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Article

Steroidal Glycosides from the Soft Coral *Sinularia crassa*

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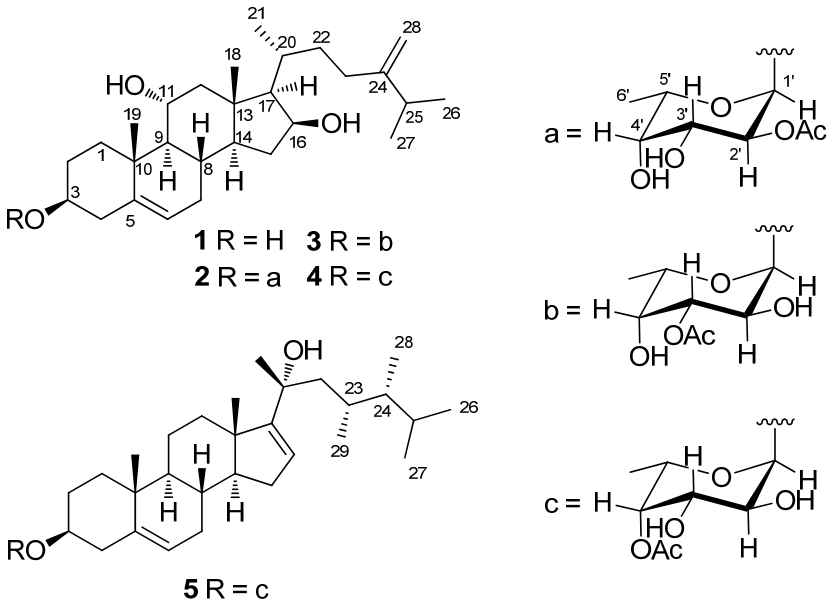
Abstract: One new sterol, crassarosterol A (**1**), and four new steroidal glycosides, crassarosterols B–E (**2**–**5**) were isolated from a Formosan soft coral *Sinularia crassa*. The absolute configuration of **1** was determined using the Mosher's method. The absolute configurations for the sugar moieties of **2**–**5** were determined by HPLC analysis on the *o*-tolylthiocarbamates derived from the liberated sugar after acid hydrolysis. Compounds **2** and **4** could significantly inhibit the expression of pro-inflammatory iNOS protein at 10 μM. Steroids **1** and **4** also showed cytotoxicity toward the selected human liver cancer cells.

Keywords: *Sinularia crassa;* crassarosterols A–E; anti-inflammatory activity; *o*-tolylthiocarbamate

1. Introduction

Soft corals were proven to be a rich source of terpenoids[1]. We previously have isolated a series of bioactive cembrane- [2–4] and norcembrane- [5–8] diterpenoids from the Formosan soft corals of the genus *Sinularia.* Previous chemical investigations on the soft coral *Sinularia crassa* (Tixier-Durivault, 1951) have led to the isolation of structurally unique steroids and cembranoids, of which some have been shown to exhibit anti-inflammatory [9,10] and 5α-reductase inhibitiory activities, [11] respectively. Our continuing chemical investigation on *S. crassa* has led to the isolation of one new sterol, crassarosterol A (**1**), and four new steroidal glycosides, crassarosterols B–E (**2**–**5**) (Figure 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) spectroscopic analysis, chemical methods, and HPLC analysis. The anti-inflammatory activity of **1**–**4** to inhibit up-regulation of the pro-inflammatory iNOS (inducible nitric oxide synthase) and COX-2 (cyclooxygenase-2) proteins in LPS (lipopolysaccharide)-stimulated RAW264.7 macrophage cells and the cytotoxicity of compounds **1**–**5** against a panel of cancer cell lines including human liver carcinoma (HepG2 and HepG3), human breast carcinoma (MCF-7 and MDA-MB-231), and human lung carcinoma (A-549) were evaluated in order to discover bioactive natural products.

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



2. Results and Discussion

The HRESIMS of crassarosterol A (**1**) exhibited a pseudomolecular ion peak at *m/z* 459.2509 $[M+Na]^+$, consistent with a molecular formula of $C_{28}H_{46}O_3$ and requiring six degrees of unsaturation. The ^{13}C NMR and DEPT spectroscopic data (Table 1) displayed 28 carbon signals, including five methyls, nine methylenes, ten methines, and four quaternary carbons. The IR spectrum revealed the presence of hydroxy group (3389 cm^{-1}). The carbon resonances at δ 141.2 (C), 121.3 (CH), 156.9 (C), and 106.3 (CH) suggested the presence of two double bonds. The above data coupled with the characteristic ^{1}H NMR signals for methyl groups at δ 0.92 (3H, s), 1.18 (3H, s), 1.04 (3H, d, J = 6.8 Hz), 1.03 (3H, d, J = 7.2 Hz), and 1.03 (3H, d, J = 7.2 Hz) and signals for olefinic protons at δ_H 5.41

