marine drugs

MDPI

Article

Bioactive Steroids with Methyl Ester Group in the Side Chain from a Reef Soft Coral *Sinularia brassica* Cultured in a Tank

Chiung-Yao Huang [1], Jui-Hsin Su [2,3], Chih-Chuang Liaw [1], Ping-Jyun Sung [1,2], Pei-Lun Chiang [4], Tsong-Long Hwang [5,6,7], Chang-Feng Dai [8] and Jyh-Horng Sheu [1,9,10,11,*]

1 Department of Marine Biotechnology and Resources, National Sun Yat-sen University, Kaohsiung 804, Taiwan; huangcy@mail.nsysu.edu.tw (C.-Y.H.); ccliaw@mail.nsysu.edu.tw (C.-C.L.)
2 National Museum of Marine Biology & Aquarium, Pingtung 944, Taiwan; x2219@nmmba.gov.tw (J.-H.S.); pjsung@nmmba.gov.tw (P.-J.S.)
3 Institute of Marine Biotechnology, National Dong Hwa University, Pingtung 944, Taiwan
4 Department of Biochemistry, University of Toronto, Toronto, ON M5G2R3, Canada; sharonjiang1996@hotmail.com
5 Graduate Institute of Natural Products, College of Medicine, Chang Gung University, Taoyuan 333, Taiwan; htl@mail.cgu.edu.tw
6 Research Center for Chinese Herbal Medicine, Research Center for Food and Cosmetic Safety, and Graduate Institute of Health Industry Technology, College of Human Ecology, Chang Gung University of Science and Technology, Taoyuan 333, Taiwan
7 Department of Anesthesiology, Chang Gung Memorial Hospital, Taoyuan 333, Taiwan
8 Institute of Oceanography, National Taiwan University, Taipei 112, Taiwan; corallab@ntu.edu.tw
9 Department of Medical Research, China Medical University Hospital, China Medical University, Taichung 404, Taiwan
10 Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung 807, Taiwan
11 Frontier Center for Ocean Science and Technology, National Sun Yat-sen University, Kaohsiung 804, Taiwan
* Correspondence: sheu@mail.nsysu.edu.tw; Tel.: +886-7-525-2000 (ext. 5030); Fax: +886-7-525-5020

Received: 11 August 2017; Accepted: 30 August 2017; Published: 1 September 2017

Abstract: A continuing chemical investigation of the ethyl acetate (EtOAc) extract of a reef soft coral *Sinularia brassica*, which was cultured in a tank, afforded four new steroids with methyl ester groups, sinubrasones A–D (**1**–**4**) for the first time. In particular, **1** possesses a *β*-D-xylopyranose. The structures of the new compounds were elucidated on the basis of spectroscopic analyses. The cytotoxicities of compounds **1**–**4** against the proliferation of a limited panel of cancer cell lines were assayed. The anti-inflammatory activities of these new compounds **1**–**4** were also evaluated by measuring their ability to suppress superoxide anion generation and elastase release in *N*-formyl-methionyl-leucyl-phenylalanine/cytochalasin B (fMLP/CB)-induced human neutrophils. Compounds **2** and **3** were shown to exhibit significant cytotoxicity, and compounds **3** and **4** were also found to display attracting anti-inflammatory activities.

Keywords: soft coral; *Sinularia brassica*; steroid; cytotoxic activity; anti-inflammatory activity

1. Introduction

Soft corals of the genus *Sinularia* have yielded series of natural products of differing chemical types [1]. Many of these compounds have been shown to exhibit interesting bioactivities, such as cytotoxic [2–8], antifouling [9], and anti-inflammatory [10–18] activities. Previous chemical investigation of the wild-type soft coral *Sinularia brassica* (May 1898) has led to the isolation of only two new steroids from this marine organism [19]. In contrast, during the course of our investigation of the bioactive substances obtained from this reef soft coral which was later cultured in a tank,

12 new withanolide-based steroidal metabolites, sinubrasolides A–L, were discovered [20,21]. As most withanolides were isolated from terrestrial plants, and have attracted considerable attention due to their versatile bioactivities [22,23], marine withanolides discovered from reef soft corals *Minabea* sp. [24] and *Paraminabea acronocephala* [25], as well as the cultured *S. brassica*, have also been found to possess unusual withanolide-based structures and/or exhibit interesting biological activities, we further continued our exhaustive investigation of the cultured *S. brassica* with the aim of discovering new bioactive metabolites for further biomedical application. This study led to the discovery of four new non-withanolidal steroids, sinubrasones A–D (**1**–**4**) (Figure 1). The structure of **1** is unusual as it possesses a *β*-D-xylopyranose at C-22 of the side-chain. The structures of **1**–**4** were established by extensive spectroscopic analyses, including 2D nuclear magnetic resonance (NMR) spectroscopy. The in vitro cytotoxicities of **1**–**4** against four cancer cell lines, murine macrophage-like (P388D1), human T-lymphoid (MOLT-4), human erythroleukemia (K-562), and human colon carcinoma (HT-29), were measured. The abilities of new compounds **1**–**4** to inhibit superoxide anion generation and elastase release in *N*-formyl-methionyl-leucyl-phenylalanine/cytochalasin B (fMLP/CB)-induced neutrophils were also assayed. Herein, we report the isolation, structure elucidation and biological activities of these compounds.

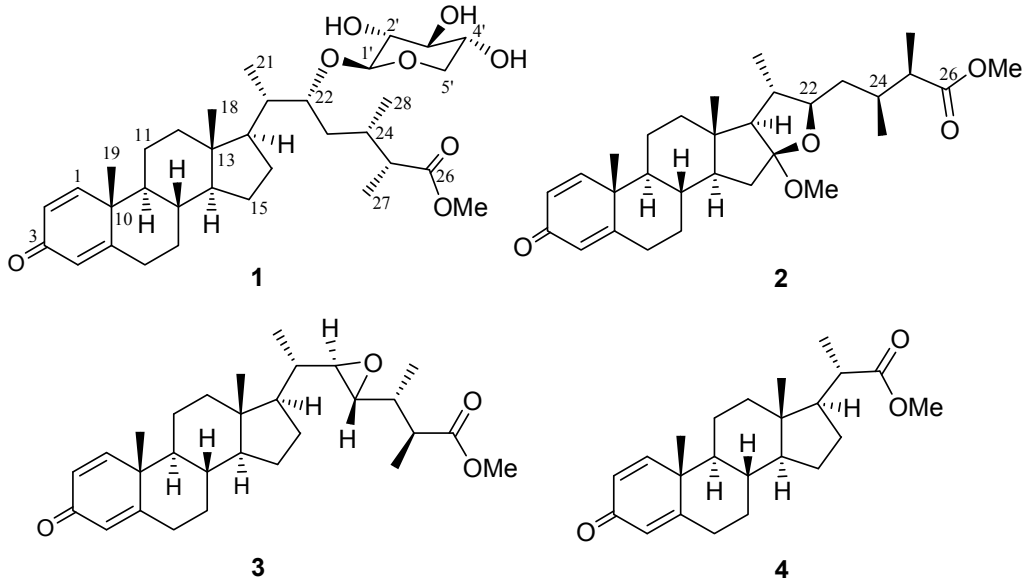


Figure 1. Structures of compounds **1**–**4**.

