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Anti-inflammatory steroids from the octocoral *Dendronephthya griffini*

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Abstract

Five new steroids, griffinisterones A–E (**1**–**5**), were isolated from the octocoral *Dendronephthya griffini*. The structures of these compounds were elucidated by extensive spectroscopic analysis. The single-crystal X-ray crystallography on **1** allowed the determination of the 24*R* configuration in **1**. The absolute stereochemistry of **5** was determined by the application of PGME method. Compounds **3** and **4** were found to significantly inhibit the accumulation of the pro-inflammatory iNOS protein of the LPS-stimulated RAW264.7 macrophage cells.

Keywords: Octocoral; Steroid; Griffinisterones A–E; Anti-inflammatory effect

1. Introduction

Previous chemical investigations on the octocoral of the genus *Dendronephthya* have led to the isolation of structurally unique steroids, of which some have been shown to exhibit cytotoxic[1,2] and antifouling[3,4] activities. Our current chemical investigation on *Dendronephthya griffini* has led to the isolation of five new steroids, griffinisterones A–E (**1**–**5**) (Fig. 1). The structures of **1**–**5** have been established by extensive spectroscopic analysis, including 2D NMR (^{1}H–^{1}H COSY, HMQC, HMBC, and NOESY) spectroscopy. In addition to the use of 2D NMR, the structures of **1** and its 24-epimer **2** were also identified by the assistance of a single-crystal X-ray crystallography on **1**. The absolute configuration at C-20 in **5** was determined by the application of PGME method. The ability of **1**–**4** to inhibit the expression of the pro-inflammatory iNOS (inducible nitric oxide synthase) and COX-2 (cyclooxygenase-2) proteins in LPS (lipopolysaccharide)-stimulated RAW264.7 macrophage cells has been evaluated.

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2. Results and discussion

Griffinisterone A (**1**) was obtained as colorless crystals. The HRESIMS of **1** exhibited a $[M+H]^+$ peak at *m*/*z* 451.3189 and established a molecular formula $C_{28}H_{44}O_3$, implying seven degrees of unsaturation. The ^{13}C NMR and DEPT spectroscopic data (Table 1) displayed 28 carbon signals, including five methyls, eight methylenes, eleven methines, and four quaternary carbons. The IR spectrum of **1** revealed the presence of hydroxy (ν_{max} 3409 cm^{-1}) and carbonyl (ν_{max} 1675 cm^{-1}) groups. The latter was identified as an α,β-unsaturated ketone from the carbon resonances at δ_C 200.2 (qC), 158.4 (CH), and 127.4 (CH).[2,4] The above data, coupled with the characteristic methyl signals at δ_H 0.83 (3H, s), 1.02 (3H, s), 0.86 (3H, d, *J*=6.9 Hz), 0.87 (3H, d, *J*=6.9 Hz), and 0.98 (3H, d, *J*=6.9 Hz) (Table 2), suggested that **1** might be an ergost-1-en-3-one derivative.[2,4,5] In addition, an *E* geometry of 1,2-disubstituted double bond [δ_H 5.73 (dd, *J*=15.6, 8.7 Hz), 5.53 (dd, *J*=15.6, 8.1 Hz)] was also observed in ^{1}H NMR spectrum of **1**. The above data accounted for three of the seven degrees of unsaturation and revealed a tetracyclic nature of **1**. The gross structure of metabolite **1** was further established by the

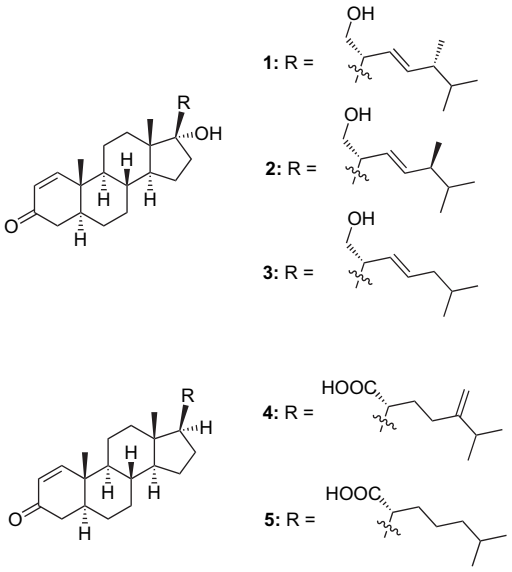

Figure 1. Structures of metabolites **1**–**5**.

