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Hirsutalins I–M, eunicellin-based diterpenoids from the soft coral *Cladiella hirsuta*

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ABSTRACT

New eunicellin-base diterpenoids, hirsutalins I–M (**1**–**5**), were isolated from the soft coral *Cladiella hirsuta*. Their structures were elucidated by spectroscopic methods, particularly in 1D and 2D NMR experiments. The absolute configuration of **2** was determined by Mosher's method. These compounds did not exhibit cytotoxicity toward a limited panel of cancer cell lines but showed nitric oxide inhibitory activity in LPS-stimulated RAW264.7 macrophage cells. Among them, compound **3** was found to possess the strongest NO inhibitory activity with an IC_{50} value of 9.8 μg/mL. Furthermore, **3** was shown to significantly reduce the expression iNOS protein in the same cells.

1. Introduction

Previous reports on the chemical constituents of soft corals belonging to the genus *Cladiella*[1–16] have shown that eunicellin-based diterpenoids are major secondary metabolites in these marine organisms. During the course of our investigation into new natural substances from the soft coral *Cladiella hirsuta*, new eunicellin-type metabolites hirsutalins A–H,[17] and several polyoxygenated steroids, hirsutosterols A–G,[18] were isolated. In our continuing effort toward the discovery of new and bioactive substances from marine invertebrates, the chemical constituents of the soft coral *C. hirsuta* were further studied. This investigation again led to the isolation of five new eunicellin-based metabolites, hirsutalins I–M (**1**–**5**). The structures with relative configuration of compounds **1**–**5** were established by extensive spectroscopic analysis, including 2D NMR (^{1}H–^{1}H COSY, HSQC, HMBC, and NOESY) spectroscopy. The absolute configuration of **2** was further determined by Mosher's method. Cytotoxicity of metabolites **1**–**5** against a limited panel of human tumor cell lines including human liver carcinoma (Hep G2), human breast carcinoma (MDA-MB-231) and human lung carcinoma (A-549) and the anti-inflammatory activity of **1**–**5** to inhibit NO (nitric oxide) production and up-regulation of the pro-inflammatory iNOS (inducible nitric oxide synthase) in LPS (lipopolysaccharide)-stimulated RAW264.7 macrophage cells were studied.

2. Results and discussion

The frozen bodies of the octocoral *C. hirsuta* were minced and extracted exhaustively with acetone. The combined organic extract was concentrated to an aqueous suspension, which was partitioned between EtOAc and H_2O. The EtOAc-soluble fraction was concentrated under reduced pressure and the residue was repeatedly purified by chromatography to yield metabolites **1**–**5** (Scheme 1).

Hirsutalin I (**1**) was isolated as a colorless oil. The HRESIMS of **1** exhibited an $[M+Na]^+$ peak at *m*/*z* 505.2780, which established a molecular formula of $C_{26}H_{42}O_8$, implying six degrees of unsaturation. The IR spectrum of **1** revealed the presence of hydroxy and carbonyl functionalities from absorptions at 3414, 1738, and 1715 cm^{-1}, respectively. The ^{13}C NMR spectroscopic data of **1** included 26 carbon signals (Table 1), which were assigned by the assistance of a DEPT spectrum into 6 methyls, 8 methylenes (including 1 oxymethylene), 6 methines (including 2 oxymethines), 3 carbonyls, and 3 sp^3 quaternary carbons. The ^{13}C NMR spectrum of **1** showed the presence of a ketone (δ_C 211.3). Two ester carbonyl carbons (δ_C 172.8 and 169.6) were correlated with the methylenes

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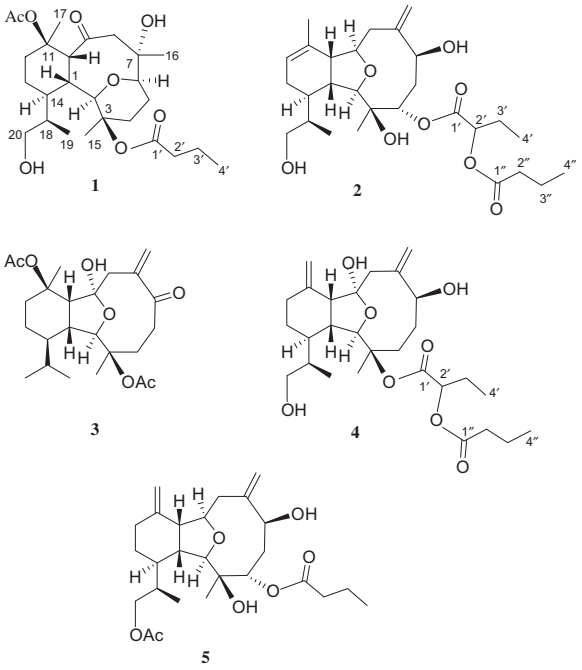

Scheme 1. Structures of metabolites **1**–**5**.

