

Article

Steroidal Carboxylic Acids from Soft Coral *Paraminabea acronocephala*

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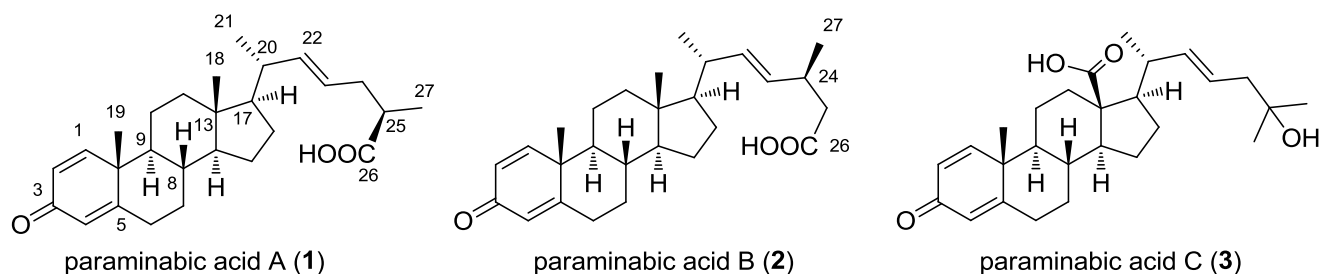
Abstract: Three new steroidal carboxylic acids, paraminabic acids A–C (**1–3**) were isolated from a Formosan soft coral *Paraminabea acronocephala*. The structures of these compounds were established by extensive spectroscopic analysis and chemical methods. Application of the PGME method allowed the establishment of the absolute configurations at C-25 and C-24 for **1** and **2**, respectively. Compound **3** showed potent cytotoxicity toward Hep3B, MDA-MB-231, MCF-7, and A-549 cancer cell lines, with IC₅₀ values ranging from 2.05 to 2.83 µg/mL. Compounds **2** and **3** were found to inhibit the accumulation of the pro-inflammatory iNOS protein.

Keywords: *Paraminabea acronocephala*; paraminabic acid; soft coral; cytotoxicity; anti-inflammatory activity

1. Introduction

Marine withanolides, with potent pro-inflammatory inducible nitric oxide synthase (iNOS) inhibitory activity, have previously been reported from two species of soft corals, *Paraminabea acronocephala* [1] and *Minabea* sp. [2]. These compounds possess a different A-ring structure (1,4-dien-3-one or 4-en-3-one) from those of plant origin [1–3]. Our previous chemical investigation of the soft coral *P. acronocephala* led to the isolation of novel withanolides with a 24 β ,25 β -dimethyl- γ -lactone or a 24 β ,25 α -dimethyl- γ -lactone in the steroidal side chain moiety [1]. As part of our continuing search for bioactive, structurally interesting metabolites from this coral, three steroidal carboxylic acids (**1–3**) were isolated and their structures were elucidated (Figure 1). The cytotoxicity of compounds **1–3** against human liver carcinoma (HepG2 and HepG3), human breast carcinoma (MCF-7 and MDA-MB-231), and human lung carcinoma (A-549) cell lines and the ability of **1–3** to inhibit up-regulation of the pro-inflammatory iNOS and COX-2 (cyclooxygenase-2) proteins in LPS (lipopolysaccharide)-stimulated RAW264.7 macrophage cells were also evaluated.

Figure 1. The structures of paraminabic acids A–C (**1–3**).



2. Results and Discussion

The ethanolic extract of the soft coral *P. acronocephala* was partitioned between EtOAc and H₂O to afford the EtOAc-soluble fraction. It was then subjected to silica gel column chromatography. The fractions containing steroids were selected for further study, based on characteristic methyl signals in the ¹H NMR spectrum. These fractions were subsequently subjected to a series of chromatographic separations to afford three new steroidal carboxylic acids, paraminabic acids A–C (**1–3**).

