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Cytotoxic and PPARs transcriptional activities of sterols from the Vietnamese soft coral *Lobophytum laevigatum*

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ABSTRACT

A new unusual sterol, named lobophytosterol (**1**), and five known metabolites (**2**–**6**) were isolated from the methanol extract of the soft coral *Lobophytum laevigatum*. Their chemical structures were elucidated by extensive spectroscopic analysis and comparison with those reported in the literature. The absolute stereochemistry of **1** was determined using a modified Mosher's method. Compounds **1**–**3** showed cytotoxic activity against HCT-116 cells with IC_{50} values of 3.2, 6.9 and 18.1 μM, respectively. Compound **1** additionally displayed cytotoxic effects on A549 and HL-60 cells with IC_{50} values of 4.5 and 5.6 μM, respectively. Treatment of these cells with compound **1** resulted in an induction of apoptosis evident by chromatin condensation in treated cells. Besides, compounds **2**, **4**, and **6** significantly upregulated PPARs transcriptional activity dose-dependently in Hep-G2 cells. Taken together, these data suggest that compound **1** might inhibit the growth of the cancer cells by the induction of apoptosis, and compounds **2**, **4**, and **6** might act as specific agonists for PPARα, PPARδ, and PPARγ and may therefore regulate cellular glucose, lipid, and cholesterol metabolism.

Marine organisms are a natural source of various classes of secondary metabolites with diverse structures and biological activities. The soft corals of the genus *Lobophytum* have been reported to produce mainly diterpenoids and steroids, that showed cytotoxic,[1–4] anti-inflammatory,[3,5] and anti-HIV activities.[6] However, little is known about the chemical components and biological activity of the soft coral *Lobophytum laevigatum* (Alcyoniidae). As a part of our search for bioactive compounds from Vietnamese marine organisms,[4,7–9] a chemical investigation of the methanol extract of *L. laevigatum* led to the isolation of six sterols,[10] including a new one (**1**), lobophytosterol, and five known metabolites: (22*S*,24*S*)-24-methyl-22,25-epoxyfurost-5-ene-3β,20β-diol (**2**),[11] (24*S*)-24-methylcholest-5-ene-3β,25-diol (**3**),[12] (24*S*)-ergost-5-ene-3β,7α-diol (**4**),[13] cholesterol (**5**),[14] and pregnenolone (**6**).[15] The structures of these compounds were established by extensive spectroscopic analysis and comparison with those reported in the literature. The absolute configuration of compound **1** was determined using the modified Mosher's method.

We initially assessed the cytotoxicity of compounds **1**–**6** against lung (A549), colon (HCT-116), and leukemia (HL-60) cancer cell lines. Apoptosis, a process of cell death stimulated in response to cellular stress, results in cell shrinkage followed by chromatin condensation, which is associated with characteristic internucleosomal DNA cleavage. This cleavage results in the production of nucleosomes of DNA fragments complexed with core histones, which exist in discrete multiples of a 180 base pair subunit. One of the major modes of action of chemotherapeutic anti-cancer drugs on malignant cells is via the induction of apoptosis.[16] As compound **1** showed cytotoxicity against A549, HCT-116, and HL-60 cells, we selected this compound to assess its ability to induce apoptosis in these cell lines.

The peroxisome proliferator-activated receptors (PPARs) form a subfamily of the nuclear receptor superfamily, of which three isoforms, PPARα, PPARδ, and PPARγ have been identified. PPARs regulate the expression of genes involved in the regulation of glucose, lipid, and cholesterol metabolism by binding to specific peroxisome proliferator response elements (PPREs) in the enhancer sites of regulated genes.[17–20] Accordingly, compounds that modulate the function of PPARs are attractive for the treatment of type 2 diabetes, obesity, metabolic syndrome, inflammation, and cardiovascular disease.[21] Thus we investigated the effects of compounds **1**–**6** on PPARs transcriptional activity in human hepatocarcinoma (Hep-G2) cells.