(δ_H 2.43 m, 2H and 1.73 m, 2H) of an *n*-butyrate and an acetate methyl (δ_H 2.00 s, 3H) on the basis of HMBC analysis, respectively. The remaining three degrees of unsaturation identified **1** as a tricyclic compound. The planar structure of metabolite **1** was elucidated by analysis of ^1H–^1H COSY and HMBC correlations, as shown in Fig. 1. Key HMBC correlations from H-2 to C-1 and C-6; H-1, H_2-8, and H-10 to C-9; H_3-15 to C-2, C-3 and C-4; H_3-16 to C-6, C-7 and C-8; H_3-17 to C-10, C-11 and C-12; and both H_3-19 and H_2-20 to C-14 and C-18, permitted the assembly of the carbon skeleton of **1**. Therefore, the planar structure of **1** was established. In the NOESY spectrum of **1** (Fig. 2), observation of the NOE correlations among H-10 and both H-1 and one proton (δ_H 2.77) at C-8, which was assigned as H-8β (δ_H 2.77); H_3-16 with H-8β and one proton (δ_H 1.33) at C-5, but not with H-6, suggested the β-orientations of H-1, H-10, and H_3-16 and α-orientation of H-6. Also, H-2 did not correlate with both H-1 and H-10, but correlated with both H-14 and H_3-15, and H-14 correlated with H-12α (δ_H 1.31), which was further correlated with H_3-17, suggesting all of H-2, H-14, H_3-15, and H_3-17 are α-oriented. Besides, both H-2 and H-6 showed NOE correlations with H-4α, confirming the α-orientation of H-6. Furthermore, the asymmetric center at C-18 was suggested to be *R**-configured on the basis of NOE correlations between the β-oriented H-1 and H_3-19 and between the α-oriented H-2 and H-18. Thus, the relative configuration of diterpene **1** was established.

The HRESIMS of **2** exhibited a pseudomolecular ion peak at *m*/*z* 531.2931 $[M+Na]^+$, consistent with a molecular formula of $C_{28}H_{44}O_8$. The ^{13}C NMR data of **2** revealed the presence of one

Table 1
^1H NMR data for compounds **1**–**5**

Position	**1**[a]	**2**[a]	**3**[a]	**4**[a]	**5**[a]
1	48.9 (CH)[b]	39.3 (CH)	41.3 (CH)	41.7 (CH)	43.2 (CH)
2	77.9 (CH)	88.2 (CH)	89.1 (CH)	89.8 (CH)	91.2 (CH)
3	81.0 (C)	74.7 (C)	84.6 (C)	86.0 (C)	73.6 (C)
4	27.6 (CH_2)	73.3 (CH_2)	32.6 (CH_2)	27.5 (CH_2)	73.7 (CH)
5	20.4 (CH_2)	36.7 (CH_2)	36.4 (CH_2)	35.7 (CH_2)	41.1 (CH_2)
6	80.4 (CH)	72.9 (CH)	206.2 (C)	71.7 (CH)	70.1 (CH)
7	85.5 (C)	147.3 (C)	146.4 (C)	150.7 (C)	150.6 (C)
8	49.5 (CH_2)	40.0 (CH_2)	47.5 (CH_2)	46.8 (CH_2)	38.1 (CH_2)
9	211.3 (C)	82.7 (CH)	105.2 (C)	108.7 (C)	80.4 (CH)
10	55.6 (CH)	43.9 (CH)	47.9 (CH)	48.5 (CH)	46.8 (CH)
11	83.2 (C)	132.5 (C)	82.8 (C)	145.3 (C)	145.3 (C)
12	30.3 (CH_2)	121.1 (CH_2)	34.3 (CH_2)	33.1 (CH_2)	31.6 (CH_2)
13	20.2 (CH_2)	21.7 (CH_2)	18.0 (CH_2)	25.1 (CH_2)	25.8 (CH_2)
14	32.1 (CH)	33.8 (CH)	41.3 (CH)	38.6 (CH)	39.7 (CH)
15	24.2 (CH_3)	21.6 (CH_3)	22.2 (CH_3)	21.8 (CH_3)	23.4 (CH_3)
16	22.9 (CH_3)	115.2 (CH_2)	120.2 (CH_2)	117.3 (CH_2)	117.9 (CH_2)
17	23.5 (CH_3)	22.0 (CH_2)	24.0 (CH_3)	112.4 (CH_2)	112.0 (CH_2)
18	35.9 (CH)	35.3 (CH)	28.1 (CH)	36.5 (CH)	32.7 (CH)
19	9.9 (CH_3)	16.2 (CH_3)	15.0 (CH_3)	10.6 (CH_3)	10.4 (CH_3)
20	67.3 (CH_2)	66.2 (CH_2)	21.7 (CH_3)	66.6 (CH_2)	68.0 (CH_2)
n-Butyrate	172.8 (C)				173.8 (C)
	37.2 (CH_2)				36.4 (CH_2)
	18.5 (CH_2)				18.4 (CH_2)
	13.7 (CH_3)				13.6 (CH_3)
2-Butyryloxybutanoate					
1′		171.4 (C)		169.2 (C)	
2′		74.2 (CH)		73.6 (CH)	
3′		24.3 (CH_2)		24.4 (CH_2)	
4′		9.3 (CH_3)		9.6 (CH_3)	
1″		174.2 (C)		173.5 (C)	
2″		35.8 (CH_2)		35.7 (CH_2)	
3″		18.3 (CH_2)		18.3 (CH_2)	
4″		13.6 (CH_3)		13.6 (CH_3)	
3-OAc			170.2 (C)		
			22.7 (CH_3)		
11-OAc	169.6 (C)		170.5 (C)		
	22.4 (CH_3)		24.0 (CH_3)		
20-OAc					171.3 (C)
					21.1 (CH_3)