The HRESIMS and ¹³C NMR spectroscopic data of paraminabic acid A (**1**) suggested a molecular formula of C₂₇H₃₈O₃, appropriate for nine degrees of unsaturation. The ¹³C NMR and DEPT spectroscopic data (Table 1) displayed 27 carbon signals, including 4 methyls, 7 methylenes, 11 methines, and 5 quaternary carbons. A broad O–H stretching absorption in the region of 3400–2600 cm^{−1} is ascribable to a carboxylic acid, which was evidenced by the carbon resonance at δ_C 180.6 (C). The same steroidal nucleus as that of paraminabeolides D and E was deduced for **1** by detailed comparison of their NMR spectroscopic data [1]. The side chain moiety of **1** resembles that of a known steroidal carboxylic acid, (25*S*)-3-oxocholesta-1,4-dien-26-oic acid [4], isolated from the Indonesian soft coral *Minabea* sp. However, **1** varied from (25*S*)-3-oxocholesta-1,4-dien-26-oic acid in the respective side chain. The proton resonances at δ_H 5.50 (1H, dt, J = 15.6, 6.4 Hz, H-23) and 5.44 (1H, dd, J = 15.6, 8.8 Hz, H-22), measured in C₅D₅N, were due to the presence of a *trans* C-22/C-23 double bond, which was confirmed by the HMBC correlations from H₃-21 to C-17, C-20,

and C-22. The absolute configuration at C-25 was determined by the application of Kusumi's method (PGME method) [5–7]. The chemical shift differences of (*S*)-PGME amide (**1a**) and (*R*)-PGME amide (**1b**) ($\Delta\delta = \delta_{(S)} - \delta_{(R)}$) were summarized in Figure 2 and established the *R* configuration at C-25.

Table 1. ^{13}C NMR spectroscopic data of compounds **1–3**.

Position	1 ^a , δ_{C} , mult.	2 ^a , δ_{C} , mult.	3 ^a , δ_{C} , mult.
1	156.1, CH	156.1, CH	155.9, CH
2	127.4, CH	127.4, CH	127.5, CH
3	186.5, C	186.5, C	186.5, C
4	123.8, CH	123.7, CH	123.8, CH
5	169.5, C	169.6, C	169.3, C
6	32.9, CH ₂	32.9, CH ₂	32.7, CH ₂
7	33.7, CH ₂	33.7, CH ₂	33.4, CH ₂
8	35.5, CH	35.5, CH	37.1, CH
9	52.4, CH	52.4, CH	52.4, CH
10	43.6, C	43.6, C	43.6, C
11	22.8, CH ₂	22.8, CH ₂	24.6, CH ₂
12	39.3, CH ₂	39.3, CH ₂	35.1, CH ₂
13	42.6, C	42.5, C	55.8, C
14	55.6, CH ₂	55.5, CH ₂	55.7, CH ₂
15	24.4, CH ₂	24.3, CH ₂	25.0, CH ₂
16	28.4, CH ₂	28.3, CH ₂	25.3, CH ₂
17	55.5, CH	55.5, CH	55.3, CH
18	12.2, CH ₃	12.2, CH ₃	176.8, C ^b
19	18.7, CH ₃	18.7, CH ₃	18.7, CH ₃
20	40.0, CH	39.9, CH	38.4, CH
21	20.6, CH ₃	20.6, CH ₃	22.0, CH ₃
22	139.6, CH	136.1, CH	136.7, CH
23	123.8, CH	131.3, CH	124.8, CH
24	36.4, CH ₂	33.7, CH	46.8, CH ₂
25	39.3, CH	41.6, CH ₂ ^b	71.7, C
26	180.6, C ^b	176.9, C ^b	27.6, CH ₃
27	16.3, CH ₃ ^b	20.6, CH ₃	30.5, CH ₃

^a Spectra were measured in CDCl₃ (100 MHz); ^b values obtained from the relevant HMBC or HSQC correlation peaks.

Paraminabic acid B (**2**) gave the same molecular formula, C₂₇H₃₈O₃, as that of **1**, based on the analysis of the HRESIMS and ^{13}C NMR spectroscopic data (Table 1). The NMR spectroscopic data of **2** are similar to those of **1**, but differences were observed in their side chains. The HMBC correlations from H₃-21 to C-17, C-20, and C-22 allowed the assignment of a C-22/C-23 double bond. A large coupling constant (15.2 Hz, C₅D₅N) between H-22 and H-23 suggested the *E* geometry of this double bond. The H-23 signal appeared as a doublet of doublets, revealing that the adjacent carbon (C-24) should be a methine. This might be due to the attachment of a methyl group (δ_{H} 1.03, 3H, d, *J* = 6.4 Hz, H₃-27) at C-24 (Table 2). This was confirmed by the HMBC correlations from H₃-27 to C-23, C-24, and C-25 as well as from H₂-25 to the carboxyl carbon (C-26). The absolute configuration at C-24 of **2**

was determined by the application of Kusumi's method developed for chiral β,β -disubstituted propionic acid derivatives [6,7]. The ^1H NMR shift differences ($\Delta\delta = \delta_{(R)} - \delta_{(S)}$) between the diastereomeric (*R*)- and (*S*)-PGME amides, **2a** and **2b**, respectively, are summarized in Figure 2 and establish the 2*S* configuration for **2**.