2. Results and Discussion

A reef soft coral *S. brassica* further cultured in a tank was collected by hand from a cultivation pool of at the National Museum of Marine Biology and Aquarium, Taiwan, in January 2010. The organisms were further stored in a freezer until extraction. The frozen bodies were minced and extracted exhaustively with CH_2Cl_2 and MeOH (0.5 L × 6), as previously described [20]. Fractions 12 and 18, which contained terpenoids, as revealed by ^{1}H-NMR spectra, were further purified by column chromatography and reversed-phase high-performance liquid chromatography (HPLC) to afford **1**–**4** (see Section 3).

Sinubrasone A (**1**) was isolated as an amorphous solid. The high-resolution electrospray ionization mass spectrometry (HRESIMS) spectrum of **1** exhibited a $[M + Na]^+$ peak at m/z 611.35545, indicating the molecular formula $C_{34}H_{52}O_8$, requiring nine degrees of unsaturation. The infrared (IR) spectrum revealed the presence of a hydroxy group (3396 cm^{-1}), an ester (1735 cm^{-1}) group, and a conjugated enone (1664 cm^{-1}). The ^{13}C NMR spectroscopic data of **1** exhibited 34 carbon signals (Table 1), which were assigned by the assistance of a distortionless enhancement by polarization transfer (DEPT)

spectra to six methyls (including a methoxy), eight sp^3 methylenes, 12 sp^3 methines (including five oxymethines), three sp^2 methines, three sp^2, and two sp^3 non-protonated carbons (including an ester carbonyl and a ketone). The above data accounted for four of the nine degrees of unsaturation, indicating a pentacyclic structure for **1**. Proton signals (Table 1) resonating at δ_H 7.05 (1H, d, J = 10.0 Hz), 6.23 (1H, d, J = 10.0 Hz), and 6.07 (1H, s), as well as carbon signals appearing at δ_C 186.5 (C), 169.3 (C), 156.0 (CH), 127.5 (CH), and 123.8 (CH), indicated the presence of a 1,4-dien-3-one structural unit in ring A of the steroids [25,26]. The molecular framework of **1** was further established by correlation spectroscopy (COSY) and heteronuclear multiple bond correlation (HMBC) correlations (Figure 2). By comparison of the NMR data of **1** with those of cladophenol glycoside A [12], it was found that the presence of an anomeric proton at δ_H 4.30 (1H, d, J = 7.0 Hz) to arise from a β-xylopyranose moiety prossessing carbon signals at δ_C 104.5 (CH, C-1′), 73.4 (CH, C-2′), 75.9 (CH, C-3′), 69.5 (CH, C-4′), and 64.9 (CH, C-5′) (Table 1), as confirmed by HMBC correlation from H-5′ to C-1′ and the NOE correlations of H-1′/H-3′ and H-2′/H-4′. Moreover, the β-xylopyranose residue attached at C-22 was assigned according to an HMBC correlation from the anomeric proton (H-1′) to C-22. Thus, the planar structure of **1** was established.

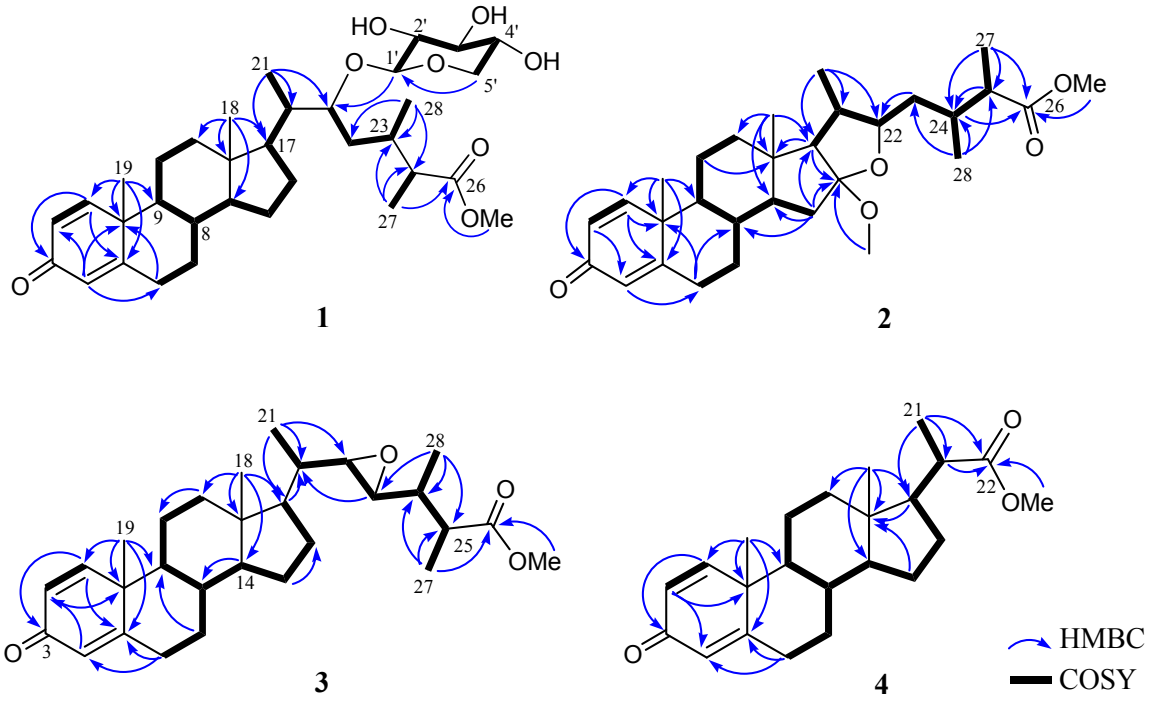


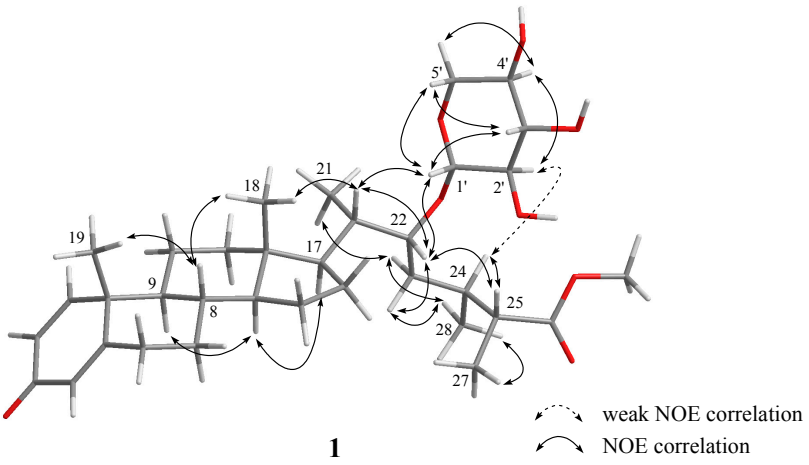
Figure 2. Selected correlation spectroscopy (COSY) and heteronuclear multiple bond correlations (HMBC) of **1**–**4**.