[a] Spectra recorded at 100 MHz in $CDCl_3$ at 25 °C.
[b] Multiplicities deduced by DEPT.

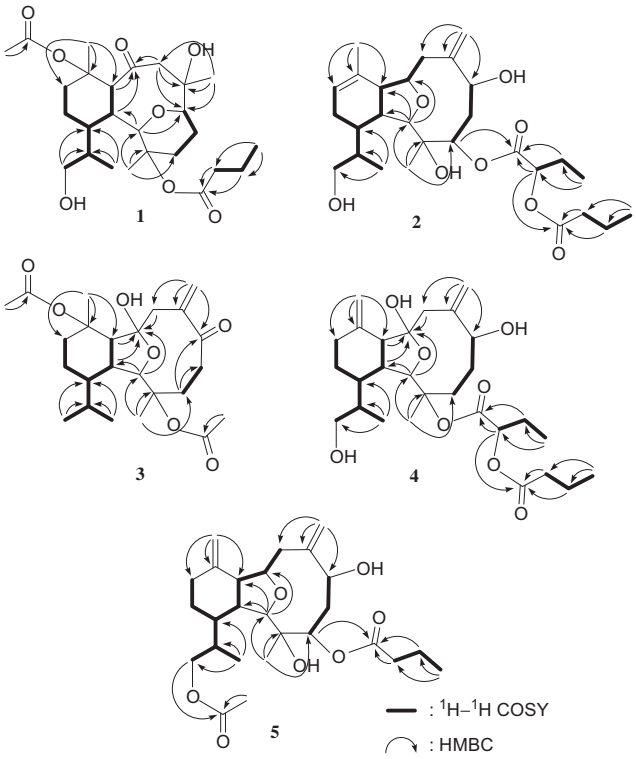

Fig. 1. Key ^{1}H–^{1}H COSY and HMBC correlations for **1**–**5**.

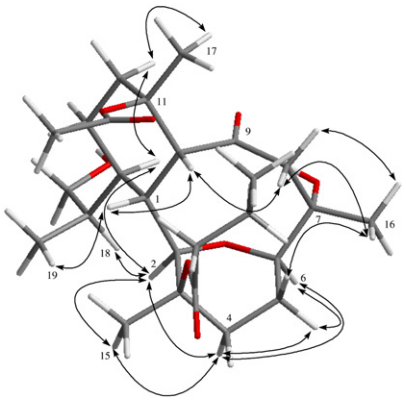

Fig. 2. Key NOESY correlations of **1**.