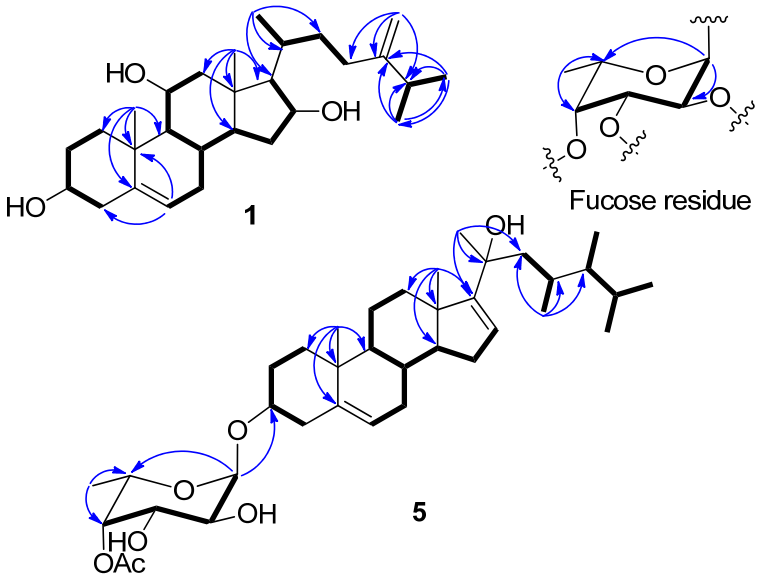
Table 1. ^{13}C NMR sectroscopic data of cmpounds **1**–**5**.

position	**1**[a]	**2**[b]	**3**[b]	**4**[a]	**5**[a]
1	39.1, CH_2	39.2, CH_2	39.2, CH_2	39.2, CH_2	37.1, CH_2
2	31.8, CH_2	29.7, CH_2	29.5, CH_2	29.7, CH_2	29.6, CH_2
3	71.8, CH	78.2, CH	77.8, CH	78.2, CH	78.3, CH
4	42.7, CH_2	39.1, CH_2	39.0, CH_2	39.1, CH_2	38.8, CH_2
5	141.2, qC	140.6, qC	140.7, qC	140.5, qC	140.3, qC
6	121.3, CH	121.7, CH	121.6, CH	121.8, CH	122.1, CH
7	31.9, CH_2	31.8, CH_2	31.8, CH_2	31.8, CH_2	31.5, CH_2
8	31.4, CH	31.3, CH	31.3, CH	31.3, CH	30.4, CH
9	56.9, CH	56.8, CH	56.9, CH	56.8, CH	50.3, CH
10	38.1, qC	38.3, qC	38.3, qC	38.3, qC	36.9, qC
11	68.9, CH	68.9, CH	68.9, CH	68.9, CH	21.0, CH_2
12	51.4, CH_2	51.4, CH_2	51.4, CH_2	51.4, CH_2	36.2, CH_2
13	42.9, qC	42.9, qC	42.9, qC	42.9, qC	47.4, qC
14	53.7, CH	53.6, CH	53.6, CH	53.6, CH	57.9, CH
15	36.3, CH_2	36.3, CH_2	36.3, CH_2	36.3, CH_2	31.0, CH_2
16	72.5, CH	72.5, CH	72.5, CH	72.5, CH	123.8, CH
17	61.2, CH	61.2, CH	61.2, CH	61.2, CH	160.9, CH
18	14.0, CH_3	14.1, CH_3	14.1, CH_3	14.1, CH_3	18.1, CH_3
19	19.1, CH_3	19.0, CH_3	19.0, CH_3	19.0, CH_3	19.3, CH_3
20	29.6, CH	29.6, CH	29.6, CH	29.6, CH	76.0, qC
21	18.2, CH_3	18.2, CH_3	18.2, CH_3	18.2, CH_3	29.6, CH_3
22	34.8, CH_2	34.8, CH_2	34.7, CH_2	34.7, CH_2	49.1, CH_2
23	31.2, CH_2	31.2, CH_2	31.2, CH_2	31.2, CH_2	29.6, CH
24	156.9, qC	156.9, qC	156.9, qC	156.9, qC	45.5, CH
25	34.1, CH	34.0, CH	34.0, CH	34.0, CH	30.9, CH
26	21.8, CH_3	21.8, CH_3	21.8, CH_3	21.8, CH_3	20.9, CH_3
27	21.9, CH_3	21.9, CH_3	21.9, CH_3	21.9, CH_3	21.5, CH_3
28	106.3, CH_2	106.3, CH_2	106.3, CH_2	106.4, CH_2	11.6, CH_3
29					15.7, CH_3
1’		97.4, CH	94.7, CH	97.2, CH	97.2, CH
2’		74.0, CH	68.6, CH	70.1, CH	70.1, CH
3’		66.9, CH	72.1, CH	69.5, CH	69.5, CH
4’		70.9, CH	72.4, CH	73.0, CH	73.0, CH
5’		65.8, CH	65.3, CH	65.2, CH	65.2, CH
6’		16.0, CH_3	16.1, CH_3	16.2, CH_3	16.2, CH_3
OAc		170.9, qC	171.5, qC	171.3, qC	171.3, qC
		21.2, CH_3	21.1, CH_3	20.8, CH_3	20.8, CH_3