2D NMR studies, particularly in ^{1}H–^{1}H COSY, HMQC, and HMBC experiments. The correlations of ^{1}H–^{1}H COSY revealed three separated proton sequences (a–c), as depicted in Figure 2. A tertiary hydroxy group attached at C-17 [δ_C 86.1 (qC)] was deduced from the HMBC correlations from H_3-18 to C-12, C-13, C-14, and C-17. The detailed analysis of the ^{1}H–^{1}H COSY and HMBC correlations (Fig. 2) further established the planar structure of **1**. The NOE correlations between H-8 and both H_3-18 and H_3-19, and H_3-18 and both H-20 and H-21a further established the stereochemistry of the nucleus as shown in the formula of **1**. A single-crystal X-ray diffraction analysis (Fig. 3)[6] was used to confirm the above elucidation and determine the stereochemistry of the side chain in **1**. Thus, the 24*R* configuration of **1** was established.

Table 1
^{13}C NMR spectroscopic data for compounds **1**–**5**

Position	**1**[a]	**2**[a]	**3**[a]	**4**[b]	**5**[b]
1	158.4 (CH)[c]	158.4 (CH)	158.4 (CH)	158.3 (CH)	158.3 (CH)
2	127.4 (CH)	127.4 (CH)	127.4 (CH)	127.4 (CH)	127.4 (CH)
3	200.2 (qC)	200.2 (qC)	200.2 (qC)	200.2 (qC)	200.2 (qC)
4	41.0 (CH_2)	41.0 (CH_2)	41.0 (CH_2)	40.9 (CH_2)	40.9 (CH_2)
5	44.2 (CH)	44.2 (CH)	44.2 (CH)	44.3 (CH)	44.3 (CH)
6	27.6 (CH_2)	27.6 (CH_2)	27.6 (CH_2)	27.5 (CH_2)	27.5 (CH_2)
7	31.2 (CH_2)	31.2 (CH_2)	31.3 (CH_2)	31.3 (CH_2)	31.2 (CH_2)
8	35.9 (CH)	35.9 (CH)	35.9 (CH)	35.6 (CH)	35.6 (CH)
9	49.5 (CH)	49.5 (CH)	49.5 (CH)	50.0 (CH)	49.9 (CH)
10	38.9 (qC)	38.9 (qC)	38.9 (qC)	39.0 (qC)	38.9 (qC)
11	21.1 (CH_2)	21.1 (CH_2)	21.1 (CH_2)	21.2 (CH_2)	21.2 (CH_2)
12	32.0 (CH_2)	32.0 (CH_2)	32.0 (CH_2)	37.5 (CH_2)	37.5 (CH_2)
13	47.6 (qC)	47.6 (qC)	47.6 (qC)	42.3 (qC)	42.3 (qC)
14	50.8 (CH)	50.8 (CH)	50.8 (CH)	55.9 (CH)	55.7 (CH)
15	23.4 (CH_2)	23.4 (CH_2)	23.4 (CH_2)	23.4 (CH_2)	23.5 (CH_2)
16	39.0 (CH_2)	39.2 (CH_2)	39.1 (CH_2)	27.2 (CH_2)	27.1 (CH_2)
17	86.1 (qC)	86.1 (qC)	86.1 (qC)	52.5 (CH)	52.5 (CH)
18	14.9 (CH_3)	14.9 (CH_3)	14.9 (CH_3)	12.3 (CH_3)	12.3 (CH_3)
19	13.0 (CH_3)	13.0 (CH_3)	13.0 (CH_3)	13.0 (CH_3)	13.0 (CH_3)
20	50.0 (CH)	50.0 (CH)	49.9 (CH)	47.6 (CH)	47.0 (CH)
21	64.4 (CH_2)	64.4 (CH_2)	64.4 (CH_2)	181.7 (qC)	181.2 (qC)
22	127.1 (CH)	127.3 (CH)	132.9 (CH)	30.8 (CH_2)	32.1 (CH_2)
23	138.5 (CH)	138.6 (CH)	129.4 (CH)	32.2 (CH_2)	25.0 (CH_2)
24	43.2 (CH)	43.5 (CH)	42.1 (CH_2)	155.3 (qC)	38.8 (CH_2)
25	32.9 (CH)	33.1 (CH)	28.4 (CH)	33.6 (CH)	27.8 (CH)
26	19.8 (CH_3)	19.7 (CH_3)	22.2 (CH_3)	21.8 (CH_3)	22.3 (CH_3)
27	19.9 (CH_3)	20.2 (CH_3)	22.4 (CH_3)	21.9 (CH_3)	22.7 (CH_3)
28	17.5 (CH_3)	18.1 (CH_3)		106.9 (CH_2)	

[a] Spectra recorded at 75 MHz in $CDCl_3$ at 25 °C.
[b] Spectra recorded at 125 MHz in $CDCl_3$ at 25 °C.
[c] Multiplicity deduced by DEPT. The chemical shifts referenced to residual signal of $CDCl_3$ at δ 77.0 ppm.