trisubstituted and one 1,1-disubstituted carbon–carbon double bonds [δ_C 132.5 (C) and 121.1 (CH); 147.3 (C) and 115.2 (CH_2)]. The planar structure of **2** was established by 2D NMR experiments, in particular by analysis of ^{1}H–^{1}H COSY and HMBC correlations (Fig. 1). Two ester carbonyl carbons (δ_C 174.2 and 171.4) were correlated in the HMBC spectrum with the methine proton (H-2′, δ_H 4.81 t, J=6.0 Hz) of a 2-butyryloxybutanoate unit. The placement of the above butanoate at C-4 was proven by the HMBC correlation from H-4 (δ 5.28) to the carbonyl carbon resonating at δ 171.4 (C). In the NOESY spectrum of **2** (Fig. 3), observation of the NOE correlations among H-10 and both H-1 and H-4; and H-1 and H_3-19, suggested that H-1, H-4, H-10, and H_3-19 are all β-oriented. Also, correlations of H-2 with both H_3-15 and H-14; H-9 with H-14; and H-6 with H_3-15, suggested that H-2, H-6, H-9, H-14, and H_3-15 are all α-oriented. Furthermore, the asymmetric center at C-18 was suggested to be R^*-configured on the basis of NOE correlations between the β-oriented H-1 and H_3-19 and between the α-oriented H-2 and H-18. Thus, the relative configuration of diterpene **2** was established. In order to resolve the absolute structure of **2**, we

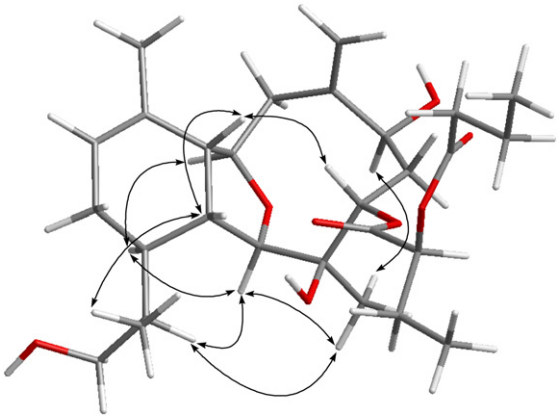

Fig. 3. Key NOESY correlations of **2**.

determined the absolute configuration at C-6 using Mosher's method.[19,20] The *S*- and *R*-α-methoxy-α-trifluoromethyl-phenylacetic (MTPA) esters of **2** (**2a** and **2b**, respectively) were prepared by using the corresponding *R*-(−)- and *S*-(+)-α-methoxy-α-(trifluoromethyl) phenylacetyl chlorides, respectively. The values of $\Delta\delta$ [δ(*S*-MTPA ester)−δ(*R*-MTPA ester)] for H-8, H-9, and H_2-16 were positive, while the values of $\Delta\delta$ for H-4, and H_2-5 were negative, revealing the *S*-configuration at C-6 (Fig. 4). On the basis of its HRESIMS (*m*/*z* 459.2357 [M+Na]$^+$), the molecular formula of hirsutalin K (**3**) was established as $C_{24}H_{36}O_7$. By comparison of the NMR data of **3** with those of palmonine F,[21] it was found that the C-6 hydroxy-bearing methine in palmonine F was oxidized to a ketone (δ_C 206.2) in **3**. Moreover, the signal of oxymethine group (δ_C 78.8, C-9) in palmonine F was replaced by that of a hemiketal (δ_C 105.2, CH, C-9) in **3**, as confirmed by HMBC correlations from H-1, H-2, H_2-8, and H-10 to C-9 (Fig. 1). On the other hand, comparison of the NMR data of **3** with those of palmonine D[22] revealed that the only difference between both compounds was the replacement of H-9 in palmonine D by the hydroxy group in **3**. The relative configurations for all asymmetric carbons in **3** were elucidated by the analysis of NOE correlations, as shown in Fig. 5. When the ^{1}H NMR spectrum of **3** was measured in C_5D_5N, it was found that a significant pyridine-induced downfield shift ($\Delta\delta$=δ($CDCl_3$)−δ(C_5D_5N)) was experienced by H_3-17 ($\Delta\delta$=−0.29 ppm), which could be achieved only when H_3-17 and 9-OH are α-oriented on the same face of the molecule.[5,23,24]

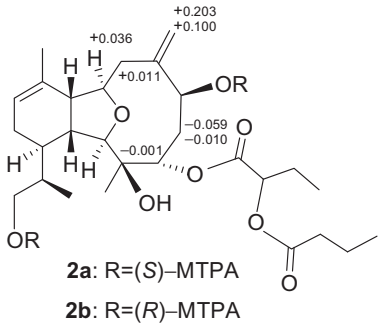

Fig. 4. ^{1}H NMR chemical shift differences $\Delta\delta$ (δ_S−δ_R) in parts per million (ppm) for the MTPA esters of **2**.