[a] Spectra were measured in $CDCl_3$ (100 MHz); [b] Spectra were measured in $CDCl_3$ (125 MHz).

(1H, d, J = 5.6 Hz), 4.76 (1H, s), and 4.70 (1H, s) (Table 2) suggested **1** to be a member of 24-methylenecholesterol class [12,13]. The ^{1}H–^{1}H COSY correlations allowed the assignment of four separated spin systems (Figure 2). The presence of sp^{2} methylene substituent at C-24 was further confirmed by the HMBC correlations from H_2-28 to C-23, C-24, and C-25 (Figure 2). Likewise, the steroidal nucleus was confirmed by the HMBC correlations from H_3-18 to C-12, C-13, C-14, and C-17 and H_3-19 to C-1, C-5, C-9, and C-10. The NOE correlations between H_3-19/H-1β, H-3/H-1α, and H_3-19/H-11 suggested the α and β orientations for H-3 and H-11, respectively. The absence of an NOE correlation between H_3-18/H-17 and the presence of the correlation between H-17/H-16, H-16/H-14, and H-14/H-9 suggested the α orientation for H-9, H-14, H-16, and H-17. Moreover, the β orientation for H-20 was evidenced from the NOE correlations between H_3-18/H-20, H_3-18/H-12a, and H_3-21/H-12a. The absolute configuration of **1** was determined by the application of Mosher's method. Analysis of the ^{1}H NMR data of esters **1a** and **1b** resulted in the determination of a 3*S* configuration (Figure 3).

Figure 2. Selected ^{1}H–^{1}H COSY (▬) and HMBC (→) correlations of **1** and **5** and the fucose residue in **1**–**5**.

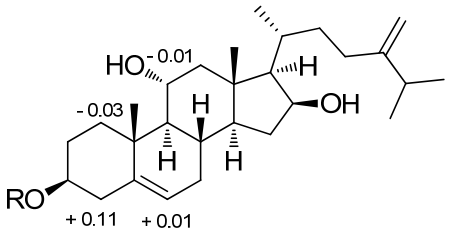


Analysis of the HREIMS and ^{13}C NMR spectroscopic data of crassarosterol B (**2**) suggested a molecular formula of $C_{36}H_{58}O_8$. The IR spectrum of **2** showed the presence of hydroxy (3461 cm^{-1}) and carbonyl (1741 cm^{-1}) groups. The latter was identified as an acetoxy group according to the carbon resonances at δ 170.9 (C) and 21.2 (CH_3) (Table 1). The ^{1}H NMR spectroscopic data of **2** showed characteristic methyl signals at δ 0.92 (3H, s), 1.18 (3H, s), 1.04 (3H, d, J = 6.8 Hz), 1.03 (3H, d, J = 7.2 Hz), 1.03 (3H, d, J = 7.2 Hz), 5.41 (1H, d, J = 5.6 Hz), 4.76 (1H, s), and 4.70 (1H, s) (Table 2), revealing that **2** has the same 24-methylenecholesterol skeleton as that of **1**. By excluding the steroidal moiety and the acetoxy group, the remaining six carbons [δ 97.4 (CH), 74.0 (CH), 66.9 (CH), 70.9 (CH), 65.8 (CH), and 16.0 (CH_3)] were ascribed to the presence of a 6'-deoxyhexose residue. The sugar residue was deduced as an α-fucopyranose on the basis of 2D NMR analysis (Figure 2) and the coupling constants of $^{3}J_{H\text{-}1',H\text{-}2'}$ (4.0 Hz), $^{3}J_{H\text{-}2',H\text{-}3'}$ (10.0 Hz), $^{3}J_{H\text{-}3',H\text{-}4'}$ (3.5 Hz), and $^{3}J_{H\text{-}4',H\text{-}5'}$ (< 1 Hz) (Table 2) [12,14,15]. The acetoxy group attached at C-2' of the α-fucose residue was evidenced from the downfield chemical shift of H-2' (δ 5.07). The HMBC correlation from H-1' to C-3 disclosed that

the α-fucose residue was attached at C-3 of the steroidal aglycone. The absolute configuration of the sugar moiety in **2** was determined by reversed phase HPLC analysis of its *o*-tolylthiocarbamate [16]. The liberated fucose from acid hydrolysis of **2** was treated with L-cysteine methyl ester followed by reaction with *o*-tolylisothiocyanate to afford the corresponding *o*-tolylthiocarbamate derivative. The retention time of the liberated sugar derivative by HPLC analysis was found to be consistent with that of standard L-fucose derivative.