Table 2. ^1H NMR spectroscopic data of compounds 1–3.

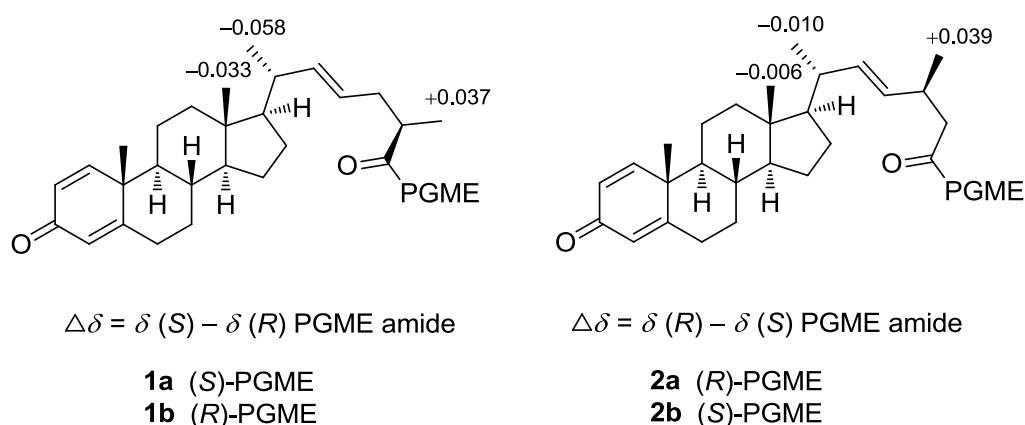
#	1, δ_{H} (J in Hz) ^a	2, δ_{H} (J in Hz) ^a	3, δ_{H} (J in Hz) ^a
1	7.06, d (10.0)	7.06, d (10.0)	7.03, d (10.0)
2	6.23, dd (10.0, 1.6)	6.24, d (10.0)	6.23, d (10.0)
4	6.07, s	6.07, s	6.07, s
6	a: 2.45, m b: 2.35, m	a: 2.46, td (13.6, 4.4) b: 2.35, m	a: 2.46, td (13.4, 4.4) b: 2.35, m
7	a: 1.93, m b: 1.02, m	a: 1.93, m b: 1.02, m	a: 2.04, m b: 1.07, m
8	1.60, m	1.59, m	1.69, m
9	1.04, m	1.03, m	1.10, m
11	1.67, m	1.67, m	1.85, m 1.69, m
12	a: 2.00, m b: 1.18, m	a: 1.99, m b: 1.17, m	a: 2.66, br d (14.0) b: 1.03, m
14	1.12, m	1.11, m	1.30, m
15	a: 1.55, m b: 1.08, m	a: 1.52, m b: 1.02, m	a: 1.91, m b: 1.66, m
16	a: 1.65, m b: 1.22, m	a: 1.62, m b: 1.20, m	a: 1.78, m b: 1.70, m
17	0.99, m	0.99, m	1.62, m
18	0.74, s	0.74, s	
19	1.23, s	1.23, s	1.15, s
20	2.03, m	2.00, m	2.36, m
21	0.99, d (6.8)	0.98, d (6.4)	1.05, d (6.4)
22	5.27–5.30 ^b	5.23–5.26 ^b	5.39 dd (15.2, 8.8)
23	5.27–5.30 ^b	5.23–5.26 ^b	5.48, ddd (15.2, 8.8, 5.2)
24	a: 2.33, m b: 2.10, m	2.59, m	2.18, dd (14.0, 5.2) 2.11, dd (14.0, 8.8)
25	2.49, m	2.30, d (7.2)	
26			1.25, s
27	1.15, d (7.2)	1.03, d (6.4)	1.25, s

^a Spectra were measured in CDCl_3 (400 MHz); ^b overlapped signals.