Griffinisterone B (**2**) was found to possess the same molecular formula $C_{28}H_{44}O_3$ as that of **1** from the HRESIMS and NMR spectroscopic data. The ^{13}C NMR and ^{1}H NMR spectroscopic data (Tables 1 and 2) of **2** were found to be quite similar to those of **1**, except for the C-28 carbon resonating at δ 17.5 (CH_3) in **1** and at δ 18.1 (CH_3) in **2**, revealing the 24*R*/*S* isomers of both compounds. Since the 24*R* configuration has been assigned for **1**, the 24*S* configuration was then determined for **2**. On the basis of above findings and by NOE correlations between H_3-18 and H-20 as well as between H-8 and both H_3-18 and H_3-19, the structure of **2** was fully established.

Griffinisterone C (**3**) was obtained as an amorphous solid. The formula of **3** was found to be $C_{27}H_{42}O_3$, 14 mass units less than that of **1**, as deduced from HRESIMS and NMR spectroscopic data. The ^{1}H NMR spectrum of **3** was found to be quite similar to that of **1** (Table 2), but with the absence of C-28 methyl. The *E* geometry of 22,23-disubstituted double bond and the presence of C-24 methylene were elucidated by the coupling constants and multiplicity of H-23 proton (δ 5.60, 1H, ddd, J=15.4, 8.2, 6.8 Hz). Above data and further inspection of the 2D NMR correlations (Fig. 2) established the structure of this metabolite as revealed by formula **3**.

Griffinisterone D (**4**) was isolated as an amorphous solid. The HRESIMS of **4** established a molecular formula $C_{28}H_{42}O_3$, implying eight degrees of unsaturation. The comparison of NMR spectroscopic data of **4** with those of dendronesterone B[2] revealed that **4** might be a derivative of dendronesterone B. The C-21 carboxyl functionality in **4** was assigned on the basis of the carbon resonance at δ 181.7 (qC)[7] and the IR absorption at 1699 cm^{-1}. The gross structure of **4** was further established by the 2D NMR studies, particularly in ^{1}H–^{1}H COSY, HMQC, and HMBC experiments. The correlations of ^{1}H–^{1}H COSY revealed four separated proton sequences, as shown in Figure 2. The HMBC correlations from H_3-19 to C-1, C-5, C-9, and C-10, and from H_3-18 to C-12, C-13, C-14, and C-17, as well as other correlations illustrated in Figure 2 established the planar structure of **4**. In the NOESY spectrum of **4** (Fig. 4), the NOE correlations between H-8 and both H_3-18 and H_3-19, and H-5 and H-9 revealed that the stereochemistry of the nucleus is the same as that of dendronesterone B. Similar to those found in **1**–**3**, H_3-18 was found to show NOE response with H-20, but not with H-17, suggesting the α position of the carboxyl functionality.

Griffinisterone E (**5**) was obtained as an amorphous solid, which gave a $[M+Na]^+$ peak at *m*/*z* 437.3027 in the HRESIMS. Thus, a molecular formula of $C_{27}H_{42}O_3$ was established. The NMR spectra of **5** were almost identical with those of **4** except that the NMR data of **5** showed the absence of an sp^2 methylene