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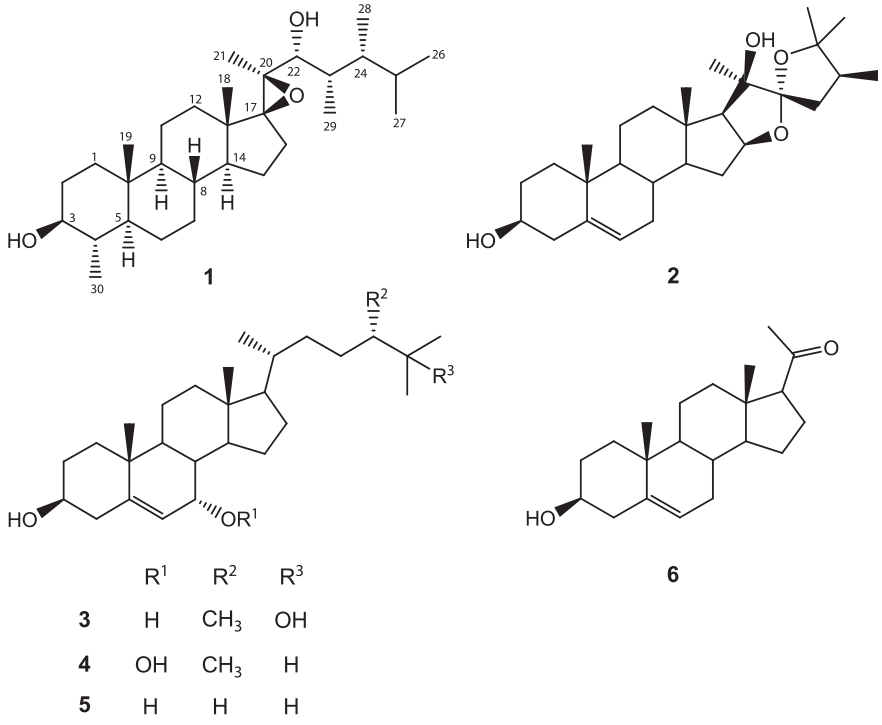

Figure 1. Structures of the compounds **1**–**6**.

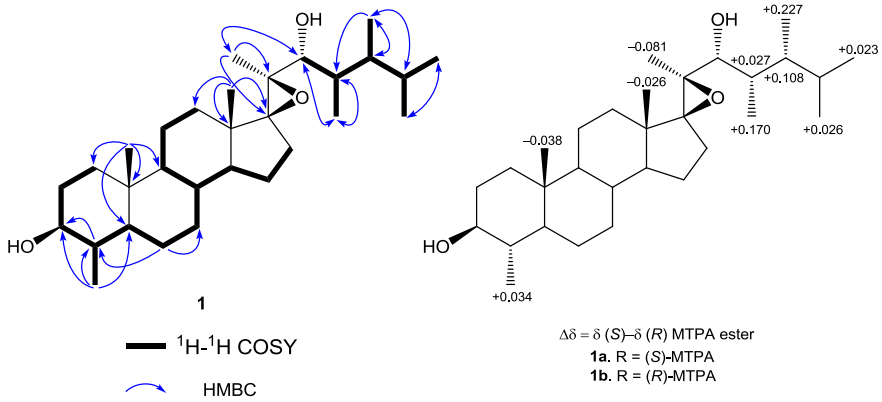

Figure 2. Selective ^{1}H–^{1}H COSY (—), HMBC correlations (H → C) and ^{1}H NMR chemical shift differences of MTPA esters of **1**.