The HRESIMS and ^{13}C NMR spectroscopic data of paraminabic acid C (**3**) established a molecular formula of $\text{C}_{27}\text{H}_{38}\text{O}_4$ and nine degrees of unsaturation. The IR absorptions at 3419 and 1714 cm^{-1} suggested the presence of hydroxy and carbonyl groups, respectively. Both ^1H and ^{13}C NMR spectra of **3** lacked signals of the angular methyl group, which might be replaced by a carboxylic acid according to the carbon signal at δ_{C} 176.8 (C) (Table 1). The carboxylic acid attached at C-13 was further confirmed by the HMBC correlations from both H_2 -12 and H-17 to C-18. The *trans* C-22/C-23 double

bond was deduced by the HMBC correlations from H₃-21 to C-17, C-20, and C-22 as well as *J* value (15.2 Hz) (Table 2) between H-22 and H-23. In addition, the downfield-shifted quaternary carbon at δ_C 69.9 was ascribable to a hydroxy group attached at C-25, which was correlated by H₂-24, H₃-26, and H₃-27 in the HMBC spectrum.

Figure 2. ¹H NMR chemical shift differences of PGME amides of **1** and **2**.



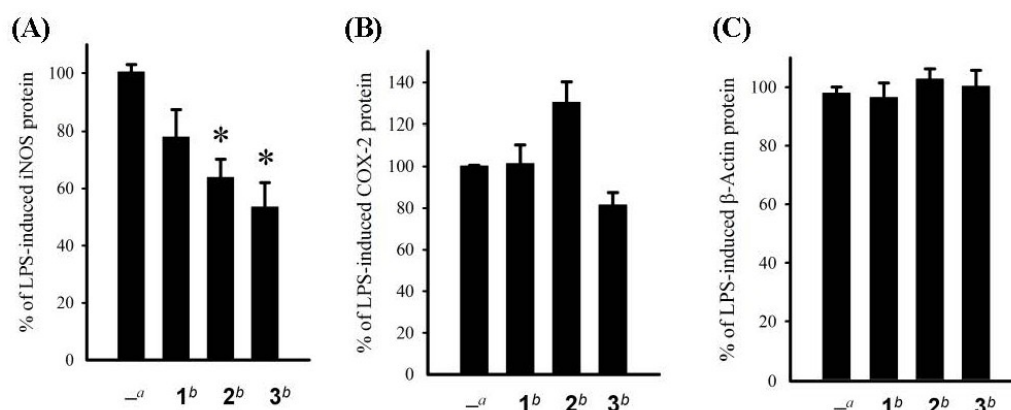
The cytotoxicity of compounds **1–3** against HepG2, Hep3B, MDA-MB-231, MCF-7, and A-549 cancer cells was studied and shown in Table 3. Compound **3** showed potent cytotoxicity toward Hep3B, MDA-MB-231, MCF-7, and A-549 cancer cell lines, with IC₅₀ values ranging from 2.05 to 2.83 $\mu\text{g/mL}$. We also investigated the inhibition of these compounds toward LPS-induced pro-inflammatory protein (iNOS and COX-2) expression in RAW264.7 macrophage cells by Western blot analysis. At a concentration of 10 μM , compounds **2** and **3** reduced the levels of iNOS to $63.9 \pm 6.3\%$ and $53.5 \pm 8.6\%$, respectively; whereas, compound **2** enhanced the expression of COX-2 ($130.5 \pm 9.8\%$) in comparison with those of control cells stimulated with LPS only (100% for both iNOS and COX-2). Also, compound **3** could inhibit the expression of iNOS protein but did not induce cytotoxicity in macrophage cells as determined through internal control β -actin expression, as shown in Figure 3. These results indicate that **3** possesses moderate anti-inflammatory activity and potent cytotoxicity, and might be useful for further medicinal study.

Table 3. Cytotoxicity data of compounds **1–3**.

Compound	Cell lines IC ₅₀ ($\mu\text{g/mL}$)				
	Hep G2	Hep 3B	MDA-MB-231	MCF-7	A549
1	15.21	—	19.66	—	—
2	19.77	—	—	—	—
3	13.57	2.83	2.25	2.23	2.05
doxorubicin	0.31	0.40	1.32	0.68	1.33

(—): Compound is considered inactive with IC₅₀ > 20 $\mu\text{g/mL}$.