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

Position	**1**[a]	**2**[a]	**3**[a]	**4**[b]	**5**[b]
1	7.15 d (10.0)[c]	7.15 d (10.0)	7.15 d (10.0)	7.07 d (10.0)	7.08 d (10.0)
2	5.86 d (10.0)	5.86 d (10.0)	5.86 d (10.0)	5.81 d (10.0)	5.83 d (10.0)
4β	2.37 dd (18.0, 14.0)	2.37 dd (18.0, 14.0)	2.37 dd (18.0, 14.0)	2.36 dd (17.5, 14.0)	2.36 dd (17.5, 14.5)
4α	2.22 dd (18.0, 4.5)	2.22 dd (18.0, 4.5)	2.22 dd (18.0, 4.5)	2.22 dd (17.5, 3.5)	2.22 dd (17.5, 3.5)
5	1.94 m	1.94 m	1.94 m	1.88 m	1.91 m
6	1.42 m	1.42 m	1.42 m	1.42 m	1.42 m
7	1.73 m	1.73 m	1.73 m	1.72 m	1.72 m
	1.01 m	1.01 m	1.02 m	0.94 m	0.96 m
8	1.50 m	1.50 m	1.50 m	1.45 m	1.45 m
9	1.06 m	1.06 m	1.05 m	0.96 m	0.98 m
11	1.84 m	1.84 m	1.84 m	1.68 m	1.70 m
	1.48 m	1.48 m	1.49 m	1.38 m	1.40 m
12β	1.80 m	1.80 m	1.80 m	1.79 m	1.74 m
12α	1.72 m	1.72 m	1.72 m	1.15 m	1.17 m
14	1.79 m	1.80 m	1.81 m	1.09 m	1.10 m
15	1.65 m	1.65 m	1.70 m	1.62 m	1.64 m
	1.09 m	1.10 m	1.13 m	1.10 m	1.10 m
16	1.83 m	1.83 m	1.86 m	1.90 m	1.89 m
	1.47 m	1.48 m	1.50 m	1.30 m	1.30 m
17				1.67 m	1.67 m
18	0.83 s	0.82 s	0.83 s	0.76 s	0.76 s
19	1.02 s	1.02 s	1.02 s	0.98 s	0.99 s
20	2.33 m	2.32 m	2.36 m	2.25 m	2.26 br dd (7.5, 3.5)
21a	4.01 m	4.01 m	4.02 m		
21b	3.79 m	3.77 m	3.78 m		
22	5.73 dd (15.6, 8.7)	5.75 dd (15.6, 9.3)	5.79 dd (15.4, 8.7)	1.63 m	1.48 m
23	5.53 dd (15.6, 8.1)	5.49 dd (15.6, 8.4)	5.60 ddd (15.4,8.2, 6.8)	2.03 m	1.27 m
				1.97 m	
24	1.99 m	1.99 m	1.97 m		1.17 m
25	1.53 m	1.54 m	1.65 m	2.20 m	1.51 m
26	0.86 d (6.9)	0.86 d (6.9)	0.90 d (5.7)	1.00 d (6.5)	0.855 d (7.0)
27	0.87 d (6.9)	0.88 d (6.9)	0.90 d (5.7)	1.01 d (6.5)	0.862 d (7.0)
28	0.98 d (6.9)	0.98 d (6.9)		4.75 s	
				4.67 s	

[a] Spectra recorded at 300 MHz in $CDCl_3$ at 25 °C.
[b] Spectra recorded at 500 MHz in $CDCl_3$ at 25 °C.
[c] *J* values (in Hz) in parentheses. The chemical shifts referenced to TMS at δ 0.0 ppm.

substituent at C-24 and revealed the association of two protons with C-24 in **5**. The careful inspection of the 2D NMR spectroscopic data of **5** has led to the establishment of its planar structure. The relative stereochemistry of compound **5** was also established by comparison of NOESY correlations (Fig. 4) with those of **4**. To determine the absolute configuration at C-20 for **5**, it was treated with (*S*)- or (*R*)-PGME (phenylglycine methyl ester) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl), 4-(dimethylamino)pyridine (4-DMAP), and 4-(dimethylamino)pyridine hydrochloride (4-DMAP·HCl)[8] to yield the corresponding (*S*)- or (*R*)-PGME amide (**5a** or **5b**), respectively. The chemical shift differences of (*S*)-PGME amide (**5a**) and (*R*)-PGME amide (**5b**) were summarized in Figure 5 and established the *R*-configuration at C-20.[9,10]

The in vitro anti-inflammatory effect of the sterones **1**–**4** was tested. In this assay, the up-regulation of the pro-inflammatory iNOS and COX-2 proteins of the LPS-stimulated RAW264.7 macrophage cells was evaluated using the immunoblot analysis. Although these four compounds did not inhibit the COX-2 expression at 10 μM, however, it was found that compounds **1**, **2**, and **4** reduced the levels of the iNOS protein at this concentration to 49.7±8.6%, 48.9±16.2%, and 29.8±7.4%, respectively, relative to the control cells stimulated with LPS. Moreover, at the same concentration compound **3** showed more potent inhibition of iNOS as the expression of this pro-inflammatory protein was reduced to a level of 8.1±5.9% (Fig. 6). The toxicity of **1**–**4** to RAW264.7 cells was also assessed by trypan blue staining. It was found that **1**–**4** were not cytotoxic, as the cell survival was not changed in the presence of **1**–**4** at the concentration of 10 μM (Fig. 6).

It has to be noted that the 24*R*/*S* isomers, **1** and **2**, represent the first two examples of 17,21-dihydroxyergost-1,22-dien-3-one derivatives. The NMR spectroscopic data of these two isomers were found to have the following main differences, which might be useful for the differentiation of isomers of this type: (1) in the case of 24*R* isomer **1**, the value of chemical shift difference between H-22 and H-23 ($\Delta=\delta_{H\text{-}22}-\delta_{H\text{-}23}$) was found to be 0.20 ppm, which was changed to 0.26 ppm as observed for 24*S* isomer **2**; (2) the carbon resonance of C-28 was observed at δ 17.5 for **1** and 18.1 for **2**, as measured in $CDCl_3$.

