRNA expression in Hep-G2 cells.

pro-inflammatory agents such as TNFα activate the NF-κB pathway.[22] Hep-G2 cells transfected with the NF-κB luciferase reporter plasmid exhibited an approximately fourfold increase in the luciferase signal after treatment with 10 ng/mL TNFα, indicating an increase in transcriptional activity, compared with untreated cells. The results (Fig. 4) showed that compounds **1**, **2**, **8**, and **9** exhibited dose-dependently inhibitory effects on TNFα-induced NF-κB transcriptional activity in Hep-G2 cells with IC_{50} values of 9.4, 9.7, 6.9, and 6.7 μM, respectively.

Since NF-κB is an important transcription factor involved in the regulation of the expression of inflammatory NF-κB target genes such as iNOS and COX-2,[23,24] we examined the effect of compounds **1**, **2**, **8**, and **9** on the expression of these genes in TNFα-stimulated Hep-G2 cells using RT-PCR. Hep-G2 cells treated with 10 ng/mL TNFα significantly up-regulated the mRNA expression of the NF-κB target genes COX-2 and iNOS, by approximately 2.2- and 8.5-fold, respectively. Consistent with their inhibitory activity towards NF-κB, compounds **1**, **2**, **8**, and **9** significantly inhibited the induction of COX-2 and iNOS mRNA in a dose-dependent manner, indicating that these compounds attenuated the synthesis of these transcripts at the transcriptional level (Fig. 5). These primary results suggested that compounds **1**, **2**, **8**, and **9** might be useful anti-inflammatory agents for human.

Cell viability, as measured by the MTT assay, showed that compounds **1**, **2**, **8**, and **9** had no significant cytotoxicity in Hep-G2 cells at concentrations that were effective for inhibition of NF-κB activation and induction of COX-2 and iNOS mRNA (data not shown).

3. Experimental

3.1. General experimental procedures

Optical rotation was determined using a Jasco DIP-370 digital polarimeter. IR spectra were measured using a Perkin–Elmer 577

spectrometer (Perkin Elmer, Waltham, MA, USA). Electrospray ionization (ESI) mass spectra were obtained using an Agilent 1200 LC-MSD Trap spectrometer. High resolution mass spectra were obtained using a HR-FTICR-MS 910 spectrometer. The NMR spectra were recorded on a Bruker DRX 400 NMR spectrometer (Bruker, Billerica, MA, USA) and a Jeol ECA 600 spectrometer using TMS as an internal standard. TLC was performed on Kieselgel 60 F254 (1.05715; Merck, Darmstadt, Germany) or RP-18 F254s (Merck) plates. Spots were visualized by spraying with 10% aqueous H_2SO_4 solution, followed by heating. Column chromatography (CC) was performed on silica gel (Kieselgel 60, 70–230 mesh and 230–400 mesh, Merck) and YMC RP-18 resins.

3.2. Animal material

The soft coral *L. laevigatum* was collected by hand using scuba at Khanh Hoa province, Viet Nam, in February 2009, and was stored in a freezer until being extracted. The scientific name was identified by Dr. Do Cong Thung, Institute of Marine Resources and Environment, Viet Nam Academy of Science and Technology (VAST). A voucher specimen (No. 20090214) was deposited in the Institute of Marine Biochemistry, VAST.

3.3. Extraction and isolation

The frozen bodies of *L. laevigatum* (2 kg, wet wt) were minced and exhaustively extracted with MeOH. After concentration, the MeOH extract (80 g) was suspended in water and then partitioned with chloroform to give chloroform and water fractions (fractions A and B, respectively). Fraction A was chromatographed over silica gel, eluting with EtOAc in *n*-hexane (0–100%, step-wise), yielding nine fractions (fractions A1–A9). Fraction A5 was chromatographed over silica gel column eluting with *n*-hexane–EtOAc (6:1) to afford four subfractions (A5A–A5D). Subfraction A5C was chromatographed over silica gel column eluting with *n*-hexane–acetone (8:1) and further separated by column chromatography over silica gel, using CH_2Cl_2–acetone (15:1) to obtain **5** (35 mg) and **7** (15 mg). Subfraction A5D was further separated by a silica gel column using CH_2Cl_2–EtOAc (10:1) to give **1** (15 mg) and **6** (5 mg). Fraction A6 was chromatographed on a silica gel column eluting with CH_2Cl_2–EtOAc (13:1) to afford three subfractions (A6A–A6C). Subfraction A6B was then purified by YMC reverse-phase chromatography, using acetone–water (3:1) as eluent to afford **2** (12.0 mg). Subfraction A6C was chromatographed on a silica gel column eluting with *n*-hexane–acetone (5:1) and further separated by column chromatography over silica gel, using CH_2Cl_2–acetone (8:1) as eluent to obtain **8** (10 mg), **9** (8 mg), and **10** (7 mg). Fraction A8 was separated by column chromatography over silica gel, eluting with *n*-hexane–acetone (4:1) to give four subfractions (A8A–A8D). Subfraction A8C was separated on a silica gel column, eluting with CH_2Cl_2–acetone (7:2) and further purified by YMC reverse-phase chromatography using MeOH–H_2O (4:1) as eluent to afford **3** (16 mg) and **4** (5 mg).