Figure 3. Effect of compounds 1–3 at 10 μ M on the LPS-induced pro-inflammatory iNOS and on COX-2 protein expression of RAW264.7 macrophage cells by immunoblot analysis (A) Quantification of immunoblots of iNOS; (B) Quantification of immunoblots of COX-2. The values are means $\pm$ SEM ($n = 6$). The relative intensity of the LPS alone stimulated group was taken as 100%. * Significantly different from LPS alone stimulated group (* $P < 0.05$). ^a Stimulated with LPS. ^b Stimulated with LPS in the presence of 1–3 (10 μ M); (C) Quantification of immunoblots of β -actin.



3. Experimental Section

3.1. General Experimental Procedures

Optical rotations were determined with a JASCO P1020 digital polarimeter. The IR spectra were obtained on a JASCO FT/IR-4100 spectrophotometer. The NMR spectra were recorded on a Bruker AVANCE 300 FT-NMR (or Varian MR-400 NMR) instrument at 300 MHz (or 400 MHz) for ^1H (referenced to TMS for both CDCl_3 and $\text{C}_5\text{D}_5\text{N}$) and 75 MHz (or 100/125 MHz) for ^{13}C (referenced to δ_{C} 77.0 for CDCl_3 and to internal TMS for $\text{C}_5\text{D}_5\text{N}$). ESIMS were recorded by ESI FT-MS on a Bruker APEX II mass spectrometer. Silica gel 60 (230–400 mesh, Merck, Darmstadt, Germany) and LiChroprep RP-18 (Merck, 40–63 μm) were used for column chromatography. Precoated silica gel plates (Kieselgel 60 F254, 0.25 mm, Merck, Darmstadt, Germany) and precoated RP-18 F254S plates (Merck, 1.05560) were used for TLC analyses. High-performance liquid chromatography was performed on a Hitachi L-7100 pump equipped with a Hitachi L-7400 UV detector at 210 nm and a semi-preparative reversed-phase column (Hibar Purospher RP-18e, 5 μm , 250 $\times$ 10 mm, Merck, Darmstadt, Germany).

3.2. Animal Material

The soft coral *P. acronocephala* was collected by scuba divers, off the western coast of Pingtung county, in May 2009, at a depth of 10 m, and was stored in a freezer until being extracted. This soft coral was identified by Prof. Chang-Fong Dai, Institute of Oceanography, National Taiwan University. A voucher specimen (specimen No. 200905PA) was deposited in the Department of Marine Biotechnology and Resources, National Sun Yat-sen University.

3.3. Extraction and Isolation

The soft coral *P. acronocephala* (3.8 kg fresh wt) was collected and freeze-dried. The freeze-dried material was minced and extracted exhaustively with EtOH (6 × 2 L). The organic extract was concentrated to an aqueous suspension and was further partitioned between EtOAc and water. The EtOAc extract (30 g) was fractionated by open column chromatography on silica gel using *n*-hexane–EtOAc and EtOAc–MeOH mixtures of increasing polarity to yield 28 fractions. Fraction 21 (3.6 g), eluted with *n*-hexane–EtOAc (1:6), was further separated by silica gel column chromatography with gradient elution (*n*-hexane–acetone, 5:1 to 2:1) to yield eight subfractions (21A to 21H). Subfraction 21D was fractionated by RP-18 open column (MeOH–H₂O, 50% to 100%) to afford six subfractions (21D1 to 21D6). Compounds **1** (1.9 mg) and **2** (2.6 mg) were obtained from subfraction 21D5 using RP-18 HPLC (MeOH–H₂O, gradient 75% to 85%). Compound **3** (5.1 mg) was obtained from fraction 25 (0.85 g) using repeatedly column chromatography over silica gel (*n*-hexane–EtOAc, 1:3 to 0:1) and RP-18 gel (MeOH–H₂O, 50% to 100%), and subsequently separated by RP-18 HPLC (MeOH–H₂O, gradient, 65%–75%).