Crassarosterol C (**3**) gave the same molecular formula as that of **2** based on the analysis of the HRESIMS and ^{13}C NMR spectroscopic data (Table 1). The NMR spectroscopic data of **3** were similar to those of **2**, with some exceptions for those of sugar residue. An HMBC correlation from the anomeric proton at δ 5.12 (H-1') to the carbon signal at δ 77.8 (C-3) connected the fucose residue to C-3 of the steroidal aglycone. The downfield proton chemical shift at δ 4.87 (1H, dd, J = 10.5, 4.0 Hz) was ascribed to the presence of an acetoxy group at C-3' (Table 2). The detailed 2D NMR analysis confirmed the above elucidation. Likewise, the L-fucose residue was deduced according to RP HPLC analysis of the corresponding *o*-tolylthiocarbamate as described above.

Figure 3. ^{1}H NMR chemical shift differences of MTPA esters of **1**.



$\triangle\delta = \delta\,(S) - \delta\,(R)$ MTPA ester

1a R = (*S*)-MTPA
1b R = (*R*)-MTPA

Crassarosterol D (**4**) was assigned the same molecular formula as those of **2** and **3**. A comparison of NMR spectroscopic data of **4** with those of **2** and **3** revealed that an acetoxy group should be located at C-4' of fucose residue (Tables 1 and 2). This was evidenced by the ^{1}H NMR shift of H-4' at 5.20 (1H, d, J = 2.8 Hz). In the same manner, RP HPLC anslysis of the corresponding *o*-tolylthiocarbamate derived from the hydrolyte of **4** allowed the determination of L-fucose moiety.

The HRESIMS and ^{13}C NMR spectroscopic data of crassarosterol E (**5**) established a molecular formula of $C_{37}H_{60}O_7$. The presence of an acetoxy group was evidenced by the ^{1}H NMR signal at δ 2.17 (3H, s) (Table 1) and ^{13}C NMR signals at δ 171.3 (C) and 20.8 (CH_3) (Table 2) as well as the IR absorption band at 1737 cm^{-1}. The NMR spectroscopic data appropriate for the sugar moiety of **5** were quite similar to those of **4**, suggesting that they shared the same 4'-*O*-acetylfucose residue. Except for the sugar moiety, the remaining 29 carbon signals as well as the characteristic methyl signals at δ 1.00 (3H, s), 1.05 (3H, s), 1.38 (3H, s), 0.86 (3H, d, J = 6.4 Hz), 0.89 (3H, d, J = 6.4 Hz), 0.76 (3H, d, J = 6.8 Hz), and 0.78 (3H, d, J = 6.4 Hz) (Table 2) revealed that the aglycone of **5** should have a C_{29} steroidal skeleton [17]. The 23,24-dimethyl-20-hydroxy side chain was deduced by the ^{1}H–^{1}H COSY correlations from H_2-22 to H_3-29 through H-23, from H-24 to both H_3-26 and H_3-27, and from H-24 to H_3-28 as well as the HMBC correlations from H_3-21 to C-17, C-20, and C-22 and H_3-29 to C-22, C-23, and C-24. This rare steroidal side chain is the same as that of sarcophytosterol isolated previously by

Table 2. ^{1}H NMR Spectroscopic Data of Compounds **1**–**5**.