3.3.1. Laevigatol A (1)

Colorless gum; $[\alpha]_D^{25}$ −23.0 (*c* 1.00, CH_2Cl_2); IR (CH_3CN) ν_{max} (neat) 3441, 2931, 2868, 1736, 1715, 1669, 1448, 1383, 1250, 1063, 1014, 909 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HR-FTICR-MS *m/z* 335.22232 $[M+H]^+$ (calcd for $C_{20}H_{31}O_4$, 335.22223).

3.3.2. Laevigatol B (2)

Colorless gum; $[\alpha]_D^{25}$ +7.7 (*c* 1.00, CH_2Cl_2); IR (CH_3CN) ν_{max} (neat) 3454, 2927, 1760, 1712, 1451, 1383, 1250, 1074 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HR-FTICR-MS *m/z* 319.22712 $[M+H]^+$ (calcd for $C_{20}H_{31}O_3$, 319.22732).

3.3.3. Laevigatol C (3)

Colorless gum; $[\alpha]_D^{25}$ +2.0 (*c* 1.00, CH_2Cl_2); IR (CH_3CN) ν_{max} (neat) 3375, 2926, 2864, 1758, 1660, 1452, 1382, 1061, 971 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HR-FTICR-MS *m/z* 333.20641 $[M+H-H_2O]^+$ (calcd for $C_{20}H_{29}O_4$, 333.20658).

3.3.4. Laevigatol D (4)

Colorless gum; $[\alpha]_D^{25}$ +2.5 (*c* 1.00, CH_2Cl_2); IR (CH_3CN) ν_{max} (neat) 3456, 2926, 2862, 1731, 1668, 1455, 1372, 1017, 999 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HR-FTICR-MS *m/z* 369.22764 $[M+H]^+$ (calcd for $C_{20}H_{33}O_6$, 369.22771).

3.3.5. (+)-Sarcophine (5)

Colorless needles; $[\alpha]_D^{25}$ +92.5 (*c* 1.00, $CHCl_3$); ESI-MS *m/z* 338.9 $[M+Na]^+$, 317.0 $[M+H]^+$, 298.7 $[M-H_2O+H]^+$.

3.3.6. 7β,8β-Epoxy-4α-hydroxycembra-1(15),2,11-trien-16,2-olide (6)

Colorless needles; $[\alpha]_D^{25}$ −5.5 (*c* 0.10, $CHCl_3$); ESI-MS *m/z* 355.2 $[M+Na]^+$.

3.3.7. Emblide (7)

Colorless gum; $[\alpha]_D^{25}$ +115.5 (*c* 0.10, $CHCl_3$); ESI-MS *m/z* 426.9 $[M+Na]^+$, 405.0 $[M+H]^+$.

3.3.8. Ximaolide F (8)

Amorphous powder; $[\alpha]_D^{25}$ +113.2 (*c* 0.50, $CHCl_3$); ESI-MS *m/z* 765.5 $[M+Na]^+$.

3.3.9. Methyl tortuoate B (9)

Amorphous powder; $[\alpha]_D^{25}$ +85 (*c* 0.10, $CHCl_3$); ESI-MS *m/z* 683.5 $[M+H]^+$.

3.3.10. Nyalolide (10)

Amorphous powder; $[\alpha]_D^{25}$ +91.7 (*c* 1.00, $CHCl_3$); ESI-MS *m/z* 743.5 $[M+H]^+$.