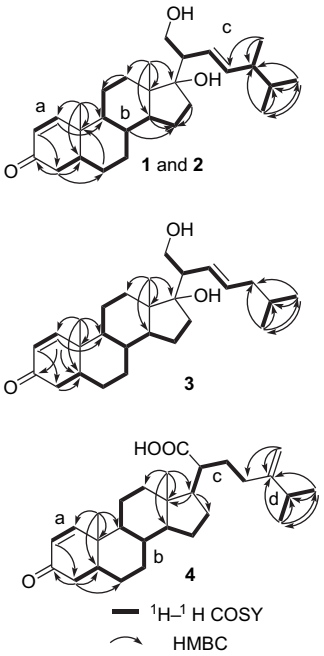

Figure 2. Selective ^{1}H–^{1}H COSY and HMBC correlations of **1**–**4**.

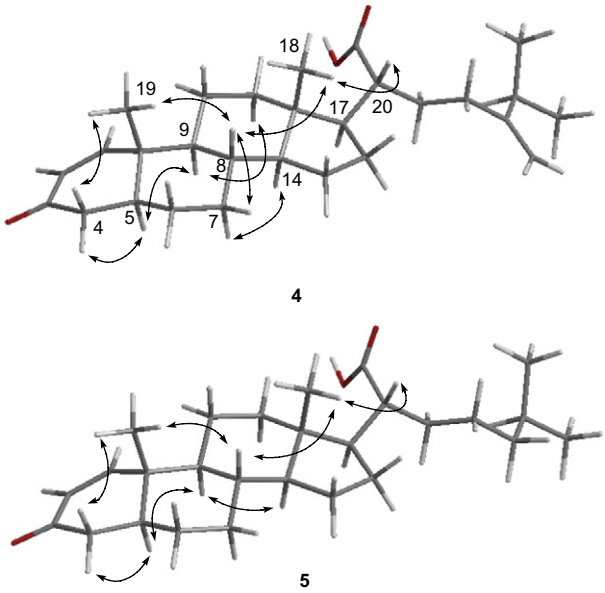

Figure 4. Selective NOSEY correlations of **4** and **5**.

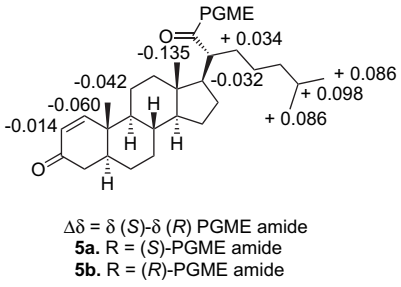

Figure 5. ^{1}H NMR chemical shift differences of PGME amides of **5**.

We also applied a modified method for the preparation of the PGME amides of **5** by treating **5** with PGME, EDC·HCl, 4-DMAP, and 4-DMAP·HCl. Comparing with the original method to prepare the PGME amide with PGME in the presence of benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBop), 1-hydroxybenzotriazole (HOBt), and *N*-methylmorpholine, the cost of our modified method is less expensive.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a Jasco P-1020 polarimeter. IR spectra were recorded on Jasco FT/IR-4100 fourier transform infrared spectrophotometer. The NMR spectra were recorded on Varian Mercury-Plus 300 FT-NMR(or Varian Unity INOVA 500 FT-NMR) instrument at 300 MHz (or 500 MHz) for ^{1}H (referenced to TMS, δ_H 0.0 ppm) and 75 MHz (or 125 MHz) for ^{13}C in $CDCl_3$ (referenced to the center line of $CDCl_3$, δ_C 77.0 ppm). LRMS and HRMS were obtained by ESI on a Bruker APEX II mass spectrometer. Silica gel 60 (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 (HPLC) was performed on a Shimadzu LC-10AT$_{VP}$ apparatus equipped with a Shimadzu SPD-10A$_{VP}$ UV detector. The columns used in HPLC separation are YMC-Pack Pro C18 (reverse-phase column, 250×10 mm, 5 μm) and Varian Dynamax, Si-60 (normal-phase column, 250×21.4 mm, 100 Å, 5 μm). (*S*)-(+)-2-Phenylglycine methyl ester hydrochloride,

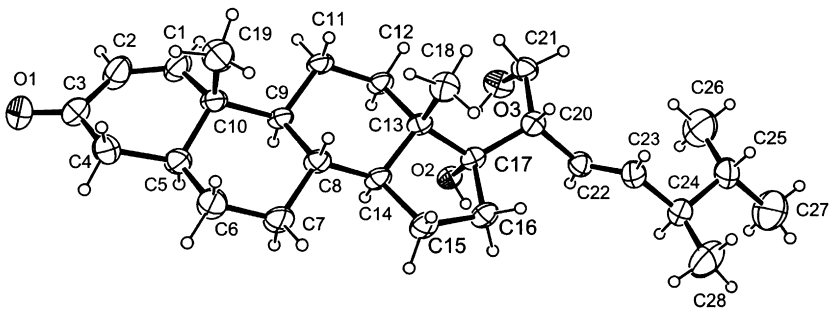

Figure 3. X-ray ORTEP drawing of **1**.