#	**1**, δ_H (J in Hz)[a]	**2**, δ_H (J in Hz)[b]	**3**, δ_H (J in Hz)[b]	**4**, δ_H (J in Hz)[a]	**5**, δ_H (J in Hz)[a]
1	a: 2.55, dt (13.6, 3.6)	a: 2.58, dt (13.5, 3.5)	a: 2.56, dt (13.5, 3.5)	a: 2.58, dt (14.0, 3.6)	a: 1.86, m
	b: 1.16, m	b: 1.16, m	b: 1.16, m	b: 1.16, m	b: 1.10, m
2	a: 1.81, m	a: 1.85, m	a: 1.79, m	a: 1.81, m	a: 1.89, m
	b: 1.58, m	b: 1.65, m	b: 1.66, m	b: 1.64, m	b: 1.60, m
3	3.53, m	3.51, m	3.43, m	3.49, m	3.49, m
4	a: 2.30, m	a: 2.36, m	2.24, m	a: 2.36, m	a: 2.36, m
	b: 2.26, m	b: 2.26, m		b: 2.26, m	b: 2.24, m
6	5.41, d (5.6)	5.41, d (5.5)	5.40, d (5.5)	5.41, d (5.6)	5.38, br d (3.2)
7	a: 1.99, m	a: 1.99, m	a: 1.98, m	a: 1.99, m	a: 2.01, m
	b: 1.54, m	b: 1.56, m	b: 1.54, m	b: 1.54, m	b: 1.61, m
8	1.50, m	1.50, m	1.49, m	1.50, m	1.66, m
9	0.99, m	0.99, m	0.97, m	0.98, m	1.01, m
11	4.07, td (10.8, 4.8)	4.07, m	4.06, m	4.07, td (10.8, 4.4)	1.59, m
12	a: 2.31, m	a: 2.31, m	a: 2.31, m	a: 2.31, m	a: 2.10, m
	b: 1.18, m	b: 1.18, m	b: 1.18, m	b: 1.18, m	b: 1.59, m
14	0.98, m	0.98, m	0.98, m	0.98, m	1.41, m
15	a: 2.24, m	a: 2.24, m	a: 2.23, m	a: 2.23, m	a: 2.08, m
	b: 1.17, m	b: 1.16, m	b: 1.17, m	b: 1.16, m	b: 1.87, m
16	4.40, m	4.40, m	4.40, m	4.40, m	5.50, br s
17	1.07, m	1.07, m	1.07, m	1.07, m	
18	0.92, s	0.92, s	0.92, s	0.92, s	1.00, s
19	1.18, s	1.18, s	1.17, s	1.18, s	1.05, s
20	1.86, m	1.86, m	1.86, m	1.87, m	
21	1.04, d (6.8)	1.04, d (6.8)	1.04, d (6.5)	1.04, d (6.4)	1.38, s
22	a: 1.68, m	a: 1.68, m	a: 1.68, m	a: 1.67, m	a: 1.59, m
	b: 1.22, m	b: 1.22, m	b: 1.22, m	b: 1.22, m	b: 1.48, m
23	a: 2.18, m	a: 2.18, m	a: 2.18, m	a: 2.18, m	1.82, m
	b: 1.95, m	b: 1.95, m	b: 1.95, m	b: 1.95, m	
24					1.06, m
25	2.24, m	2.25, m	2.25, m	2.25, m	1.42, m
26	1.03, d (7.2)	1.03, d (7.0)	1.03, d (7.0)	1.03, d (6.8)	0.86, d (6.4)
27	1.03, d (7.2)	1.03, d (7.0)	1.03, d (7.0)	1.03, d (6.8)	0.89, d (6.4)
28	a: 4.76, s	a: 4.76, s	a: 4.76, s	a: 4.76, s	0.76, d (6.8)
	b: 4.70, s	b: 4.70, s	b: 4.70, s	b: 4.70, s	
29					0.78, d (7.2)
1’		5.02, d (4.0)	5.12, d (4.0)	5.04, d (4.0)	5.04, d (4.0)
2’		5.07, dd (10.0, 4.0)	4.02, m	3.93, dd (10.0, 4.0)	3.93, dd (10.0, 4.0)