The configuration of **1** was further confirmed by analysis of their nuclear Overhauser effect (NOE) correlations. In the nuclear Overhauser enhancement spectroscopy (NOESY) spectrum of **1**, NOE correlations of H-20 with H_3-18 and H-22, but H-22 not with H_3-21, revealed the β-orientation of H-22. Moreover, correlations of H-25 with H-22 and H-24, and H_3-27 with H_3-28, revealed the 20*S*, 22*R*, 24*S*, 25*R* configuration of **1**. Finally, the anomeric proton (H-1′) was found to show NOE correlations with H-20 and H-22 as shown **1** in Figure 3, and H-2′ was also found to show weak NOE correlations with H-24, but H_2-5′ did not show NOE correlation with H-24, revealed the β-D-xylopyranose residue of the cholesterol. On the basis of the above findings and other detailed NOE correlations, the structure of **1** was established to be that of formula **1**.

Table 1. ^{1}H and ^{13}C-NMR spectroscopic data of **1**.

1					
Position	**δ_C [a] (Mult.) [b]**	**δ_H [c] (*J* in Hz)**	**Position**	**δ_C (Mult.)**	**δ_H (*J* in Hz)**
1	156.0, CH	7.05 d (10.0)	1′	104.5, CH	4.30 d (7.0)
2	127.5, CH	6.23 d (10.0)	2′	73.4, CH	3.42 dd (8.5, 7.0)
3	186.5, C		3′	75.9, CH	3.52 dd (8.5, 8.5)
4	123.8, CH	6.07 s	4′	69.5, CH	3.75 ddd (9.5, 8.5, 5.0)
5	169.3, C		5′	64.9, CH_2	4.01 dd (12.0, 5.0);
6	32.8, CH_2	2.47 ddd (12.5, 12.5, 4.5)			3.30 d (12.0, 9.5)
		2.36 br d (12.5)	26-OMe	52.1, CH_3	3.71 s
7	33.6, CH_2	1.95 m; 1.04 m			
8	35.5, CH	1.63 m			
9	52.3, CH	1.04 m			
10	43.6, C				
11	22.8, CH_2	1.70 m			
12	39.4, CH_2	2.04 m; 1.16 m			
13	43.0, C				
14	55.0, CH	0.99 m			
15	24.5, CH_2	1.66 m; 1.20 m			
16	27.5, CH_2	1.68 m; 1.34 m			
17	52.6, CH	1.06 m			
18	11.9, CH_3	0.76 s			
19	18.7, CH_3	1.23 s			
20	39.5, CH	2.04 m			
21	12.6, CH_3	0.92 d (6.5)			
22	81.5, CH	3.63 br d (11.0)			
23	32.3, CH_2	1.43 ddd (14.5, 11.0, 3.5);			
		1.28 m			
24	31.5, CH	2.29 m			
25	40.9, CH	2.62 qd (6.0, 4.0)			
26	177.7, C				
27	9.9, CH_3	1.02 d (7.0)			
28	16.3, CH_3	0.85 d (7.0)			

[a] Spectrum recorded at 100 MHz in $CDCl_3$. [b] Attached protons were deduced by distortionless enhancement by polarization transfer (DEPT) experiment. [c] Spectrum recorded at 500 MHz in $CDCl_3$.



1

Figure 3. Selected nuclear Overhauser effect (NOE) correlations for **1**.

Sinubrasone B (**2**) had the molecular formula $C_{30}H_{44}O_5$ as determined by HRESIMS and from ^{13}C-NMR data. Thus, nine degrees of unsaturation were determined for **2**. The IR absorption band at 1735 and 1662 cm^{-1} indicated the presence of an ester carbonyl group and a conjugated enone, which was further supported by NMR signals resonating at δ_C 176.8, C in **2**. The ^{1}H and ^{13}C-NMR spectra (Table 2) displayed resonances for a carbonyl (δ_C 186.3, C), three sp^2 methine double

bonds (δ_H 7.04, 1H, d, J = 10.0 Hz, δ_C 155.7; δ_H 6.23, 1H, d, J = 10.0 Hz, δ_C 127.6; δ_H 6.07, 1H, s, δ_C 123.9), an sp^2 non-protonated carbon (δ_C 168.9), an oxygenated non-protonated carbon (δ_C 117.9), two methoxy groups (δ_C 51.4, δ_H 3.67, s; and δ_C 49.3, δ_H 3.20, s), and an oxymethine (δ_C 86.9, δ_H 3.69, m). The remaining five degrees of unsaturation identified **2** as a pentacyclic triterpane. Proton signals (Table 2) resonating at δ_H 7.04 (1H, d, J = 10.0 Hz), 6.23 (1H, d, J = 10.0 Hz), and 6.07 (1H, s), as well as carbon signals appearing at δ_C 186.3 (C), 155.7 (CH), 127.6 (CH), 123.9 (CH), and 168.9 (C), indicated the presence of a 1,4-dien-3-one structural unit in ring A of the steroids [25,26]. Analysis of COSY correlations (Figure 2) of **2** indicated three consecutive proton sequences. The connection of the three partial structures was subsequently resolved by HMBC. Methoxyl and methyl ester groups were assigned at C-16 and C-25, respectively, based on HMBC correlations from the methoxyl proton (δ_H 3.20, s) to C-16, and from H_3-27 to C-24, C-25, and C-26. In consideration of the degrees of unsaturation and molecular formula, an ether linkage was placed between C-16 and C-22, which further confirmed the presence of a tetrahydrofuran (THF) ring fused to ring D (Figure 2). Accordingly, the planar structure of **2** was established.

Table 2. ^1H and ^{13}C-NMR spectroscopic data of **2**–**4**.