Paraminabic acid A (**1**): amorphous solid; $[\alpha]_D^{24} +13$ (*c* 0.09, CHCl₃); IR (KBr) $\nu_{\max}$ 3400–2600 (br), 2933, 2868, 2853, 1718, 1662, 1615, 1602, 1457, 1406, 1375, 1292, 1241 cm^{−1}; ¹³C NMR and ¹H NMR data, see Tables 1 and 2; Selected ¹H NMR (C₅D₅N, 400 MHz) of **1**: δ 7.01 (1H, d, *J* = 10.0 Hz, H-1), 6.42 (1H, dd, *J* = 10.0, 2.0 Hz, H-2), 6.26 (1H, s, H-4), 5.50 (1H, dt, *J* = 15.6, 6.4 Hz, H-23), 5.44 (1H, dd, *J* = 15.6, 8.8 Hz, H-22), 2.78 (1H, m, H-25), 2.65 (1H, m, H-24a), 2.40 (1H, m, H-24b), 1.38 (3H, d, *J* = 6.8 Hz, H₃-27), 1.10 (3H, s, H₃-19), 1.03 (3H, d, *J* = 6.4 Hz, H₃-21), 0.67 (3H, s, H₃-18); ESIMS *m/z* 433 [M + Na]⁺; HRESIMS *m/z* 433.2715 [M + Na]⁺ (calcd for C₂₇H₃₈O₃Na, 433.2718).

Paraminabic acid B (**2**): amorphous solid; $[\alpha]_D^{24} +60$ (*c* 0.16, CHCl₃); IR (KBr) $\nu_{\max}$ 3400–2600 (br), 2934, 2868, 1718, 1662, 1617, 1601, 1455, 1405, 1375, 1292, 1241 cm^{−1}; ¹³C NMR and ¹H NMR data, see Tables 1 and 2; ¹H NMR (C₅D₅N, 400 MHz) of **2**: δ 7.01 (1H, d, *J* = 10.0 Hz, H-1), 6.42 (1H, dd, *J* = 10.0, 1.6 Hz, H-2), 6.27 (1H, s, H-4), 5.51 (1H, dd, *J* = 15.2, 7.2 Hz, H-23), 5.41 (1H, dd, *J* = 15.2, 8.4 Hz, H-22), 2.97 (1H, m, H-24), 2.63 (1H, dd, *J* = 14.8, 7.6 Hz, H-25a), 2.55 (1H, dd, *J* = 14.8, 7.2 Hz, H-25b), 2.31 (1H, td, *J* = 13.6, 4.4 Hz, H-6a), 2.18 (1H, dt, *J* = 13.6, 2.4 Hz, H-6b), 2.04 (1H, m, H-20), 1.90 (1H, dt, *J* = 12.4, 3.2 Hz, H-12a), 1.71 (1H, m, H-16a), 1.70 (1H, m, H-7a), 1.54 (2H, m, H₂-11), 1.42 (1H, m, H-8), 1.39 (1H, m, H-15a), 1.25 (1H, m, H-16b), 1.21 (3H, d, *J* = 6.8 Hz, H₃-27), 1.09 (3H, s, H₃-19), 1.08 (1H, m, H-17), 1.06 (1H, m, H-12b), 1.04 (3H, d, *J* = 6.0 Hz, H₃-21), 0.99 (1H, m, H-15b), 0.88 (1H, m, H-9), 0.83 (1H, m, H-14), 0.82 (1H, m, H-7b), 0.67 (3H, s, H₃-18); ¹³C NMR (C₅D₅N, 100 MHz) of **2**: δ 185.9 (C, C-3), 175.2 (C, C-26), 169.2 (C, C-5), 156.0 (CH, C-1), 135.5 (CH, C-22), 132.7 (CH, C-23), 127.7 (CH, C-2), 124.0 (CH, C-4), 55.9 (CH, C-17), 55.7 (CH, C-14), 52.6 (CH, C-9), 43.7 (C, C-10), 43.1 (CH₂, C-25), 42.7 (C, C-13), 40.3 (CH, C-20), 39.6 (CH₂, C-12), 35.4 (CH, C-8), 34.1 (CH, C-24), 33.8 (CH₂, C-7), 32.9 (CH₂, C-6), 28.7 (CH₂, C-16), 24.4 (CH₂, C-15), 22.9 (CH₂, C-11), 20.9 (CH₃, C-21), 20.8 (CH₃, C-27), 18.7 (CH₃, C-19), 12.3 (CH₃, C-18); ESIMS *m/z* 433 [M + Na]⁺; HRESIMS *m/z* 433.2715 [M + Na]⁺ (calcd for C₂₇H₃₈O₃Na, 433.2718).