Hirsutalin L (**4**) was isolated as a colorless oil. The HRESIMS of **4** exhibited an [M+Na]$^+$ peak at *m*/*z* 531.2937, which established a molecular formula of $C_{28}H_{44}O_8$, implying seven degrees of unsaturation. Similar to hirsutalin C,[17] the ^{13}C NMR spectroscopic data of **4** (Table 1) showed the presence of one 2-butyryloxybutanoate [δ_C 169.2 (C), 73.6 (CH), 24.4 (CH_2), and 9.6 (CH_3);

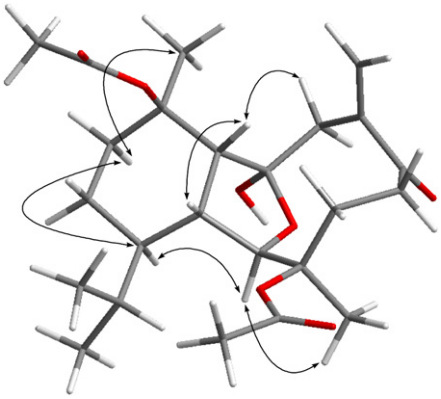

Fig. 5. Key NOESY correlations of **3**.

173.5 (C), 35.7 (CH_2), 18.3 (CH_2), and 13.6 (CH_3)]. Comparison of the 1D and 2D NMR data of **4** with those of hirsutalin C revealed that the only difference between the two compounds was the replacement of the oxymethine moiety (δ_C 80.1) at C-9 in hirsutalin C by a hemiketal group (δ_C 108.7) in **4**, as confirmed by the HMBC correlations from H-2 to C-9. The relative configuration for all asymmetric carbons in **4** was elucidated by the analysis of NOE correlations. One proton (δ_H 2.73) at C-8 showed NOE correlations with H-10, confirming the α-orientation of 9-OH as shown in Fig. 6.

A structurally related metabolite, hirsutalin M (**5**), was also isolated as a colorless oil with a molecular formula of $C_{26}H_{40}O_7$, implying seven degrees of unsaturation. The ^{13}C NMR spectroscopic data of **5** (Table 1) showed the presence of an acetate [δ_C 171.3 (C) and 21.1 (CH_3)]. Moreover, the ^1H NMR spectroscopic data (Table 2) of **5** showed the presence of one *n*-butyrate, which showed signals at δ_H 2.34 (2H, m), 1.67 (2H, m), 0.97 (3H, t, J=6.8 Hz). Comparison of the NMR data of **5** with those of hirsutalin B[17] revealed that the only difference between the compounds was the replacement of the 2-butyryloxybutanoate in hirsutalin B by an *n*-butyrate at C-4 in **5**.

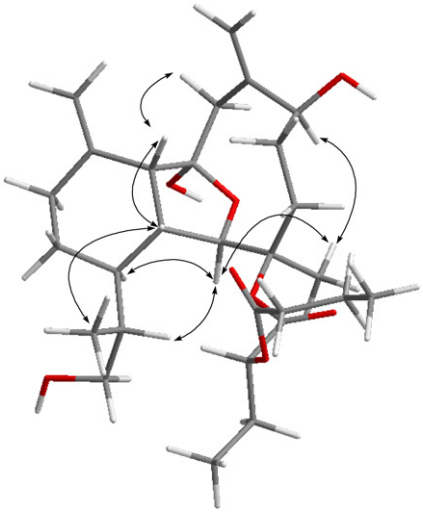

Fig. 6. Key NOESY correlations of **4**.