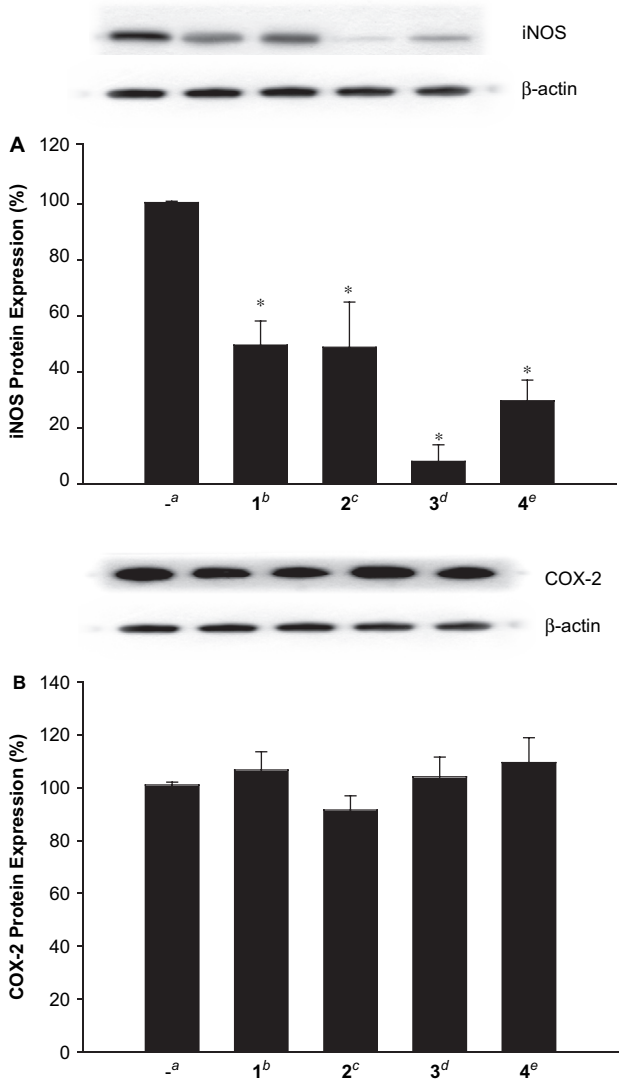

Figure 6. Effect of compounds **1**–**4** on iNOS and COX-2 protein expression of RAW264.7 macrophage cells by immunoblot analysis. (A) Immunoblots of iNOS and β-actin; (B) immunoblots of COX-2 and β-actin. The values are mean±SEM (n=6). Relative intensity of the LPS alone stimulated group was taken as 100%. Under the same experimental condition CAPE (caffeic acid phenylethyl ester, 10 μM) reduced the levels of the iNOS and COX-2 to 2.5±3.7% and 67.2±13.4%, respectively. *: Significantly different from LPS alone stimulated group (P<0.05); a: stimulated with LPS alone; b: stimulated with LPS in the presence of **1** (10 μM); c: stimulated with LPS in the presence of **2** (10 μM); d: stimulated with LPS in the presence of **3** (10 μM); e: stimulated with LPS in the presence of **4** (10 μM).

(*R*)-(−)-2-Phenylglycine methyl ester hydrochloride, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) were purchased from Aldrich and 4-(dimethylamino)pyridine (4-DMAP) was purchased from Lancaster. 4-DMAP·HCl was prepared by 4-DMAP and concd HCl in THF.

3.2. Organism

The octocoral *D. griffini* was collected unexpectedly by a bottom trawl net at depths ranged from 200 to 100 m during an ecological investigation at Taiwan Straight in December 2004. The location was about 60 km west of Singda Harbor, Kaohsiung County, Taiwan. A voucher specimen was deposited in the Department of Marine Biotechnology and Resources, National Sun Yat-sen University (specimen no. 041206A).