The soft coral *L. laevigatum* was collected by hand diving in 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, Vietnam Academy of Science and Technology (VAST) and a voucher specimen (No. 20090214) was deposited in the Institute of Marine Biochemistry, VAST. Using a combined chromatographic separation, six compounds (Fig. 1) were isolated from the methanol extract of *L. laevigatum*.[22] Compound **1** was obtained as an amorphous powder. The HR-FTICR-MS of **1** showed an intensive pseudomolecular ion peak $[M+H-H_2O]^+$ at *m*/*z* 443.38876 (calcd for $C_{30}H_{51}O_2$, 443.38891), consistent with a molecular formula of $C_{30}H_{52}O_3$, and implying five degrees of unsaturation. The ^{13}C NMR (Table 1) and DEPT spectra displayed the presence of eight methyls, eight methylenes, ten methines, and four quaternary carbons. All the carbon–proton assignments of **1** were performed based on the HSQC, HMBC, and ^{1}H–^{1}H COSY experiments (Fig. 2). The IR spectrum of **1** showed an absorption band of hydroxyl groups (ν_{max} 3450 cm^{-1}), which was further confirmed by NMR signals at δ_H 3.08 (1H, m, H-3), 3.21 (1H, d, *J* = 9.5 Hz, H-22), and δ_C 76.5 (C-3 and C-22). The HMBC cross peaks from H-4 and H_3-21 to δ_C 76.5 revealed that two hydroxyl groups were located at C-3 and C-22. The presence of a tetra-substituted epoxide was determined by the ^{13}C NMR signals at δ_C 80.0 and 69.8. The position of the epoxy functionality was assigned to C-17/C-20 by the HMBC correlations between H_3-21/C-17, H_3-21/C-20, and H_3-18/C-17. The presence of an isopropyl group was evident at δ_H 1.53 (1H, m, H-25)/δ_C 32.2 (C-25), δ_H 0.92 (3H, d, *J* = 6.5 Hz, H-26)/δ_C 20.8 (C-26), and δ_H 0.95 (3H, d, *J* = 6.5 Hz, H-27)/δ_C 21.8 (C-27). Furthermore, the NMR signals of three secondary methyls at δ_H 0.81 (3H, d, *J* = 6.5 Hz, H_3-28)/δ_C 13.1 (C-28), δ_H 0.89 (3H, d, *J* = 6.5 Hz, H_3-29)/δ_C 10.3 (C-29), and δ_H 0.96 (3H, d, *J* = 6.5 Hz, H_3-30)/δ_C 15.1 (C-30), and two tertiary methyls at δ_H 0.91 (3H, s, H_3-18)/δ_C 15.2 (C-18) and δ_H 0.83 (3H, s, H_3-19)/δ_C 13.3 (C-19) were observed. On the basis of above analysis and comparison of the NMR data of **1** (Table 1) with those of a related compound,[23] the planar structure of compound **1** was established unambiguously.

The relative configuration of **1** was determined through the analysis of NOE correlations as well as a computer-generated lower energy conformation using MM2 force field calculations (Fig. 3). In

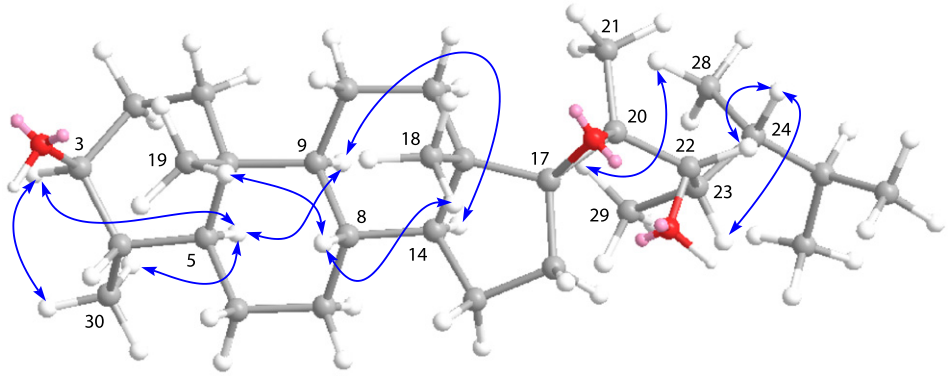

Figure 3. Key NOE correlations and computer-generated perspective model using MM2 force field calculations for **1**.

Table 1
NMR Data for compound **1**

Position	δ_C[a,b]	δ_H[a,c] (*J* in Hz)
1	36.8	1.74 (m)
		1.35 (m)
2	31.8	1.82 (m)
		0.84 (m)
3	76.5	3.08 (m)
4	39.2	1.31 (m)
5	50.8	0.75 (m)
6	31.0	1.84 (m)
		1.50 (m)
7	24.0	1.72 (m)
		1.07 (m)
8	35.0	1.44 (m)
9	54.2	0.61 (m)
10	36.0	
11	21.2	1.60 (m)
		1.29 (m)
12	36.8	1.80 (m)
		1.04 (m)
13	43.8	
14	54.8	1.17 (m)
15	23.6	1.61 (m)
		1.34 (m)
16	31.0	1.97 (m)
		1.71 (m)
17	80.0	
18	15.2	0.91 (s)
19	13.3	0.83 (s)
20	69.8	
21	15.8	1.42 (s)
22	76.5	3.21 (d, 9.5)
23	36.7	1.89 (m)
24	39.0	1.20 (m)
25	32.2	1.53 (m)
26	20.8	0.92 (d, 6.5)
27	21.8	0.95 (d, 6.5)
28	13.1	0.81 (d, 6.5)
29	10.3	0.89 (d, 6.5)
30	15.1	0.96 (d, 6.5)