Although many eunicellin-type natural products have been discovered, eunicellins possessing a 2-acyloxybutyrate and a hemiketal as in **3** and **4** were discovered for the first time. Cytotoxicity of metabolites **1**–**5** against a limited panel of human tumor cell lines including human liver carcinoma (Hep G2), human breast carcinoma (MDA-MB-231), and human lung carcinoma (A-549) was investigated in order to discover the useful biological activity of new compounds, however, none of these compounds was found to possess satisfactory cytotoxicity at 20 μM. The NO inhibitory activities of compounds **1**–**5** were further evaluated by assay of LPS-induced NO production in activated RAW264.7 cells, as shown in Fig. 7. The dosage of each compound used was 20 μg/mL and the results indicated that the metabolite **3**, inhibiting 97% NO production at a dosage of 20 μg/mL, possessed the strongest NO inhibitory activity with an IC_{50} value of 9.8 μg/mL while curcumin at 10 μg/mL was used as a positive control (Fig. 8). Meanwhile, compounds **1**–**5** were evaluated for the accumulation of pro-inflammatory iNOS and COX-2 proteins in RAW264.7 macrophage cells stimulated with LPS using immunoblot analysis. At the concentration of 2.5–10 μg/mL, compound **3** was found to significantly reduce the expression of iNOS protein, and could completely inhibit the expression of iNOS protein at 20 μg/mL relative to the control cells only stimulated with LPS as shown in Fig. 9. On the other hand, none of these compounds was found to reduce the expression of COX-2 protein. The above results revealed that the α,β-unsaturated ketone in **3** might be able to significantly reduce the expression of iNOS protein in the LPS-stimulated RAW264.7 cells. Thus, **3** is a potential anti-inflammatory compound and further biomedical investigation is necessary.

3. Experimental

3.1. General experimental procedures

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

3.2. Material

Specimens of the soft coral *C. hirsuta* were collected by hand using SCUBA off the coast of Sianglu Islet (23°32′ N, 119°38′ E) in the region of Penghu Islands, in June 2008, at a depth of 10 m, and were stored in a freezer until extraction. A voucher sample (PI-20080610-17) was deposited at the Department of Marine Biotechnology and Resources, National Sun Yat-sen University.

3.3. 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 partitioned between EtOAc and H_2O. The EtOAc layer was dried with anhydrous Na_2SO_4. After removal of 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 20, eluted with *n*-hexane/EtOAc (1:2), 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 reversed-phase HPLC (CH_3CN/H_2O, 3:1) to afford compounds **1** (1.4 mg), **3** (1.8 mg), and **5** (1.6 mg). Subfraction A3 was separated by reversed-phase HPLC (CH_3CN/H_2O, 2:3) to afford compounds **2** (5.8 mg) and **4** (1.1 mg).

*3.3.1. Hirsutalin I (**1**).* Colorless oil; $[\alpha]_D^{22}$ −21 (*c* 0.14, $CHCl_3$); IR (neat) ν_{max} 3414, 1738, and 1715 cm^{-1}; ^{13}C (100 MHz, $CDCl_3$) and ^1H

Table 2
^{1}H NMR data for compounds **1**–**5**

Position	**1**[a]	**2**[a]	**3**[a]	**4**[a]	**5**[a]
1	2.59 dd (12.8, 4.4)[b]	2.82 t (8.8)	2.20 m	2.31 m	2.46 m
2	3.86 s	3.84 d (8.4)	3.70 s	3.90 s	3.78 s
4	2.95 dd (11.6, 5.2)	5.28 m	2.12 m	2.29 m	4.86 dd (8.4, 4.0)
	1.36 m		2.68 m	1.71 m	
5	1.65 m	2.94 m	2.28 m	2.19 m	2.43 m
	1.33 m	1.74 m		1.72 m	1.96 m
6	3.83 dd (6.0, 5.2)	4.24 m		4.44 dd (10.4, 4.0)	4.41 dd (8.4, 4.4)
8	2.77 d (12.4)	2.36 m	3.02 s	2.73 d (13.6)	2.68 dd (13.6, 4.4)
	2.00 d (12.0)	2.34 m		2.53 d (13.6)	2.34 d (13.6)
9		4.18 m			4.17 dd (10.0, 2.8)
10	4.08 br d (2.8)	2.70 d (8.4)	3.54 d (7.2)	3.19 d (8.8)	3.03 (9.6, 8.8)
12	2.26 m	5.45 m	1.98 m	2.46 m	2.27 m
			1.90 m	2.30 m	2.03 m
13	1.44 m	2.16 m	1.40 m	1.65 m	1.64 m
	1.31 m	1.96 m	1.38 m	1.08 m	1.13 m
14	2.24 m	1.91 m	1.48 m	1.90 m	1.52 m
15	1.54 s	1.35 s	1.44 s	1.67 s	1.34 s
16	1.12 s	5.59 s	5.56 s	5.43 s	5.65 s
		5.21 s	5.40 s	5.20 s	5.19 s
17	1.46 s	1.68 s	1.72 s	4.94 s	4.84 s
				4.73 s	4.74 s
18	1.89 m	1.55 m	1.74 m	1.93 m	2.05 m
19	0.80 d (6.8)	0.93 d (6.4)	0.78 d (6.8)	0.81 d (6.8)	0.77 d (7.2)
20	3.63 dd (10.4, 7.6)	3.82 m	0.95 d (6.8)	3.55 m	3.95 d (7.6)
	3.50 dd (10.4, 6.0)	3.51 dd (11.2, 3.2)			
n-Butyrate	2.43 m				2.34 m
	1.73 m				1.67 m
	1.02 t (7.2)				0.97 t (6.8)
2-Butyryloxybutanoate					
2′		4.81 t (6.0)		4.62 t (5.6)	
3′		1.91 m		1.79 m	
4′		1.03 t (7.2)		0.99 t (7.2)	
2″		2.39 m		2.39 m	
3″		1.69 m		1.71 m	
4″		0.97 t (7.2)		0.97 t (7.2)	
3-OAc			2.05 s		
11-OAc	2.00 s		2.02 s		
20-OAc					2.07 s