Paraminabic acid C (**3**): amorphous solid; $[\alpha]_D^{24} +43$ (*c* 0.09, CHCl₃); IR (KBr) $\nu_{\max}$ 3400–2600 (br), 3419, 2967, 2936, 2870, 1714, 1660, 1616, 1599, 1456, 1442, 1375, 1295, 1240, 1161 cm^{−1}; ¹³C NMR and ¹H NMR data, see Tables 1 and 2; ¹H NMR (C₅D₅N, 400 MHz) of **3**: δ 7.01 (1H, d, *J* = 10.0 Hz,

H-1), 6.41 (1H, dd, $J = 10.0, 2.0$ Hz, H-2), 6.25 (1H, s, H-4), 5.73 (1H, dt, $J = 15.2, 7.4$ Hz, H-23), 5.49 (1H, dd, $J = 15.2, 8.4$ Hz, H-22), 3.00 (1H, br d, $J = 12.4$ Hz, H-12a), 2.47 (1H, m, H-20), 2.41 (2H, m, H₂-24), 2.25 (1H, m, H-15a), 2.22 (1H, m, H-6a), 2.17 (1H, m, H-6b), 2.02 (1H, m, H-8), 1.98 (1H, m, H-16a), 1.92 (2H, m, H-7a and H-11a), 1.90 (1H, m, H-16b), 1.84 (1H, m, H-11b), 1.64 (1H, m, H-15b), 1.60 (1H, m, H-17), 1.40 (3H, s, H₃-26), 1.39 (3H, d, $J = 6.4$ Hz, H₃-21), 1.39 (3H, s, H₃-27), 1.38 (1H, m, H-14), 1.22 (1H, m, H-12b), 1.06 (1H, m, H-9), 1.00 (3H, s, H₃-19), 0.80 (1H, m, H-7b); ¹³C NMR (C₅D₅N, 100 MHz) of **3**: δ 185.9 (C, C-3), 176.9 (C, C-18), 169.1 (C, C-5), 155.9 (CH, C-1), 139.3 (CH, C-22), 127.7 (CH, C-2), 125.3 (CH, C-23), 124.2 (CH, C-4), 69.9 (CH, C-25), 57.1 (C, C-13), 56.4 (CH, C-14), 56.0 (CH, C-17), 52.6 (CH, C-9), 48.2 (CH₂, C-24), 43.7 (C, C-10), 42.0 (CH, C-20), 37.5 (CH, C-8), 37.2 (CH₂, C-12), 33.8 (CH₂, C-7), 32.8 (CH₂, C-6), 30.5 (CH₂, C-16), 29.9 (CH₃, C-27), 29.7 (CH₃, C-26), 25.5 (CH₂, C-15), 25.2 (CH₂, C-11), 21.0 (CH₃, C-21), 18.7 (CH₃, C-19); ESIMS m/z 449 [M + Na]⁺; HRESIMS m/z 449.2666 [M + Na]⁺ (calcd for C₂₇H₃₈O₄Na, 449.2668).