Assignments were confirmed by HSQC, HMBC, ^1H–^1H COSY, and NOESY spectra.
[a] Recorded in $CDCl_3$.
[b] 125 MHz.
[c] 500 MHz.

Table 2
Cytotoxicity data for compounds **1**–**6**

Compounds	Cell lines IC_{50} (μM)		
	A549	HCT-116	HL-60
1	4.5 ± 0.5	3.2 ± 0.9	5.6 ± 0.4
2	>20[a]	6.9 ± 0.8	>20
3	>20	18.1 ± 1.2	>20
Mitoxantrone[b]	7.8 ± 0.4	7.2 ± 0.3	8.2 ± 0.9

IC_{50} values are expressed as mean ± SEM, (n = 6).
Compounds **4**–**6** were inactive for all cell lines.
[a] A compound is considered to be inactive with IC_{50} >20 μM.
[b] Positive control.

the NOESY spectrum of **1**, the NOE correlations between H-3/H-5, H-3/H_3-30, and H5/H-9, indicated α-orientations of H-5, H-9, and H_3-30. The NOE cross peaks from H-8 to H_3-18, and H_3-19 but not H-5, clearly revealed that H-8, H_3-18 and H_3-19 are positioned on the β-side. In addition, H_3-18 did not give NOE interaction with H_3-21, indicating that H_3-21 and the C-17/C-20 epoxy group are α- and β-oriented, respectively. H-22 showed a NOE cross peak with H-24 but not with H_3-21, and a NOE correlation between H-23 and H-24, suggesting β-orientations of H-22, H-23, and H-24. Based on the above analysis and other detailed NOE correlations, the relative configuration of **1** was established. Finally, the absolute configuration of **1** was determined using the modified Mosher's method.[24] The (*S*)- and (*R*)-MTPA esters of **1** (**1a** and **1b**, respectively) were prepared using (*R*)- and (*S*)-MTPA chloride, respectively.[25] The determination of $\Delta\delta$ values (δ_S–δ_R) for protons neighboring C-22 led to the assignment of the *R* configuration at C-22 in compound **1** (Fig. 1). Finally, the structure of **1** was determined to be (3*S*,4*S*,22*R*,23*S*,24*S*)-17β,20β-epoxy-4,23,24-trimethylcholest-3β,22α-diol, named lobophytosterol.

The cytotoxicity of compounds **1**–**6** against A549, HCT-116, and HL-60 cells was evaluated (Table 2) using the MTT assay.[26] Among the compounds tested, compound **1** showed the strongest cytotoxicity against A549, HCT-116, and HL-60 cells with IC_{50} values of 4.5, 3.2, and 5.6 μM, respectively. Indeed, the cytotoxicity of **1** was more potent than that of the chemotherapy drug mitoxantrone. Compound **2** and **3** exhibited significant cytotoxic activity against only HCT-116 cells with IC_{50} values of 6.9 and 18.1 μM, respectively.

Since compound **1** showed potent cytotoxicity against the cancer cell lines, we next investigated whether the cytotoxicity of compound **1** might arise from an induction of apoptosis.[27] Apoptosis is characterized by nuclear morphological changes, including chromatin condensation, membrane blebbing, and cell shrinkage. After 24 h treatment of A549, HCT-116, and HL-60 cells with the IC_{50} of compound **1**, the presence of chromatin condensation in apoptotic bodies was observed in all tested cell lines by staining with DNA-specific fluorescent dye Hoechst 33342 (Fig. 5). These data demonstrate that compound **1** might inhibit the growth of A549, HCT-116, and HL-60 cancer cells by the induction of apoptosis.