3.3. Extraction and separation

The octocoral (15 kg fresh wt) was collected and freeze-dried. The freeze-dried material was minced and extracted exhaustively with EtOH (3×10 L). The organic extract was concentrated to an aqueous suspension and was further partitioned between EtOAc and water. The EtOAc extract (100 g) was fractionated by open column chromatography on silica gel using *n*-hexane–EtOAc and EtOAc–MeOH mixtures of increasing polarity to yield 19 fractions. Fraction 7, eluted with *n*-hexane–EtOAc (2:1), was further subjected to silica gel column chromatography with gradient elution (*n*-hexane–acetone, 90:10 to 85:15), and followed by normal-phase HPLC (*n*-hexane–EtOAc, 4:1) to afford a mixture of **4** and **5**. The final purification was carried out by reverse-phase HPLC (CH_3CN–H_2O, 4:1) to obtain compounds **4** (1.5 mg) and **5** (4.5 mg). Fraction 8, eluted with *n*-hexane–EtOAc (1:1), was further subjected to silica gel column chromatography with gradient elution (*n*-hexane–acetone, 9:1 to 8:2), Sephadex LH-20 (acetone) and followed by normal-phase HPLC (*n*-hexane–EtOAc, 85:15) to yield a mixture of **1**–**3**. Finally, this mixture was purified by reverse-phase HPLC (CH_3CN–H_2O, 4:1) to yield compounds **1** (3.0 mg), **2** (2.5 mg), and **3** (3.0 mg), and **1** (24*R* epimer) was eluted after **2** (24*S* epimer).

3.3.1. Griffinisterone A (*1*)

Colorless crystals; mp 184–185 °C; $[\alpha]_D^{22}$ −20 (c 0.24, $CHCl_3$); IR (KBr) ν_{max} 3409, 1675, and 1382 cm^{-1}; ^{13}C and ^1H NMR data, see Tables 1 and 2; ESIMS m/z 451 $[M+Na]^+$; HRESIMS m/z 451.3186 $[M+Na]^+$ (calcd for $C_{28}H_{44}O_3Na$, 451.3188).

3.3.2. Griffinisterone B (*2*)

Amorphous solid; $[\alpha]_D^{22}$ +3 (c 0.45, $CHCl_3$); IR (KBr) ν_{max} 3360, 1674, and 1382 cm^{-1}; ^{13}C and ^1H NMR data, see Tables 1 and 2; ESIMS m/z 451 $[M+Na]^+$; HRESIMS m/z 451.3189 $[M+Na]^+$ (calcd for $C_{28}H_{44}O_3Na$, 451.3188).

3.3.3. Griffinisterone C (*3*)

Amorphous solid; $[\alpha]_D^{22}$ −5 (c 0.58, $CHCl_3$); IR (KBr) ν_{max} 3383, 1674, and 1382 cm^{-1}; ^{13}C and ^1H NMR data, see Tables 1 and 2; ESIMS m/z 437 $[M+Na]^+$; HRESIMS m/z 437.3033 $[M+Na]^+$ (calcd for $C_{27}H_{42}O_3Na$, 437.3031).

3.3.4. Griffinisterone D (*4*)

Amorphous solid; $[\alpha]_D^{22}$ −7 (c 0.32, $CHCl_3$); IR (KBr) ν_{max} 1699 and 1682 cm^{-1}; ^{13}C and ^1H NMR data, see Tables 1 and 2; ESIMS m/z 449 $[M+Na]^+$; HRESIMS m/z 449.3029 $[M+Na]^+$ (calcd for $C_{28}H_{42}O_3Na$, 449.3031).

*3.3.5. Griffinisterone E (**5**)*

Amorphous solid; $[\alpha]_D^{22}$ −8 (*c* 0.38, $CHCl_3$); IR (KBr) ν_{max} 1700 and 1683 cm^{-1}; ^{13}C and ^1H NMR data, see Tables 1 and 2; ESIMS *m/z* 437 $[M+Na]^+$; HRESIMS *m/z* 437.3027 $[M+Na]^+$ (calcd for $C_{27}H_{42}O_3Na$, 437.3031).

*3.3.6. Preparation of (S) and (R)-PGME amides of **5***

To a stirred solution of compound **5** (1 mg) and (*S*)-PGME (2 mg) in a 1 mL mixture of $CHCl_3$–DMF (10:1) were successively added DMAP (2 mg) and 4-DMAP·HCl (2 mg). After the mixture was stirred at 0 °C for 5 min, EDC·HCl (2 mg) was added. The reaction mixture was then moved to a freezer at 4 °C for overnight. The mixture was then stirred at room temperature for 3 h. Subsequently, ethyl acetate was added, and the resulting solution was successively washed with 5% HCl, saturated $NaHCO_{3(aq)}$, and brine. The organic layer was dried over anhydrous Na_2SO_4 and concentrated to give a residue, which was chromatographed on silica gel using *n*-hexane–EtOAc (8:1) as eluent to afford the (*S*)-PGME amide (**5a**) (0.8 mg). The same procedure was used to prepare the (*R*)-PGME amide (**5b**) (0.6 mg from 1 mg of **5**) with (*S*)-PGME. Selective ^1H NMR ($CDCl_3$) of **5a**: δ_H 7.355 (5H, br s, Ph), 7.044 (1H, d, *J*=10.0 Hz, H-1), 6.467 (1H, d, *J*=7.2 Hz, NH), 5.831 (1H, d, *J*=10.0 Hz, H-2), 5.610 (1H, d, *J*=7.2 Hz, CH–N), 3.735 (3H, s, OCH_3), 1.650 (1H, m, H-17), 1.443 (2H, m, H-22), 1.479 (1H, m, H-25), 0.946 (3H, s, H_3-19), 0.834 (6H, d, *J*=6.3 Hz, H_3-26 and H_3-27), 0.620 (3H, s, H_3-18). Selective ^1H NMR ($CDCl_3$) of **5b**: δ_H 7.339 (5H, br s, Ph), 7.104 (1H, d, *J*=10.0 Hz, H-1), 6.460 (1H, d, *J*=7.2 Hz, NH), 5.845 (1H, d, *J*=10.0 Hz, H-2), 5.653 (1H, d, *J*=7.2 Hz, CH–N), 3.749 (3H, s, OCH_3), 1.682 (1H, m, H-17), 1.409 (2H, m, H-22), 1.381 (1H, m, H-25), 0.988 (3H, s, H_3-19), 0.755 (3H, s, H_3-18), 0.748 (6H, d, *J*=6.3 Hz, H_3-26 and H_3-27).