3.4. Preparation of (S) and (R)-PGME amides of **1** and **2**

To a stirred solution of compound **1** (0.5 mg) and (S)-PGME (2 mg) in a 1 mL mixture of CHCl₃–DMF (10:1) were successively added DMAP (2 mg) and 4-DMAP·HCl (2 mg) [5]. 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 refrigerator 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 NaHCO₃ (aq), and brine. The organic layer was dried over anhydrous Na₂SO₄ and concentrated to give a residue, which was chromatographed on silica gel using *n*-hexane–EtOAc (5:1) as eluent to afford the (S)-PGME amide (**1a**) (0.3 mg). The same procedure was used to prepare the (R)-PGME amide (**1b**) (0.3 mg from 0.5 mg of **1**) with (R)-PGME. Selective ¹H NMR (CDCl₃, 300 MHz) of **1a**: δ 7.343 (5H, br s, Ph), 7.049 (1H, d, $J = 10.2$ Hz, H-1), 6.384 (1H, d, $J = 7.0$ Hz, NH), 6.228 (1H, d, $J = 10.2$ Hz, H-2), 6.068 (1H, s, H-4), 5.586 (1H, d, $J = 7.0$ Hz, CH-N), 5.207 (2H, overlapped, H-22 and H-23), 3.730 (3H, s, OMe), 1.225 (3H, s, H₃-19), 1.143 (3H, d, $J = 6.3$ Hz, H₃-27), 0.911 (3H, d, $J = 6.4$ Hz, H₃-21), 0.711 (3H, s, H₃-18); selective ¹H NMR (CDCl₃, 300 MHz) of **1b**: δ 7.345 (5H, br s, Ph), 7.052 (1H, d, $J = 9.7$ Hz, H-1), 6.405 (1H, d, $J = 7.4$ Hz, NH), 6.226 (1H, d, $J = 9.7$ Hz, H-2), 6.066 (1H, s, H-4), 5.568 (1H, d, $J = 7.4$ Hz, CH-N), 5.286 (2H, overlapped, H-22 and H-23), 3.726 (3H, s, OMe), 1.229 (3H, s, H₃-19), 1.106 (3H, d, $J = 6.0$ Hz, H₃-27), 0.969 (3H, d, $J = 6.5$ Hz, H₃-21), 0.744 (3H, s, H₃-18). The same procedure was applied on **2** (0.5 mg) to prepare the (R)-PGME amide **2a** (0.4 mg) and the (S)-PGME amide **2a** (0.4 mg from 0.5 mg of **2**). Selective ¹H NMR (CDCl₃, 300 MHz) of **2b**: δ 7.357 (5H, br s, Ph), 7.050 (1H, d, $J = 10.1$ Hz, H-1), 6.362 (1H, d, $J = 7.4$ Hz, NH), 6.224 (1H, d, $J = 10.1$ Hz, H-2), 6.067 (1H, s, H-4), 5.597 (1H, d, $J = 7.4$ Hz, CH-N), 5.241 (2H, overlapped, H-22 and H-23), 3.727 (3H, s, OMe), 1.225 (3H, s, H₃-19), 1.016 (3H, d, $J = 6.6$ Hz, H₃-27), 0.947 (3H, d, $J = 6.5$ Hz, H₃-21), 0.716 (3H, s, H₃-18); selective ¹H NMR (CDCl₃, 300 MHz) of **2a**: δ 7.341 (5H, br s, Ph), 7.051 (1H, d, $J = 10.2$ Hz, H-1), 6.455 (1H, d, $J = 6.9$ Hz, NH), 6.223 (1H, d, $J = 10.2$ Hz, H-2), 6.066 (1H, s, H-4), 5.579 (1H, d, $J = 6.9$ Hz, CH-N), 5.238 (2H, overlapped, H-22 and H-23), 3.722 (3H, s, OMe), 1.226 (3H, s, H₃-19), 0.977 (3H, d, $J = 6.2$ Hz, H₃-27), 0.957 (3H, d, $J = 6.0$ Hz, H₃-21), 0.722 (3H, s, H₃-18). It has to be noted that the chemical shifts of H-22 and H-23 in both PGME amides of **1** and **2** were overlapped seriously, that might interfere the correct assignment of the corresponding

protons. Fortunately, we afford the $\Delta\delta$ values of the H₃-21 and H₃-18 of (*S*) and (*R*)-PGME amides of both **1** and **2** which could be used for configuration assignment of C-25 in **1** and C-24 in **2**, respectively.

3.5. Cytotoxicity Testing

Cell lines were purchased from the American Type Culture Collection (ATCC). Compounds were assayed for cytotoxicity against human liver carcinoma (HepG2 and HepG3), human breast carcinoma (MCF-7 and MDA-MB-231), and human lung carcinoma (A-549) cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method [8]. Freshly trypsinized cell suspensions were seeded in 96-well microtiter plates at densities of 5000–10,000 cells per well with tested compounds added from DMSO-diluted stock. After 3 days in culture, attached cells were incubated with MTT (0.5 mg/mL, 1 h) and subsequently dissolved in DMSO. The absorbency at 550 nm was then measured using a microplate reader. The IC₅₀ is the concentration of agent that reduced cell growth by 50% under the experimental conditions.

3.6. In Vitro Anti-Inflammatory Assay

Macrophage (RAW264.7) cell was purchased from ATCC. *In vitro* anti-inflammatory activities of tested compounds were measured by examining the inhibition of lipopolysaccharide (LPS) induced upregulation of iNOS and COX-2 proteins in macrophage cells using Western blotting analysis [9,10].

4. Conclusions

Our previous investigation on *P. acronocephala* has successfully discovered marine withanolides with potent anti-inflammatory activity. In this study, we reported three steroidal carboxylic acids, of which **3** exhibited potent cytotoxicity toward Hep3B, MDA-MB-231, MCF-7, and A-549 cancer cell lines. Compound **2**, the second member of 27-norergostan-26-oic acid obtained from nature [11,12], was isolated from the soft coral for the first time. Our present investigation demonstrated that the soft coral, *P. acronocephala*, is a useful source for the discovery of bioactive substances.

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