	2		**3**		**4**	
Position	δ_C [a] **(Mult.)** [b]	δ_H [c] **(*J* in Hz)**	δ_C [d] **(Mult.)**	δ_H [e] **(*J* in Hz)**	δ_C [a] **(Mult.)**	δ_H [c] **(*J* in Hz)**
1	155.7, CH	7.04 d (10.0)	156.0, CH	7.05 d (10.4)	155.8, CH	7.05 d (10.0)
2	127.6, CH	6.23 d (10.0)	127.5, CH	6.23 d (10.4)	127.5, CH	6.23 d (10.0)
3	186.3, C		186.5, C		186.4, C	
4	123.9, CH	6.07 s	123.8, CH	6.07 s	123.9, CH	6.07 s
5	168.9, C		169.4, C		169.2, C	
6	32.7, CH_2	2.46 ddd (12.0, 12.0, 4.0) 2.36 br d (12.0)	32.9, CH_2	2.47 m 2.37 m	32.8, CH_2	2.46 ddd (13.0, 13.0, 4.0) 2.36 m
7	33.5, CH_2	1.90 m; 1.07 m	33.6, CH_2	1.96 m; 1.05 m	33.5, CH_2	1.94 m; 1.05 m
8	35.1, CH	1.76 m	35.5, CH	1.60 m	35.5, CH	1.64 m
9	52.2, CH	1.09 m	52.4, CH	1.06 m	52.2, CH	1.08 m
10	43.6, C		43.6, C		43.5, C	
11	22.4, CH_2	1.69 m	22.8, CH_2	1.66 m	22.8, CH_2	1.71 m
12	38.8, CH_2	1.71 m 1.21 m	39.3, CH_2	1.99 m 1.21 m	39.2, CH_2	1.97 ddd (13.0, 3.0, 3.0) 1.28 m
13	40.9, C		43.0, C		42.7, C	
14	54.6, CH	1.36 dd (12.0, 5.5)	55.0, CH	1.02 m	55.0, CH	1.08 m
15	33.5, CH_2	1.96 dd (12.0, 5.5) 1.31 dd (12.0, 12.0)	24.6, CH_2	1.63 m 1.17 m	24.4, CH_2	1.62 m 1.19 m
16	117.9, C		26.9, CH_2	1.93 m; 1.60 m	27.0, CH_2	1.70 m; 1.30 m
17	70.9, CH	1.65 m	55.8, CH	1.30 m	52.7, CH	1.60 m
18	15.3, CH_3	0.83 s	12.2, CH_3	0.73 s	12.2, CH_3	0.76 s
19	19.2, CH_3	1.24 s	18.7, CH_3	1.23 s	18.7, CH_3	1.23 s
20	38.1, CH	1.74 m	38.5, CH	1.30 m	42.4, CH	2.43 m
21	18.8, CH_3	1.02 d (7.0)	15.9, CH_3	0.99 d (7.2)	17.0, CH_3	1.18 d (7.0)
22	86.9, CH	3.69 m	63.9, CH	2.59 dd (4.8, 2.4)	177.1, C	
23	38.5, CH_2	1.53 m; 1.48 m	59.1, CH	2.52 dd (7.6, 2.4)		
24	33.3, CH	2.07 dddq (6.5, 6.5, 6.5, 6.5)	39.2, CH	1.54 m		
25	43.6, CH	2.49 dq (6.5, 6.5)	42.9, CH	2.48 m		
26	176.8, C		175.6, C			
27	11.9, CH_3	1.08 d (6.5)	14.5, CH_3	1.21 d (7.2)		
28	16.3, CH_3	0.93 d (6.5)	14.8, CH_3	1.02 d (7.2)		
16-OMe	49.3, CH_3	3.20 s				
22-OMe					51.4, CH_3	3.65 s
26-OMe	51.4, CH_3	3.67 s	51.5, CH_3	3.69 s		

[a] Spectrum recorded at 125 MHz in $CDCl_3$. [b] Attached protons were deduced by DEPT experiment. [c] Spectrum recorded at 500 MHz in $CDCl_3$. [d] Spectrum recorded at 100 MHz in $CDCl_3$. [e] Spectrum recorded at 400 MHz in $CDCl_3$.

The configuration of **2** was further confirmed by NOE correlations (Figure 3). It was found that H_3-18 (δ_H 0.83, s) showed NOE interactions with H-20 (δ_H 1.74, m) and one of the methylene protons at C-23 (δ_H 1.48, m); therefore, assuming the β-orientation of H_3-18, H-20 and the above H-23 should also be positioned on the β-face, while the other (δ_H 1.53, m) was assigned as H-23α. Furthermore, H-22 was found to show NOE correlations with H_3-21 (δ_H 1.02, d, J = 7.0 Hz), H-23α and H-24 (δ_H 2.07, dddq, J = 6.5, 6.5, 6.5, 6.5 Hz), and H_3-27 showed correlation with H_3-28 (δ_H 0.93, d, J = 6.5 Hz), reflecting

the α-orientations of H-22, H-24 and H-25, and the 25*R* configuration of **2**. On the basis of the above findings and other observed correlations (Figure 3), the structure of sinubrasone B (**2**) was suggested.

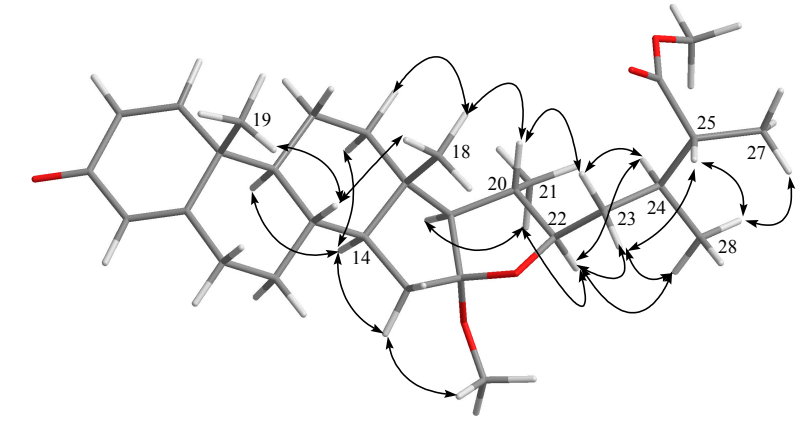
The molecular formula of sinubrasone C (**3**) was found to be $C_{29}H_{42}O_4$, as deduced from HRESIMS and ^{13}C-NMR data, appropriate for nine degrees of unsaturation. The IR spectrum of **3** showed the presence of carbonyl groups of an ester and a conjugated enone (ν_{max} 1736 and 1662 cm^{-1}), and the ^{13}C-NMR (Table 2) and DEPT spectra also showed a signal of an ester carbonyl group at δ_C 175.6, C. Inspection of the ^{1}H and ^{13}C-NMR data of **3** suggested the presence of a 1,2-disubstituted epoxide (δ_C 63.9, CH; 59.1, CH; δ_H 2.59, dd, *J* = 4.8, 2.4 Hz; 2.52, dd, *J* = 7.6, 2.4 Hz). These data suggested that **3** possessed a 22,23-epoxide, which was corroborated by the HMBC correlations from H_3-21 to C-22, and H_3-28 to C-23, as well as the COSY correlation between H-22 and H-23. Additionally, H_3-27 and H_3-OMe displayed HMBC correlation to carbonyl carbon C-26 (Figure 2).

The stereochemistry of **3** was determined on the basis of NOE correlations and by comparison of NMR spectroscopic data. A small coupling constant (2.4 Hz) between H-22 and H-23 suggested a *trans* conformation for both protons [27]. The NOE interactions of H_3-18 with H-20, H-17 with H_3-21, H-22 with H-20 and H-24, and H-23 with H_3-28, revealed the β-orientations of H-22 and H-24. Further, the 22*R* and 23*R* configurations, as opposed to 22*S* and 23*S*, were confirmed by comparing the δ values of H-20 (δ_H 1.32), H_3-21 (δ_H 1.00), H-22 (δ_H 2.59) and H-23 (δ_H 2.46) of known compound (22*R*,23*R*,24*R*)-3β-acetoxy-24-methyl-22,23-epoxy-5α-holestan-6-one [28] with the corresponding H-20 (δ_H 1.30), H_3-21 (δ_H 0.99), H-22 (δ_H 2.59) and H-23 (δ_H 2.52) of **3**, while (22*S*,23*S*,24*R*)-3β-acetoxy-24-methyl-22,23-epoxy-5α-cholestan-6-one [28] with 22*S* and 23*S* configurations showed corresponding NMR signals for H-20 (δ_H 1.17), H_3-21 (δ_H 1.09), H-22 (δ_H 2.39) and H-23 (δ_H 2.67). In addition, the NOESY spectrum of **3** showed NOE correlations between H_3-28 with H-25, and H-24 with H_3-27, but not between H_3-28 and H_3-27, revealing the 24*R* and 25*S* configurations of **3** (Figure 4). Thus, the absolute configuration of **3** was determined.