*3.4. X-ray diffraction analysis of griffinisterone A (**1**)*

A suitable colorless crystal (0.8×0.8×0.2 mm^3) of **1** was grown by slow evaporation of the acetone containing few drops of benzene at 0 °C. 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**: $C_{28}H_{44}O_3$ (formula weight 428.63), approximate crystal size, 0.8×0.8×0.2 mm^3, monoclinic, space group, $P2_1$ (#4), *T*=298(2) K, *a*=12.467(4) Å, *b*= 7.636(2) Å, *c*=14.244(5) Å, β=110.18(3)°, *V*=1272.7(7) $Å^3$, D_c=1.118 Mg/m^3, *Z*=2, *F*(000)=472, $\mu_{(Mo\ K\alpha)}$=0.070 mm^{-1}. A total of 3426 reflections were collected in the range 1.88°<θ<26.00°, with 2695 independent reflections [*R*(int)= 0.0420], completeness to θ_{max} was 99.9%; psi-scan empirical absorption correction applied; full-matrix least-squares refinement on F^2, the number of data/restraints/parameters were 2695/1/293; goodness-of-fit on F^2=1.019; final *R* indices [$I>2\sigma(I)$], R_1=0.0436, wR_2=0.1106; *R* indices (all data), R_1= 0.0656, wR_2=0.1225, largest difference peak and hole, 0.139 and −0.227 e/$Å^3$.

3.5. In vitro anti-inflammatory assay

The anti-inflammatory assay was modified from Ho et al.[11] and Park et al.[12] Murine RAW264.7 macrophages were obtained from the American Type Culture Collection (ATCC, no. TIB-71) and cultured in Dulbecco's modified essential medium (DMEM) containing 10% heat-inactivated fetal bovine serum, at 37 °C in a humidified 5% CO_2–95% air incubator under standard conditions. The macrophage cells were plated in 60 mm culture dishes (3×10^6 cells), and test compounds were added to the culture medium for 16 h. Then, cells were washed with ice-cold PBS, lysed in ice-cold lysis buffer, and then centrifuged at 20,000*g* for 30 min at 4 °C. The supernatant was decanted from the pellet and retained for Western blot analysis. Protein concentrations were determined by the DC protein assay kit (Bio-Rad) modified by the method of Lowry et al.[13] Samples containing equal quantities of protein were subjected to SDS–polyacrylamide gel electrophoresis, and the separated proteins were electrophoretically transferred to polyvinylidene difluoride membranes (PVDF; Immobilon-P, Millipore, 0.45 μm pore size). The resultant PVDF membranes were incubated with blocking solution and incubated for 180 min with antibody against inducible nitric oxide synthase (iNOS; 1:1000 dilution; Transduction Laboratories) and cyclooxygenase-2 (COX-2; 1:1000 dilution; Cayman Chemical) protein. The blots were detected using ECL detection reagents (Perkin–Elmer, Western Blot Chemiluminescence Reagent Plus) according to the manufacturer instructions. The membranes were reprobed with a monoclonal mouse anti-β-actin antibody (1:2500, Sigma) as the loading control. For the immunoreactivity data, the intensity of each drug-treated band is expressed as the integrated optical density (IOD), calculated with respect to the average optical density of the corresponding control (LPS-only treatment) band. For statistical analysis, all the data were analyzed by a one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls post hoc test for multiple comparisons. A significant difference was defined as a *P* value of <0.05.

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6. Crystallographic data for compound **1** have been deposited with the Cambridge Crystallographic Data Centre (deposition number CCDC 656887).

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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