The effects of compounds **1**–**6** on the activation of PPARs were also evaluated using a PPRE luciferase reporter assay.[28] The results (Fig. 4) showed that compounds **2**, **4**, and **6** activated the PPARs

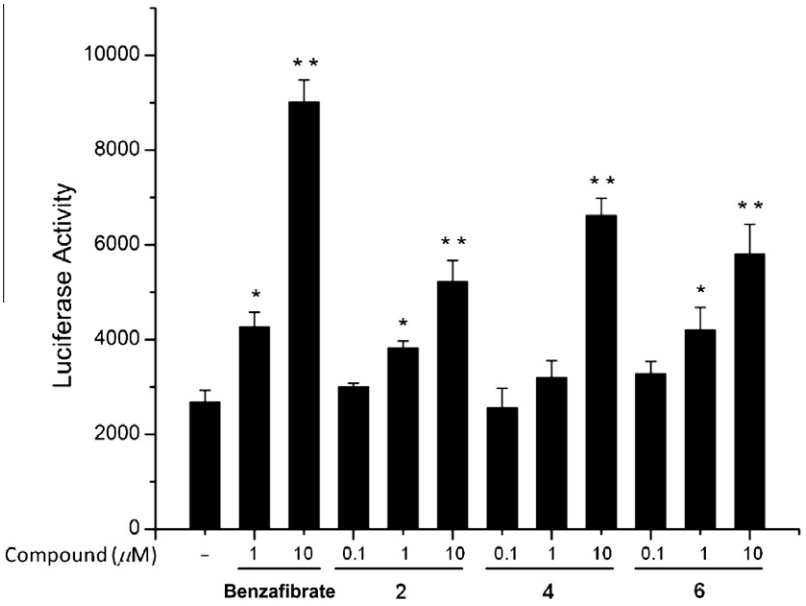

Figure 4. PPARs transcriptional activities of compounds **2**, **4**, and **6**. Benzafibrate: positive control. (−): negative control. Values are means ± SEM, $n = 6$ experiments. Statistical significance is indicated as $^{*}(p < 0.05)$ or $^{**}(p < 0.01)$ as determined by Dunnett's multiple comparison test.