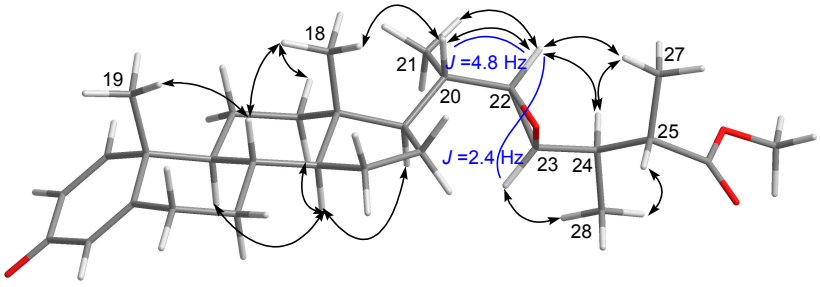
Sinubrasone D (**4**) had the molecular formula of $C_{23}H_{32}O_3$, as determined by HRESIMS. It was also found to possess the same A–D rings as compounds **1**–**3** by comparison of NMR spectroscopic data (Tables 1 and 2). The gross structure of **4** was determined by detailed analysis of COSY and HMBC correlations (Figure 2). The HMBC experiment of **4** further revealed the connectivity from H_3-21 (δ_H 1.18, d, *J* = 7.0 Hz) and the methoxyl (δ_H 3.65, s) to the carbonyl carbon (δ_C 177.1). The relative configurations at C-8, C-9, C-10, C-13, C-14, C-17, and C-20 in **4** were found to be the same as those of compounds **1**–**3** by comparison of NMR data and NOE correlations.

As previous studies revealed that steroids from soft corals might possess attracting biological activities [29–33], we further evaluated the biological activities of these isolated steroids. Compounds **1**–**4** were evaluated in terms of their cytotoxic activities against P388D1, MOLT-4, K-562, and HT-29 cell lines using the Alamar Blue assay. Compounds **2** and **3** were found to show significant cytotoxicity against all cell lines. Compounds **1** and **4** exhibited only weak cytotoxic activity against P388D1, MOLT-4, K-562, and HT-29 cell lines (Table 3).

The anti-inflammatory activities of new compounds **1**–**4** on neutrophil pro-inflammatory responses were evaluated by measuring their ability to suppress fMLP/CB-induced superoxide anion ($O_2^{-\bullet}$) generation and elastase release in human neutrophils, and the results are shown in Table 4. From the results, **4** showed significant inhibitory effect (53.6 ± 1.8%) against superoxide anion generation at 10 μM. Compounds **3** and **4** also exhibited inhibitory activities against elastase release, with the inhibition rate of 58.8 ± 4.0 and 66.3 ± 6.0% in the fMLP/CB-stimulated cells at the same concentration. The IC_{50} values of compound **4** for the superoxide anion generation and compounds **3** and **4** for inhibition of elastase release were also measured and were found to be lower than 10 μM.



2



3

Figure 4. Selected NOE correlations for **2** and **3**.

Table 3. Cytotoxicity (IC_{50} μM) of compounds **1**–**4**.

	Cell lines IC_{50} (μM)			
Compound	**P388D1**	**MOLT-4**	**K-562**	**HT-29**
1	37.2 ± 4.0	37.8 ± 5.6	— [b]	— [b]
2	9.7 ± 1.2	6.0 ± 0.4	5.2 ± 0.8	7.6 ± 2.3
3	5.7 ± 1.8	5.3 ± 1.3	12.1 ± 2.4	10.4 ± 2.2
4	24.4 ± 4.8	31.2 ± 7.0	21.3 ± 3.7	36.5 ± 7.9
5-Fluorouracil [a]	6.2 ± 0.7	6.9 ± 1.3	33.1 ± 8.9	7.7 ± 0.8

[a] Clinical anticancer drug used as a positive control. [b] —: IC_{50} > 40 μM.

Table 4. Inhibitory (% Inh) effects of compounds **1**–**4** on superoxide anion generation and elastase release in fMLP/CB-induced human neutrophils at 10 μM.

Compounds	**Superoxide Anion**			**Elastase Release**		
	IC_{50} (μM) [a]	**Inh %** [b]		**IC_{50} (μM)** [a]	**Inh %** [b]	
1	>10	24.8 ± 6.5	*	>10	35.6 ± 1.3	***
2	>10	19.4 ± 5.0	*	>10	39.0 ± 2.3	***
3	>10	27.7 ± 1.3	***	6.6 ± 1.7	58.8 ± 4.0	***
4	8.4 ± 1.1	53.6 ± 1.8	***	6.5 ± 1.1	66.3 ± 6.0	***
Idelalisib	0.07 ± 0.01	102.8 ± 2.2	***	0.3 ± 0.1	99.6 ± 4.2	***

[a] Concentration necessary for 50% inhibition (IC_{50}). [b] Percentage of inhibition (Inh%) at 10 μM concentration. Results are presented as mean ± S.E.M. (n = 3–4). * $p < 0.05$, *** $p < 0.001$ compared with the control value.

3. Experimental Section

3.1. General Experimental Procedures

Optical rotations of the isolates were measured on a JASCO P1020 digital polarimeter (JASCO Corporation, Tokyo, Japan) and on a Horiba High Sensitivity Polarimeter SEPA-300 (Horiba Ltd., Kyoto, Japan). Ultraviolet spectra were recorded on a JASCO V-650 spectrophotometer (JASCO Corporation). IR spectra were recorded on a JASCO FT/IR-4100 infrared spectrophotometer (JASCO Corporation). NMR spectra were recorded on a Varian 400MR FT-NMR or Varian Unity INOVA500 FT-NMR (Varian Inc., Palo Alto, CA, USA) instrument at 400 MHz (or 500 MHz) for ^{1}H and 100 MHz (or 125 MHz) for ^{13}C in $CDCl_3$, and the chemical shifts were referenced to residual signals of TMS (δ_H 0.00 ppm) and the $CDCl_3$ (δ_C 77.0 ppm). ESIMS and HRESIMS data were obtained with a Bruker APEX II mass spectrometer (Bruker, Bremen, Germany). Silica gel (230–400 mesh, Merck, Darmstadt, Germany) was used for column chromatography. Pre-coated silica gel plates (Merck, Kieselgel 60 F-254, 0.2 mm) were used for analytical TLC. High-performance liquid chromatography was performed on a Hitachi L-2455 HPLC apparatus (Hitachi Ltd., Tokyo, Japan) with a Supelco C18 column (250 × 21.2 mm, 5 μm).