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

To a solution of **1** (2 mg) in CH_2Cl_2 (0.4 mL) was added (*R*)-MTPA chloride (15 μL) and 5 mg of DMAP, and the mixture was allowed to react overnight at room temperature. The reaction product was purified by preparative TLC silica gel with *n*-hexane–EtOAc (4:1) to give the (*S*)-MTPA ester, **1a** (1.5 mg). The same procedure was used to prepare the (*R*)-MTPA ester, **1b** (1.6 mg from 2 mg of **1**), with (*S*)-MTPA chloride. Selected ^1H NMR ($CDCl_3$, 400 MHz) of **1a**: δ_H 7.28–7.46 (5H, m, Ph), 5.11 (1H, d, *J* = 11.2 Hz, H-3), 5.02 (1H, dd, *J* = 9.6, 5.6 Hz, H-11), 4.96 (1H, d, *J* = 11.2 Hz, H-2), 2.50 (1H, t, *J* = 4.0 Hz, H-7), 1.84 (1H, m, H-14a), 1.77 (3H, s, H_3-18), 1.64 (1H, m, H-14b), 1.47 (3H, s, H_3-20), 1.21 (3H, s, H_3-17), and 1.19 (3H, s, H_3-19). Selected ^1H NMR ($CDCl_3$, 400 MHz) of **1b**: δ_H 7.28–7.44 (5H, m, Ph), 5.00 (1H, dd, *J* = 10.4, 6.4 Hz, H-11), 4.90 (1H, d, *J* = 11.2 Hz, H-2), 4.80 (1H, d, *J* = 11.2 Hz, H-3), 2.44 (1H, t, *J* = 4.0 Hz, H-7), 1.92 (1H, m, H-14a), 1.82 (1H, m, H-14b), 1.70 (3H, s, H_3-18), 1.46 (3H, s, H_3-20), 1.37 (3H, s, H_3-17), and 1.18 (3H, s, H_3-19).

3.4. Cytotoxic assay

Human cancer cell lines including leukemia (HL-60), lung (A549), colon (HCT-116), breast (MCF-7), and liver (Hep-G2) were purchased from the Korea Cell Line Bank (KCLB, Seoul, Korea). Cytotoxic assay were performed using the MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method.[25]

3.5. In vitro anti-inflammatory assay

3.5.1. NF-κB-luciferase assay

The luciferase vector was first transfected into Hep-G2 cells. After a limited amount of time, the cells were lysed and luciferin, the substrate of luciferase, was introduced into the cellular extract along with Mg and an excess of ATP. Under these conditions, luciferase enzymes expressed by the reporter vector could catalyze the oxidative carboxylation of luciferin. Cells were seeded at 2×10^5 cells per well in 12-well plates and grown for 24 h. All cells were transfected using Lipofectamine LTX (Invitrogen, Carlsbad, CA), as described by the manufacturer. Luciferase activity was assayed using an LB 953 Autolumat (EG&G Berthold, Nashua, NH).[26] The transfected Hep-G2 cells were pretreated for 1 h with either vehicle (DMSO) and compounds, followed by 1 h of treatment with 10 ng/mL TNFα. Unstimulated Hep-G2 cells were used as a negative control (−). Cells were then harvested, and luciferase activity was assayed. The NF-κB-Luciferase plasmid was kindly provided by Dr. Kyoon E. Kim (Chungnam National University, Daejeon, Korea).

3.5.2. RNA preparation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted using Easy-blue reagent (Intron Biotechnology, Seoul, Korea). Approximately 2 μg total RNA was subjected to reverse transcription using Moloney murine leukemia virus (MMLV) reverse transcriptase and oligo-dT primers (Promega, Madison, WI) for 1 h at 42 °C. PCR for synthetic cDNA was performed using a Taq polymerase pre-mixture (TaKaRa, Japan). The PCR products were separated by electrophoresis on 1% agarose gels and stained with EtBr. PCR was conducted with the following primer pairs: iNOS sense 5′-TCATCCGCTATGCTGGCTAC-3′, iNOS antisense 5′-CTCAGGGTCACGGCCATTG-3′, COX-2 sense 5′-GCCCAGCACTTCACGCATCAG-3′, COX-2 antisense 5′-GACCAGGCACCAGACCAAAGACC-3′, GAPDH sense 5′-TGTTGCCATCAATGACCCCTT-3′, and GAPDH antisense 5′-CTCCACGACGTACTCAGCG -3′. The specificity of products generated by each set of primers was examined using gel electrophoresis and further confirmed by a melting curve analysis.

Hep-G2 cells were pretreated in the absence and presence of compounds for 1 h, then exposed to 10 ng/mL TNFα for 6 h. Total mRNA was prepared from the cell pellets using Easy-blue. The levels of mRNA were assessed by RT-PCR.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.03.009.

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