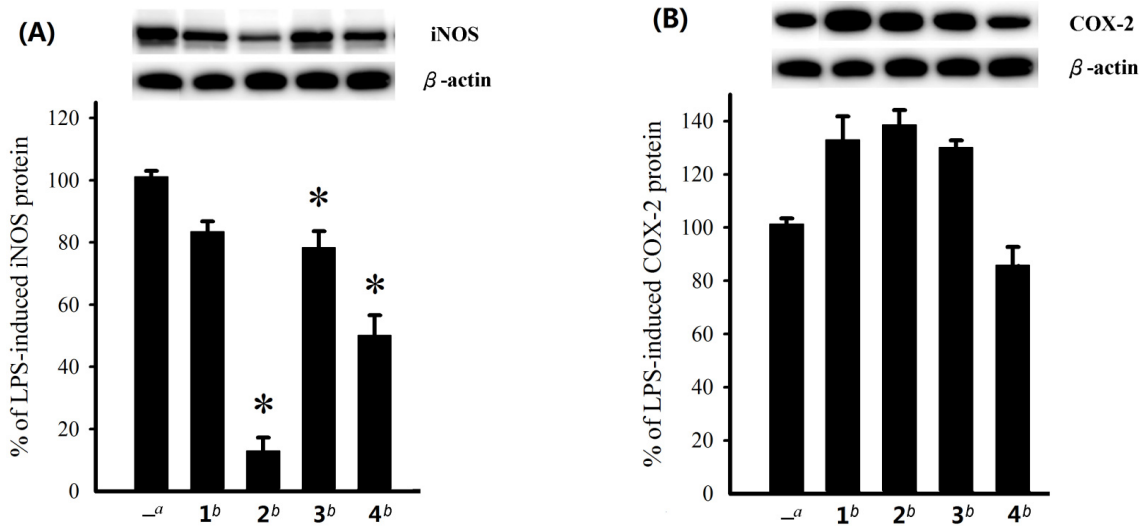
3’	3.91, ddd (11.5,10.0, 3.5)	4.87, dd (10.5, 4.0)	3.74, br d (10.0)	3.74, dd (10.0, 2.4)
4’	3.84, br s	3.82, br s	5.20, d (2.8)	5.21, d (2.4)
5’	4.11, q (7.0)	4.12, q (6.5)	4.12, q (6.4)	4.12, q (6.4)
6’	1.25, d (7.0)	1.27, d (6.5)	1.13, d (6.4)	1.14, d (6.4)
OAc	2.18, s	2.15, s	2.17, s	2.17, s
3’-OH	1.90, d (11.5)			
4’-OH	1.89, br s	2.18, br s		

[a] Spectra were measured in $CDCl_3$ (400 MHz); [b] Spectra were measured in $CDCl_3$ (500 MHz).

us from the soft coral *Lobophytum sarcophytoides* [17]. The NMR spectroscopic data for the aglycone moiety of **5** are almost the same as those of sarcophytosterol, except for some minor variations in ^{1}H and ^{13}C chemical shifts at C-2, C-3, and C-4 between both compounds. This is due to the attachment of the sugar residue at C-3 of the steroidal aglycone. Similarly, HPLC analysis of the relevant *o*-tolylthiocarbamate derived from the hydrolysis of **5** suggested the presence of L-fucose.

The absolute configuration of sterol **1** has been established by Mosher’s method in the present work. On the basis of biogenesis, the steroidal moieties of the glycosides **2**–**5** should possess the same absolute configurations as shown in the formulae. Cytotoxicity of steroids **1**–**5** against HepG2, HepG3, MCF-7, MDA-MB-231, and A-549 cancer cell lines was evaluated. The results showed that **1** exhibited cytotoxicity toward HepG2 cancer cell line with an IC_{50} value of 14.9 μM, while **4** also showed cytotoxicity toward HepG2 and HepG3 cell lines with IC_{50} values of 17.6 and 18.9 μM, respectively. The other compounds were found to be inactive (IC_{50} > 20 μM) toward the above cancer cell lines after 72 h exposure. The anti-inflammatory activity of steroids **1**–**4** against the accumulation of pro-inflammatory iNOS and COX-2 proteins in RAW264.7 macrophage cells stimulated with LPS was also evaluated using immunoblot analysis. At a concentration of 10 μM (Figure 4), steroid **2** was found to significantly reduce the level of iNOS protein to 12.9 ± 4.3% and **4** could reduce the iNOS espression to 50.1 ± 6.3%.

Figure 4. Effect of compounds **1**–**4** at 10 μM on the LPS-induced pro-inflammatory iNOS and on COX-2 protein expression of RAW264.7 macrophage cells by immunoblot analysis. (A) Immunoblots for iNOS and *β*-actin, and relative density of iNOS. (B) Immunoblots for COX-2 and *β*-actin, and relative density of COX-2. The values are means ± SEM (n = 6). The relative intensity of the LPS alone stimulated group was taken as 100%. Under the same experimental condition, 10 μM CAPE (caffeic acid phenethyl ester; Sigma Chemical. Company, St. Louis, MO) reduced the levels of the iNOS and COX-2 protein to 0.8 ± 4.5 % and 75.6 ± 12.2 %, respectively, relative to the control cells stimulated with LPS. *Significantly different from LPS alone stimulated group (*P < 0.05).



3. Experimental Section

3.1. General Experimental Procedures

The melting point was determined using a Fisher-Johns melting point apparatus. Optical rotations were determined with a JASCO P1020 digital polarimeter. IR spectrum was obtained on a JASCO FT/IR-4100 spectrophotometer. The NMR spectra were recorded on a Varian 400 MR NMR or Varian Unity INOVA 500 FT-NMR instrument at 400 or 500 MHz for ^{1}H (referenced to TMS, δ_H 0.00 ppm for $CDCl_3$) and 100 or 125 MHz for ^{13}C (referenced to δ_C 77.0 for $CDCl_3$). ESIMS were recorded by ESI FT-MS on a Bruker APEX II mass spectrometer. Silica gel 60 (Merck, 230–400 mesh) and LiChroprep RP-18 (Merck, 40–63 μm) were used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60 F_{254}, 0.25 mm) and precoated RP-18 F_{254S} plates (Merck, 1.05560) were used for TLC analyses. High-performance liquid chromatography (HPLC) 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 (Merck, Hibar Purospher RP-18e, 5 μm, 250 × 10 mm).