[a] Recorded at 400 MHz in $CDCl_3$ at 25 °C.
[b] *J* values in hertz in parentheses.

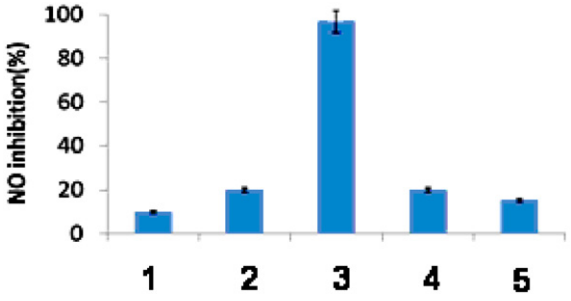

Fig. 7. Inhibitory effects of compounds **1**–**5** at 20 μg/mL on nitric oxide (NO) production in LPS-stimulated RAW264.7 cells.

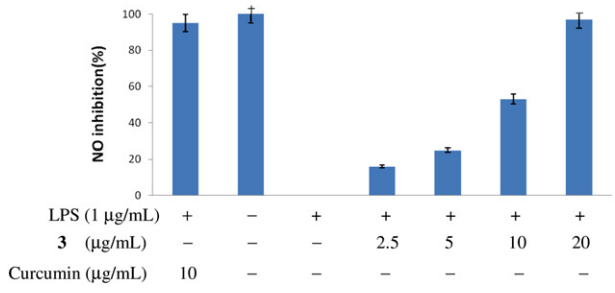

Fig. 8. Inhibitory effects of compound **3** at 2.5, 5, 10, and 20 μg/mL on nitric oxide (NO) production in LPS-stimulated RAW264.7 cells.

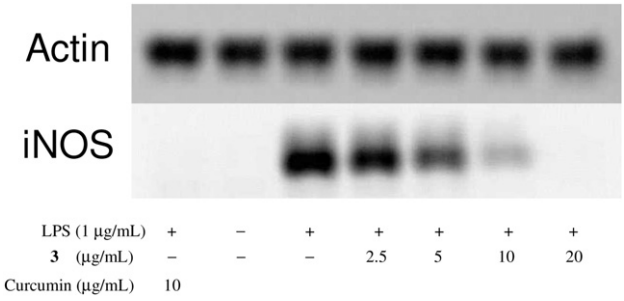

Fig. 9. Effects of **3** on the expression of iNOS protein in LPS-stimulated RAW264.7 cells. The cells were incubated with or without LPS (1 μg/mL) in the presence of the indicated concentrations of **3**.

NMR (400 MHz, $CDCl_3$) data, see Tables 1 and 2; ESIMS *m*/*z* 505 $[M+Na]^+$; HRESIMS *m*/*z* 505.2780 $[M+Na]^+$ (calcd for $C_{26}H_{42}O_8Na$, 505.2777).

*3.3.2. Hirsutalin J (**2**).* Colorless oil; $[\alpha]_D^{22}$ +13 (*c* 0.58, $CHCl_3$); IR (neat) ν_{max} 3451 and 1736 cm^{-1}; ^{13}C (100 MHz, $CDCl_3$) and ^1H NMR (400 MHz, $CDCl_3$) data, see Tables 1 and 2; ESIMS *m*/*z* 531 $[M+Na]^+$; HRESIMS *m*/*z* 531.2931 $[M+Na]^+$ (calcd for $C_{28}H_{44}O_8Na$, 531.2934).