3.2. Animal Material

The soft coral *Sinularia brassica* used in this study was originally collected from a reef and cultured in an 80-ton cultivation tank (height 1.6 m) located in the National Museum of Marine Biology and Aquarium, Taiwan, for five years. The soft coral organisms were collected in January 2010 and were stored in a −20 °C freezer until extraction, while the voucher specimen (specimen no. 201001C1) was deposited in the Department of Marine Biotechnology and Resources, National Sun Yat-sen University. The soft coral was identified by one of the authors (C.-F.D.).

3.3. Extraction and Isolation

The frozen bodies of *S. brassica* (0.4 kg, wet weight) were minced and extracted exhaustively with the 1:1 mixture of CH_2Cl_2 and MeOH (0.5 L × 6). The combined extract was evaporated under reduced pressure and the residue was partitioned between EtOAc and H_2O to give the EtOAc-soluble fraction. The EtOAc extract (3.7 g) was subjected to separation using a silica gel column with a gradient of EtOAc and *n*-hexane in an increasing polarity (0–100%, stepwise), and then with MeOH in EtOAc (5–50%, stepwise) to yield 24 fractions. Fraction 12 eluting with EtOAc–*n*-hexane (1:9) was further purified with acetone–*n*-hexane (1:7) to give five subfractions (12A–12F). Subfraction 12B was further separated by reversed-phase HPLC using MeOH–H_2O (4:1) to yield **2** (0.8 mg), **3** (1.1 mg), and **4** (0.9 mg). Fraction 18 eluting with EtOAc–*n*-hexane (1:3) was separated by silica gel column chromatography with acetone–*n*-hexane (1:3) to afford four subfractions (18A–18D). Subfraction 18C was further purified by reversed-phase HPLC using MeOH–H_2O (2:1) to yield **1** (1.3 mg).

Sinubrasone A (**1**): amorphous solid; $[\alpha]_D^{23}$ −30 (*c* 0.50, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 243 (4.2); IR (neat) ν_{max} 3396, 2941, 1735 and 1664 cm^{-1}; ^{13}C and ^{1}H-NMR data, see Table 1; ESIMS *m*/*z* 611 [M + Na]$^+$; HRESIMS *m*/*z* 611.35545 [M + Na]$^+$ (calcd. for $C_{34}H_{52}O_8Na$, 611.35544).

Sinubrasone B (**2**): amorphous solid; $[\alpha]_D^{23}$ −128 (*c* 0.25, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 244 (4.3); IR (neat) ν_{max} 2930, 1735 and 1662 cm^{-1}; ^{13}C and ^{1}H-NMR data, see Table 2; ESIMS *m*/*z* 507 [M + Na]$^+$; HRESIMS *m*/*z* 507.3089 [M + Na]$^+$ (calcd. for $C_{30}H_{44}O_5Na$, 507.3086).

Sinubrasone C (**3**): amorphous solid; $[\alpha]_D^{23}$ −68 (*c* 0.25, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 245 (4.2); IR (neat) ν_{max} 2936, 2856, 1736 and 1662 cm^{-1}; ^{13}C and ^{1}H-NMR data, see Table 2; ESIMS *m*/*z* 477 [M + Na]$^+$; HRESIMS *m*/*z* 477.2978 [M + Na]$^+$ (calcd. for $C_{29}H_{42}O_4Na$, 477.2981).

Sinubrasone D (**4**): amorphous solid; $[\alpha]_D^{24}$ −65 (*c* 0.035, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 244 (4.0); IR (neat) ν_{max} 2928, 2853, 1735 and 1662 cm^{-1}; ^{13}C and ^{1}H-NMR data, see Table 2; ESIMS *m*/*z* 379 [M + Na]$^+$; HRESIMS *m*/*z* 379.2250 [M + Na]$^+$ (calcd. for $C_{23}H_{32}O_3Na$, 379.2249).

3.4. Cytotoxicity Assay

Cytotoxicity assays were performed as previous reported, using an Alamar Blue assay [34,35]. Cell lines (P388D1, MOLT-4, K-562, and HT-29) were purchased from the American Type Culture Collection (ATCC). Cancer cells were plated onto 96-well microtiter plates possessing clear flat bottoms (Thermo Scientific Nunc MicroWell plate) with densities of 5×10^3 to 1×10^4 cells per well and incubated in a humidified 5% CO_2 atmosphere at 37 °C. After 15 h of culture, the solutions of compounds in DMSO were added. After 72 h, attached cells were incubated with Alamar Blue (10 μL/well, 4 h). The absorbance at 595 nm was recorded using the ELISA reader. The IC_{50} values represented the concentrations of the compounds tested that could reduce cell growth by 50% under the experimental conditions.

3.5. Human Neutrophil Superoxide Anion Generation and Elastase Release

The human neutrophils were isolated through dextran sedimentation and Ficoll centrifugation. As previously described procedures, the assay of superoxide anion generation was measured from the SOD-inhibitable reduction of ferricytochrome C. The elastase release experiment was performed according to MeO–Suc–Ala–Ala–Pro–Val–*p*-nitroanilide as the enzyme substrate [36,37].

4. Conclusions

Our continuing investigations demonstrated that the cultured soft coral *Sinularia brassica* is a good source of bioactive withanolides and non-withanolidal steroids with methyl ester groups. Moreover, it is worthwhile to note here that **1–4** were found to be the novel steroids with a methyl ester group, and **1** with a *β*-D-xylopyranose on the side chain is quite rare. Metabolites **2** and **3** possessing a methyl ester at C-25 were shown to exhibit significant cytotoxic activities against P388D1, MOLT-4, K-562, and HT-29 cancer cell lines. Compounds **3** and **4** also exhibited notable anti-inflammatory activities in inhibition of elastase release in fMLP/CB-induced human neutrophils, and **4** could inhibit the generation of superoxide anion, too. Owing to these attractive biological activities, **2–4** might be useful lead compounds for future drug discoveries.

Supplementary Materials: HRESIMS, ^{1}H, and ^{13}C spectra of new compounds **1–4** are available online at www.mdpi.com/1660-3397/15/9/280/s1. **Figure S1**. HRESIMS spectrum of **1**; **Figure S2**. ^{1}H-NMR spectrum (500 MHz) of compound **1** in $CDCl_3$; **Figure S3**. ^{13}C-NMR spectrum (100 MHz) of compound **1** in $CDCl_3$; **Figure S4**. HRESIMS spectrum of **2**; **Figure S5**. ^{1}H-NMR spectrum (500 MHz) of compound **2** in $CDCl_3$; **Figure S6**. ^{13}C-NMR spectrum (125 MHz) of compound **2** in $CDCl_3$; **Figure S7**. HRESIMS spectrum of **3**; **Figure S8**. ^{1}H-NMR spectrum (400 MHz) of compound **3** in $CDCl_3$; **Figure S9**. ^{13}C-NMR spectrum (100 MHz) of compound **3** in $CDCl_3$; **Figure S10**. HRESIMS spectrum of **4**; **Figure S11**. ^{1}H-NMR spectrum (500 MHz) of compound **4** in $CDCl_3$; **Figure S12**. ^{13}C-NMR spectrum (125 MHz) of compound **4** in $CDCl_3$.