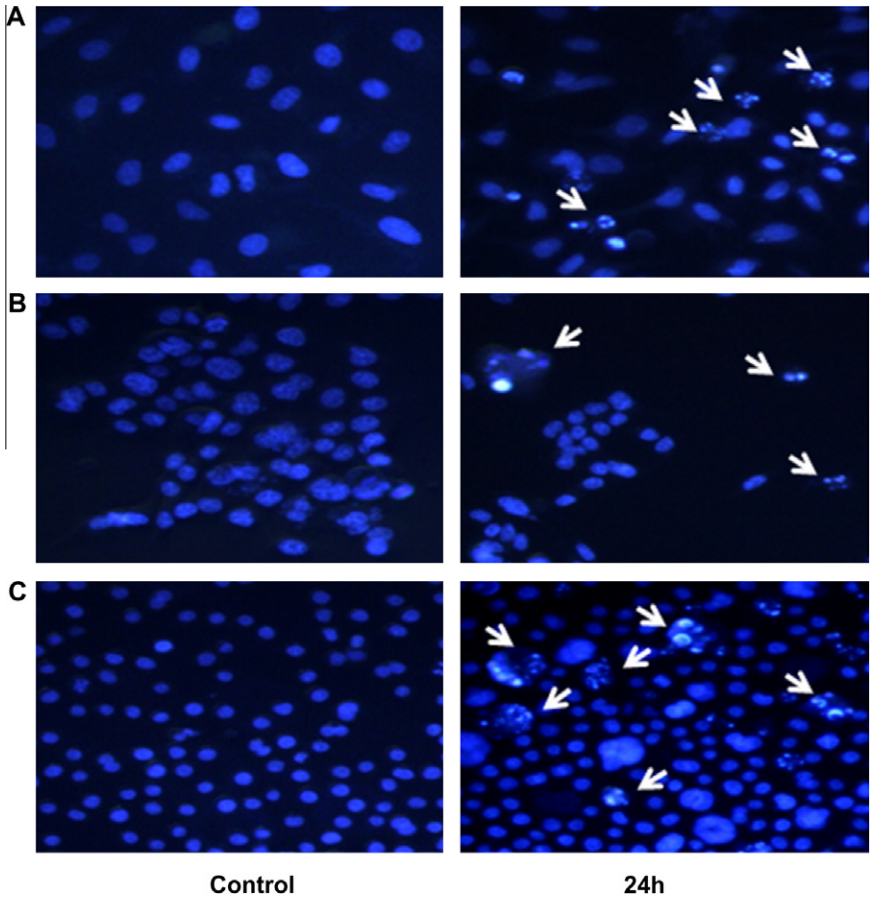

Figure 5. Compound **1** induced apoptotic characteristic in A549, HCT-116, and HL-60 cells. (A): The A549 cells (1×10^5 cells/mL) were treated with 4.5 μM of **1** for 24 h. (B): The HCT-116 cells (1×10^5 cells/mL) were treated with 3.2 μM of **1** for 24 h. (C): The HL-60 cells (4×10^5 cells/mL) were treated with 5.6 μM of **1** for 24 h.

transcriptional activity in a dose-dependent manner. At concentration of 1 μM, compounds **2**, **4**, and **6** enhanced the PPARs transcriptional activity by 1.4, 1.2, and 1.6-fold, respectively. At concentration of 10 μM, compound **4** showed the most potent stimulation of PPARs transcriptional activity, with an activation of 2.5-fold, while compounds **2** and **6** upregulated PPARs activity by 2.0 and 2.1-fold, respectively, as compared to negative control. These data suggested that compounds **2**, **4**, and **6** may act as spe-

cific agonists for PPARs isoforms (PPARα, PPARδ, and PPARγ) and may therefore regulate glucose, lipid, and cholesterol metabolism.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.03.089.