*3.3.3. Hirsutalin K (**3**).* Colorless oil; $[\alpha]_D^{22}$ +17 (*c* 0.18, $CHCl_3$); IR (neat) ν_{max} 3443, 1739, and 1697 cm^{-1}; ^{13}C (100 MHz, $CDCl_3$) and ^1H NMR (400 MHz, $CDCl_3$) data, see Tables 1 and 2; ESIMS *m*/*z* 459

$[M+Na]^+$; HRESIMS m/z 459.2357 $[M+Na]^+$ (calcd for $C_{24}H_{36}O_7Na$, 459.2359).

*3.3.4. Hirsutalin L (**4**).* Colorless oil; $[\alpha]_D^{22}$ −44 (*c* 0.11, $CHCl_3$); IR (neat) ν_{max} 3451 and 1732 cm^{-1}; ^{13}C (100 MHz, $CDCl_3$) and ^1H NMR (400 MHz, $CDCl_3$) data, see Tables 1 and 2; ESIMS m/z 531 $[M+Na]^+$; HRESIMS m/z 531.2937 $[M+Na]^+$ (calcd for $C_{28}H_{44}O_8Na$, 531.2934).

*3.3.5. Hirsutalin M (**5**).* Colorless oil; $[\alpha]_D^{22}$ −29 (*c* 0.16, $CHCl_3$); IR (neat) ν_{max} 3431 and 1733 cm^{-1}; ^{13}C (100 MHz, $CDCl_3$) and ^1H NMR (400 MHz, $CDCl_3$) data, see Tables 1 and 2; ESIMS m/z 487 $[M+Na]^+$; HRESIMS m/z 487.2673 $[M+Na]^+$ (calcd for $C_{26}H_{40}O_7Na$, 487.2672).

3.4. Preparation of (*S*)-and (*R*)-MTPA esters of 2

To a solution of **2** (1.0 mg) in pyridine (0.4 mL) was added *R*-(−)-α-methoxy-α-(trifluoromethyl)-phenylacetyl 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, **2a**. The same procedure was used to prepare the (*R*)-MTPA ester, **2b** from the reaction of (*S*)-MTPA chloride with **2** in pyridine. Selective ^1H NMR ($CDCl_3$, 400 MHz) of **2a**: 5.562 (1H, dd, *J*=11.2, 4.8 Hz, H-6), 5.510 (1H, s, H-16a), 5.268 (1H, s, H-16b), 5.031 (1H, dd, *J*=10.8, 3.6 Hz, H-4), 4.791 (1H, t, *J*=6.4, H-2′), 3.915 (1H, dd, *J*=9.2, 5.2 Hz, H-9), 2.509 (1H, d, *J*=14.0 Hz, H-8a), 2.114 (1H, m, H-5a), 2.027 (1H, m, H-5b). Selective ^1H NMR ($CDCl_3$, 400 MHz) of **2b**: 5.514 (1H, dd, *J*=11.2, 4.8 Hz, H-6), 5.307 (1H, s, H-16a), 5.168 (1H, s, H-16b), 5.032 (1H, dd, *J*=11.2, 4.0 Hz, H-4), 4.795 (1H, t, *J*=6.8, H-2′), 3.879 (1H, dd, *J*=9.6, 5.2 Hz, H-9), 2.498 (1H, d, *J*=14.0 Hz, H-8a), 2.173 (1H, m, H-5a), 2.037 (1H, m, H-5b).

3.5. Cytotoxicity testing

Cell lines were purchased from the American Type Culture Collection (ATCC). Cytotoxicity assays were performed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method.[25,26]

3.6. In vitro anti-inflammatory assay

RAW 246.7 cells were seeded in 96-well plates at a density of 2×10^5 cells/well and grown for 2 h for adherence. The cells were treated with test samples for 1 h and then incubated for 24 h in fresh DMEM with or without 1 μg/mL of LPS. The nitrite concentration in the culture medium was measured as an indicator of NO production according to the Griess reaction.[27] Briefly, 100 μL of cell culture supernatant was reacted with 100 μL of Griess reagent (1:1 mixture of 0.1% *N*-(1-naphthyl)ethylene-diamine dihydrochloride in water and 1% sulfanilamide in 5% phosphoric acid) in a 96-well plate, and absorbance at 540 nm was recorded using the ELISA reader.[28,29]

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Supplementary data

^1H and ^{13}C NMR spectra for **1**–**5**. Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.tet.2013.01.015.

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