Acknowledgments: This work was supported by grants from Ministry of Science and Technology (MOST 104-2113-M-110-006, MOST 105-2113-M-110-002 and 104-2320-B-110-001-MY2), and the National Sun Yat-sen University-Kaohsiung Medical University (NSYSU-KMU) Joint Research Project (NSYSUKMU 105-I008) awarded to J.-H.S.

Author Contributions: Jyh-Horng Sheu designed and guided the whole experiment. Chiung-Yao Huang isolated the compounds, and performed the data acquisition, structure elucidation, and cytotoxicity assay. Jui-Hsin Su, Chih-Chuang Liaw, and Ping-Jyun Sung performed the structure elucidation. Pei-Lun Chiang and Tsong-Long Hwang performed the inhibition of superoxide anion generation and elastase release assays. Chang-Feng Dai contributed to the species identification of soft coral. Chiung-Yao Huang and Jyh-Horng Sheu wrote the manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Blunt, J.W.; Copp, B.R.; Keyzers, R.A.; Munro, M.H.; Prinsep, M.R. Marine natural products. *Nat. Prod. Rep.* **2017**, *34*, 235–294 (and previous articles in this series). [CrossRef] [PubMed]

2. Chitturi, B.R.; Tatipamula, V.B.; Dokuburra, C.B.; Mangamuri, U.K.; Tuniki, V.R.; Kalivendi, S.V.; Bunce, R.A.; Yenamandra, V. Pambanolides A–C from the South Indian soft coral *Sinularia inelegans*. *Tetrahedron* **2016**, *72*, 1933–1940. [CrossRef]
3. Huang, C.Y.; Tseng, Y.J.; Chokkalingam, U.; Hwang, T.L.; Hsu, C.H.; Dai, C.F.; Sung, P.J.; Sheu, J.H. Bioactive isoprenoid-derived natural products from a Dongsha Atoll soft coral *Sinularia erecta*. *J. Nat. Prod.* **2016**, *79*, 1339–1346. [CrossRef] [PubMed]
4. Miyazato, H.; Taira, J.; Ueda, K. Hydrogen peroxide derived from marine peroxy sesquiterpenoids induces apoptosis in HCT116 human colon cancer cells. *Bioorg. Med. Chem. Lett.* **2016**, *26*, 4641–4644. [CrossRef] [PubMed]
5. Tseng, Y.J.; Yang, Y.C.; Wang, S.K.; Duh, C.Y. Numerosol A–D, new cembranoid diterpenes from the soft coral *Sinularia numerosa*. *Mar. Drugs* **2014**, *12*, 3371–3380. [CrossRef] [PubMed]
6. Wright, A.D.; Nielson, J.L.; Tapiolas, D.M.; Liptrot, C.H.; Motti, C.A. A Great Barrier Reef *Sinularia* sp. yields two new cytotoxic diterpenes. *Mar. Drugs* **2012**, *10*, 1619–1630. [CrossRef] [PubMed]
7. Arepalli, S.K.; Sridhar, V.; Rao, J.V.; Kennady, P.K.; Venkateswarlu, Y. Furano-sesquiterpene from soft coral, *Sinularia kavarittiensis*: Induces apoptosis via the mitochondrial-mediated caspase-dependent pathway in THP-1, leukemia cell line. *Apoptosis* **2009**, *14*, 729–740. [CrossRef] [PubMed]
8. Kamel, H.N.; Ferreira, D.; Garcia-Fernandez, L.F.; Slattery, M. Cytotoxic diterpenoids from the Hybrid soft coral *Sinularia maxima* × *Sinularia polydactyla*. *J. Nat. Prod.* **2007**, *70*, 1223–1227. [CrossRef] [PubMed]
9. Lai, D.W.; Geng, Z.F.; Deng, Z.W.; van Ofwegen, L.; Proksch, P.; Lin, W.H. Cembranoids from the soft coral *Sinularia rigida* with antifouling activities. *J. Agric. Food Chem.* **2013**, *61*, 4585–4592. [CrossRef] [PubMed]
10. Roy, P.K.; Ashimine, R.; Miyazato, H.; Taira, J.; Ueda, K. Endoperoxy and hydroperoxy cadinane-type sesquiterpenoids from an Okinawan soft coral, *Sinularia* sp. *Arch. Pharm. Res.* **2016**, *39*, 778–784. [CrossRef] [PubMed]
11. Tsai, T.C.; Chen, H.Y.; Sheu, J.H.; Chiang, M.Y.; Wen, Z.H.; Dai, C.F.; Su, J.H. Structural elucidation and structure–Anti-inflammatory activity relationships of cembranoids from cultured soft corals *Sinularia sandensis* and *Sinularia flexibilis*. *J. Agric. Food. Chem.* **2015**, *63*, 7211–7218. [CrossRef] [PubMed]
12. Chao, C.H.; Huang, T.Z.; Wu, C.Y.; Chen, B.W.; Huang, C.Y.; Hwang, T.L.; Dai, C.F.; Sheu, J.H. Steroidal and α-tocopherylhydroquinone glycosides from two soft corals *Cladiella hirsuta* and *Sinularia nanolobata*. *RSC Adv.* **2015**, *5*, 74256–74262. [CrossRef]
13. Lillsunde, K.E.; Festa, C.; Adel, H.; De Marino, S.; Lombardi, V.; Tilvi, S.; Nawrot, D.A.; Zampella, A.; D'Souza, L.; D'Auria, M.V.; et al. Bioactive cembrane derivatives from the Indian Ocean soft coral, *Sinularia kavarattiensis*. *Mar. Drugs* **2014**, *12*, 4045–4068. [CrossRef] [PubMed]
14. Thao, N.P.; Nam, N.H.; Cuong, N.X.; Quang, T.H.; Tung, P.T.; Dat, L.D.; Chae, D.; Kim, S.; Koh, Y.S.; Kiem, P.V.; et al. Anti-inflammatory norditerpenoids from the soft coral *Sinularia maxima*. *Bioorg. Med. Chem. Lett.* **2013**, *23*, 228–231. [CrossRef] [PubMed]
15. Putra, M.Y.; Ianaro, A.; Panza, E.; Bavestrello, G.; Cerrano, C.; Fattorusso, E.; Taglialatela-Scafati, O. Sinularioside, a triacetylated glycolipid from the Indonesian soft coral *Sinularia* sp. is an inhibitor of NO release. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 2723–2725. [CrossRef] [PubMed]
16. Shih, H.J.; Tseng, Y.J.; Huang, C.Y.; Wen, Z.H.; Dai, C.F.; Sheu, J.H. Cytotoxic and anti-inflammatory diterpenoids from the Dongsha Atoll soft coral *Sinularia flexibilis*. *Tetrahedron* **2012**, *68*, 244–249. [CrossRef]
17. Chao, C.H.; Chou, K.J.; Huang, C.Y.; Wen, Z.H.; Hsu, C.H.; Wu, Y.C.; Dai, C.F.; Sheu, J.H. Bioactive cembranoids from the soft coral *Sinularia crassa*. *Mar. Drugs* **2011**, *9*, 1955–1968. [CrossRef] [PubMed]
18. Lu, Y.; Huang, C.Y.; Lin, Y.F.; Wen, Z.H.; Su, J.H.; Kuo, Y.H.; Chiang, M.Y.; Sheu, J.H. Anti-inflammatory cembranoids from the soft corals *Sinularia querciformis* and *Sinularia granosa*. *J. Nat. Prod.* **2008**, *71*, 1754–1759. [CrossRef] [PubMed]
19. Ou, Y.F.; Xu, S.H.; Yin, P.H.; Peng, C.H.; Liao, L. NMR spectral assignments of two sterols from a soft coral *Sinularia brassica*. *Magn. Reson. Chem.* **2011**, *49*, 46–49. [CrossRef] [PubMed]
20. Huang, C.Y.; Liaw, C.C.; Chen, B.W.; Chen, P.C.; Su, J.H.; Sung, P.J.; Dai, C.F.; Chiang, M.Y.; Sheu, J.H. Withanolide-based steroids from the cultured soft coral *Sinularia brassica*. *J. Nat. Prod.* **2013**, *76*, 1902–1908. [CrossRef] [PubMed]
21. Huang, C.Y.; Ahmed, A.F.; Su, J.H.; Sung, P.J.; Hwang, T.L.; Chiang, P.L.; Dai, C.F.; Liaw, C.C.; Sheu, J.H. Bioactive new withanolides from the cultured soft coral *Sinularia brassica*. *Bioorg. Med. Chem. Lett.* **2017**, *27*, 3267–3271. [CrossRef] [PubMed]