3.2. Animal Material

The soft coral *Sinularia crassa* was collected by hand using scuba off the coast of Sansiantai, Taitung county, Taiwan, in July 2008, at a depth of 10 m, and was stored in a freezer. This soft coral was identified by one of the authors (C.-F. D.). A voucher specimen (specimen no. SST-03) was deposited in the Department of Marine Biotechnology and Resources, National Sun Yat-sen University.

3.3. Extraction and Isolation

The frozen bodies of *S. crassa* (1.1 kg fresh wt) were minced and extracted with EtOH (3 × 2 L). The organic extract was concentrated to an aqueous suspension and was further partitioned between EtOAc and H_2O. The EtOAc extract (17.0 g) was fractionated by open column chromatography on silica gel using *n*-hexane–EtOAc and EtOAc–MeOH mixtures of increasing polarity to yield 32 fractions. Fractions 25, eluting with EtOAc–MeOH (8:1), was further separated by silica gel column chromatography with gradient elution (*n*-hexane–acetone, 8:1 to 2:1) to yield four subfractions (25A–

25D). Subfraction 25B was subjected to RP-18 column chromatography (MeOH–H_2O, gradient, 50–90%), and subsequently purified by RP-18 HPLC (CH_3CN–H_2O, 65%) to obtain compounds **1** (6.6 mg) and **5** (1.2 mg). Compounds **4** (1.8 mg) was obtained from subfraction 25C using RP-18 HPLC (CH_3CN–H_2O, 65%). In the same manner, Subfraction 25D was fractionated over RP-18 gel using gradient elution (MeOH–H_2O, gradient, 50–90%) to yield two subfractions (25D-1 and 25D-2). Subfraction 25D-2 was separated by RP-18 HPLC (CH_3CN–H_2O, 85%) to yield compounds **2** (1.8 mg) and **3** (1.6 mg).

Crassarosterol A (**1**): white powder; $[\alpha]^{24}_D$ –45 (*c* 0.66, $CHCl_3$); IR (KBr) ν_{max} 3389, 2962, 2925, 2854, 1462, 1048, 1024 cm^{-1}; ^{13}C NMR and ^1H NMR data, see Tables 1 and 2; ESIMS *m/z* 453 $[M+Na]^+$; HRESIMS *m/z* 453.3342 $[M+Na]^+$ (calcd for $C_{28}H_{46}O_3Na$, 453.3344).

Crassarosterol B (**2**): white powder; $[\alpha]^{24}_D$ –34 (*c* 0.18, $CHCl_3$); IR (KBr) ν_{max} 3461, 2960, 2928, 2868, 1741, 1467, 1377, 1244, 1077, 1030 cm^{-1}; ^{13}C NMR and ^1H NMR data, see Tables 1 and 2; ESIMS *m/z* 641 $[M+Na]^+$; HRESIMS *m/z* 641.4027 $[M+Na]^+$ (calcd for $C_{36}H_{58}O_8Na$, 641.4029).

Crassarosterol C (**3**): white powder; $[\alpha]^{24}_D$ –17 (*c* 0.16, $CHCl_3$); IR (KBr) ν_{max} 3388, 2963, 2930, 2857, 1732, 1458, 1375, 1258, 1041 cm^{-1}; ^{13}C NMR and ^1H NMR data, see Tables 1 and 2; ESIMS *m/z* 641 $[M+Na]^+$; HRESIMS *m/z* 641.4026 $[M+Na]^+$ (calcd for $C_{36}H_{58}O_8Na$, 641.4029).

Crassarosterol D (**4**): white powder; $[\alpha]^{24}_D$ –52 (*c* 0.18, $CHCl_3$); IR (KBr) ν_{max} 3426, 2960, 2930, 2859, 1735, 1461, 1375, 1247, 1074, 1033 cm^{-1}; ^{13}C NMR and ^1H NMR data, see Tables 1 and 2; ESIMS *m/z* 641 $[M+Na]^+$; HRESIMS *m/z* 641.4026 $[M+Na]^+$ (calcd for $C_{36}H_{58}O_8Na$, 641.4029).