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10. *Lobophytosterol (**1**)*: white amorphous powder; $[\alpha]_D^{25}$ +9.74 (*c* 1.00, $CHCl_3$); IR (CH_3CN) ν_{max} 3450, 2915, 2360, 1458, 1373, 1226, 1015 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz): see Table 1; HR-FTICR-MS *m*/*z* 443.38876 $[M+H-H_2O]^+$ (calcd for $C_{30}H_{51}O_2$, 443.38891).
(22*S*,24*S*)-24-methyl-22,25-epoxyfurost-5-ene-3β,20β-diol (**2**): white powder; mp 213–216 °C; $[\alpha]_D^{25}$ −38.1 (*c* 0.1, $CHCl_3$); ESI-MS *m*/*z* 445 $[M+H]^+$.
(24*S*)-24-methylcholest-5-ene-3β,25-diol (**3**): white powder; mp 184–188 °C; $[\alpha]_D^{25}$ −51.3 (*c* 1.00, $CHCl_3$); ESI-MS *m*/*z* 439 $[M+Na]^+$.
(24*S*)-ergost-5-ene-3β,7α-diol (**4**): white powder; mp 210–212 °C; $[\alpha]_D^{25}$ −75.2 (*c* 0.45, $CHCl_3$); ESI-MS *m*/*z* 417 $[M+H]^+$.
Cholesterol (**5**): white powder; mp 148–149 °C; $[\alpha]_D^{25}$ −31.6 (*c* 1.00, Et_2O); ESI-MS *m*/*z* 409 $[M+Na]^+$.
Pregnenolone (**6**): colorless gum; mp 192–194 °C; $[\alpha]_D^{25}$ +28.5 (*c* 1.00, EtOH); ESI-MS *m*/*z* 317 $[M+H]^+$.
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22. 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 six fractions (fractions A1–A6). Fraction A3 was chromatographed on silica gel column eluting with *n*-hexane–EtOAc (6:1) to afford four subfractions (A3A–A3D). Subfraction A3C was separated over silica gel using CH_2Cl_2–EtOAc (15:1) as eluent to yield **5** (27 mg). Subfraction A3D was chromatographed on silica gel column eluting with CH_2Cl_2–EtOAc (10:1) and further purified by YMC reverse-phase chromatography, using acetone–water (3:1) as eluent to afford **1** (15 mg). Fraction A4 was chromatographed over silica gel using CH_2Cl_2–EtOAc (13:1) as eluent to afford three subfractions (A4A–A4C). Subfraction A4A was chromatographed over silica gel column eluting with *n*-hexane–EtOAc (4:1) and further separated by column chromatography over silica gel, using *n*-hexane–acetone (6:1) as eluent to give **2** (6 mg). Subfraction A4C was separated by column chromatography over silica gel, using CH_2Cl_2–EtOAc (6:1) as eluent to afford **3** (18 mg). Fraction A5 was chromatograped over silica gel eluting with *n*-hexane–acetone (4:1) to afford four subfractions (A5A–A5D). Subfraction A5B was chromatographed on silica gel column with CH_2Cl_2–acetone (8:1) and further separated by column chromatography over silica gel, using *n*-hexane–acetone (4:1) as eluent to obtain **4** (16 mg) and **6** (10 mg).
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25. 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 using CH_2Cl_2EtOAc (10:1) as eluent 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 7.31–7.55 (5H, Ph), 4.94 (1H, d, *J* = 10.4 Hz, H-22), 4.53 (1H, m, H-3), 1.98 (1H, m, H-23), 1.28 (3H, s, H_3-21), 1.09 (1H, m, H-24), 0.90 (3H, d, *J* = 6.8 Hz, H_3-30), 0.88 (3H, d, *J* = 6.8 Hz, H_3-27), 0.82 (3H, d, *J* = 6.8 Hz, H_3-26), 0.81 (3H, s, H_3-18), 0.80 (3H, d, *J* = 6.4 Hz, H_3-29), 0.75 (3H, d, *J* = 6.8 Hz, H_3-28), 0.74 (3H, s, H_3-19). Selected ^1H NMR ($CDCl_3$, 400 MHz) of **1b**: δ_H 7.317.59 (5H, Ph), 4.92 (1H, d, *J* = 10.4 Hz, H-22), 4.55 (1H, m, H-3), 1.95 (1H, m, H-23), 1.36 (3H, s, H_3-21), 0.98 (1H, m, H-24), 0.87 (3H, d, *J* = 6.8 Hz, H_3-30), 0.85 (3H, d, *J* = 6.8 Hz, H_3-27), 0.83 (3H, s, H_3-18), 0.80 (3H, d, *J* = 6.8 Hz, H_3-26), 0.77 (3H, s, H_3-19), 0.63 (3H, d, *J* = 6.4 Hz, H_3-29), 0.52 (3H, d, *J* = 6.8 Hz, H_3-28).
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27. A549, HCT-116, and HL-60 cells were treated with the IC_{50} of compound **1** for 24 h. Cells were washed with PBS prior to stained with 1 mg/mL DNA-specific fluorescent dye Hoechst 33342 for 30 min at 37 °C. Apoptotic bodies, with condensed and fragmented nuclei, were imaged using an inverted fluorescent microscope equipped with an IX-71 Olympus camera (magnification ×200).
28. Hep-G2 human hepatocarcinoma cells were seeded at 1.5×10^5 cells per well in 12-well plates and grown for 24 h prior to transfection. An optimized amount of DNA plasmid (0.5 μg PPRE-Luc and 0.2 μg CMV-PPARγ) was diluted in 100 μL Dulbecco's modified Eagle medium (DMEM). PLUS™ Reagent (0.5 μL) and 1 μL Lipofectamine™ LTX (Invitrogen, Carlsbad, CA) were then added to the DNA plasmid solution and mixed thoroughly. Following 30 min of incubation at room temperature, 100 μL of transfection mixture was added to the cells and mixed gently. After 24 h of transfection, the medium was replace with Opti-MEM (Invitrogen, Carlsbad, CA) containing 0.1 mM Non-Essential Amino Acids, 0.5% charcoal-stripped FBS, and either the individual compounds (test group), dimethyl sulfoxide (negative control group), or benzafibrate (positive control group). The cells were further cultured for 20 h. The cells were then washed with PBS and harvested with 200 μL of 1× passive lysis buffer. The intensity of emitted luminescence was determined using a LB 953 Autolumat (EG&G Berthold, Bad Wildbad, Germany). The luminescence intensity ratio (test group/control group) was determined for each compound, and PPARs transcriptional activity was expressed as the relative luminescence intensity of the test compound to that of the control sample.