22. Chen, L.X.; He, H.; Qiu, F. Natural withanolides: An overview. *Nat. Prod. Rep.* **2011**, *28*, 705–740. [CrossRef] [PubMed]
23. Yen, C.Y.; Chiu, C.C.; Chang, F.R.; Chen, J.Y.F.; Hwang, C.C.; Hseu, Y.C.; Yang, H.L.; Lee, A.Y.L.; Tsai, M.T.; Guo, Z.L.; et al. 4β-Hydroxywithanolide E from *Physalis peruviana* (golden berry) inhibits growth of human lung cancer cells through DNA damage, apoptosis and G(2)/M arrest. *BMC Cancer* **2010**, *10*, 46. [CrossRef] [PubMed]
24. Ksebati, M.B.; Schmitz, F.J. Minabeolides: A group of withanolides from a soft coral, *Minabea* sp. *J. Org. Chem.* **1988**, *53*, 3926–3929. [CrossRef]
25. Chao, C.H.; Chou, K.J.; Wen, Z.H.; Wang, G.H.; Wu, Y.C.; Dai, C.F.; Sheu, J.H. Paraminabeolides A–F, cytotoxic and anti-inflammatory marine withanolides from the soft coral *Paraminabea acronocephala*. *J. Nat. Prod.* **2011**, *74*, 1132–1141. [CrossRef] [PubMed]
26. Huang, C.Y.; Chang, C.W.; Tseng, Y.J.; Lee, J.; Sung, P.J.; Su, J.H.; Hwang, T.L.; Dai, C.F.; Wang, H.C.; Sheu, J.H. Bioactive steroids from the Formosan soft coral *Umbellulifera petasites*. *Mar. Drugs* **2016**, *14*, 180. [CrossRef] [PubMed]
27. Gardner, H.W.; Kleiman, R. Degradation of linoleic-acid hydroperoxides by a cysteine $FeCl_3$ catalyst as a model for similar biochemical reactions. II. Specificity in formation of fatty-acid epoxides. *Biochim. Biophys. Acta* **1981**, *665*, 113–125. [CrossRef]
28. Voigt, B.; Porzel, A.; Bruhn, C.; Wagner, C.; Merzweiler, K.; Adam, G. Synthesis of 24-epicathasterone and related brassinosteroids with modified side chain. *Tetrahedron* **1997**, *53*, 17039–17054. [CrossRef]
29. Cheng, T.C.; Din, Z.H.; Su, J.H.; Wu, Y.J.; Liu, C.I. Sinulariolide suppresses cell migration and invasion by inhibiting matrix metalloproteinase-2/-9 and urokinase through the PI3K/AKT/mTOR signaling pathway in human bladder cancer cells. *Mar. Drugs* **2017**, *15*, 238. [CrossRef] [PubMed]
30. Tsai, Y.Y.; Huang, C.Y.; Tseng, W.R.; Chiang, P.L.; Hwang, T.L.; Su, J.H.; Sung, P.J.; Dai, C.F.; Sheu, J.H. Klyflaccisteroids K–M, bioactive steroidal derivatives from a soft coral *Klyxum flaccidum*. *Bioorg. Med. Chem. Lett.* **2017**, *27*, 1220–1224. [CrossRef] [PubMed]
31. Tseng, W.R.; Huang, C.Y.; Tsai, Y.Y.; Lin, Y.S.; Hwang, T.L.; Su, J.H.; Sung, P.J.; Dai, C.F.; Sheu, J.H. New cytotoxic and anti-inflammatory steroids from the soft coral *Klyxum flaccidum*. *Bioorg. Med. Chem. Lett.* **2016**, *26*, 3253–3257. [CrossRef] [PubMed]
32. Imperatore, C.; Senese, M.; Aiello, A.; Luciano, P.; Fiorucci, S.; D'Amore, C.; Carino, A.; Menna, M. Phallusiasterol C, a new disulfated steroid from the mediterranean tunicate *Phallusia fumigata*. *Mar. Drugs* **2016**, *14*, 117. [CrossRef] [PubMed]
33. Cheung, R.C.F.; Ng, T.B.; Wong, J.H.; Chen, Y.C.; Chan, W.Y. Marine natural products with anti-inflammatory activity. *Appl. Microbiol. Biot.* **2016**, *100*, 1645–1666. [CrossRef] [PubMed]
34. Nakayama, G.R.; Caton, M.C.; Nova, M.P.; Parondoosh, Z. Assessment of the Alamar Blue assay for cellular growth and viability in vitro. *J. Immunol. Methods* **1997**, *204*, 205–208. [CrossRef]
35. O'Brien, J.; Wilson, I.; Orton, T.; Pognan, F.I. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur. J. Biochem.* **2000**, *267*, 5421–5426. [CrossRef] [PubMed]
36. Yang, S.C.; Chung, P.J.; Ho, C.M.; Kuo, C.Y.; Hung, M.F.; Huang, Y.T.; Chang, W.Y.; Chang, Y.W.; Chan, K.H.; Hwang, T.L. Propofol inhibits superoxide production, elastase release, and chemotaxis in formyl peptide-activated human neutrophils by blocking formyl peptide receptor 1. *J. Immunol.* **2013**, *190*, 6511–6519. [CrossRef] [PubMed]
37. Yu, H.P.; Hsieh, P.W.; Chang, Y.J.; Chung, P.J.; Kuo, L.M.; Hwang, T.L. 2-(2-Fluorobenzamido)benzoate ethyl ester (EFB-1) inhibits superoxide production by human neutrophils and attenuates hemorrhagic shock-induced organ dysfunction in rats. *Free Radic. Biol. Med.* **2011**, *50*, 1737–1748. [CrossRef] [PubMed]