Crassarosterol E (**5**): white powder; $[\alpha]^{24}_D$ –45 (*c* 0.12, $CHCl_3$); IR (KBr) ν_{max} 3440, 2960, 2925, 2855, 1737, 1461, 1377, 1244, 1074, 1036 cm^{-1}; ^{13}C NMR and ^1H NMR data, see Tables 1 and 2; ESIMS *m/z* 639 $[M+Na]^+$; HRESIMS *m/z* 639.4234 $[M+Na]^+$ (calcd for $C_{37}H_{60}O_7Na$, 639.4237).

*3.4. Preparation of (S)-and (R)-MTPA Esters of **1***

To a solution of **1** (1.0 mg) in pyridine (0.4 mL) was added (*R*)-MTPA chloride (25 μL), and the mixture was allowed to stand for 3 h at room temperature. The reaction was quenched by the addition of 1.0 mL of H_2O, and the mixture was subsequently extracted with EtOAc (3 × 1.0 mL). The EtOAc layers were combined, dried over anhydrous $MgSO_4$, and evaporated. The residue was subjected to short silica gel column chromatography using *n*-hexane−EtOAc (3:1) to yield the (*S*)-MTPA ester, **1a** (0.7 mg). The same procedure was used to prepare the (*R*)-MTPA ester, **1b** (1.0 mg from 1.0 mg of **1**), with (*S*)-MTPA chloride. Selected ^1H NMR ($CDCl_3$, 400 MHz) of **1a**: δ 7.41−7.52 (5H, m, Ph), 5.48 (1H, br d, *J* = 6.0 Hz, H-6), 4.89 (1H, m, H-3), 4.76 (1H, s, H-28a), 4.70 (1H, s, H-28b), 4.41 (1H, m, H-16), 4.05 (1H, m, H-11), 3.57 (3H, s, O*Me*), 2.62 (1H, br d, *J* = 14.0 Hz, H-1a), 2.48 (1H, m, H-4a), 1.85 (1H, m, H-2a), 1.17 (3H, s, H_3-19), 1.03 (6H, d, J = 7.2 Hz, H_3-26 and 27), 0.92 (3H, s, H_3-18); selected ^1H NMR ($CDCl_3$, 400 MHz) of **1b**: δ 7.41−7.53 (5H, m, Ph), 5.47 (1H, br d, *J* = 5.2 Hz, H-6), 4.89 (1H, m, H-3), 4.76 (1H, s, H-28a), 4.70 (1H, s, H-28b), 4.41 (1H, m, H-16), 4.06 (1H, m, H-11), 3.57 (3H, s, O*Me*), 2.65 (1H, br d, *J* = 13.6 Hz, H-1a), 2.37 (1H, m, H-4a), 1.77 (1H, m, H-2a), 1.17 (3H, s, H_3-19), 1.03 (6H, d, J = 7.2 Hz, H_3-26 and 27), 0.92 (3H, s, H_3-18).

3.5. Determination of Sugar Configuration

Authentic samples of D-fucose and L-cysteine methyl ester hydrochloride (each 0.5 mg) were dissolved in pyridine (0.1 mL) and heated at 60 °C for 1 h. The mixture was added *o*-tolylisothiocyanate (0.5 mg in 0.1 mL pyridine) and heated at 60 °C for additional 1 h. The reaction mixture was directly analyzed by reversed-phase HPLC (Mightysil RP-18 GP column; 4.6 × 250 nm; 25% CH_3CN in 50 mM H_3PO_4; 0.8 mL/min; 35°C) and detected at 250 nm to give the retention time of the *o*-tolylthiocarbamate of sugar. The retention of the *o*-tolylthiocarbamate derived from L-fucose, L-cysteine methyl ester, and *o*-tolylisothiocyanate was obtained by the same manner.

A solution of the glycoside (0.4 mg for each) in 0.6 M HCl/dioxane (1:1 v/v, 0.2 mL) was heated at 80 °C for 24 h. After cooling, the solution was neutralized with Amberlite IRA-400, and the resin was removed by filtration. The filtrate was extracted with EtOAc. The aqueous layer was dried *in vacuo* and the afforded residue was dissolved in pyridine (0.1 mL) containing L-cysteine methyl ester (0.5 mg), followed by heating at 60 °C for 1 h. A 0.1 mL solution of *o*-tolylisothiocyanate (0.5 mg) in pyridine was added to the mixture, which was heated at 60 °C for additional 1 h, to yield the corresponding *o*-tolylthiocarbamate derivative. Reverse phase HPLC analysis of the *o*-tolylthiocarbamate derivatives derived from the hydrolyte of the glycosides **2**–**5** showed peaks at 28.2, 28.0, 28.1, and 27.9 min, respectively, while the t_R values for standard L-fucose and D-fucose derivatives were observed at 28.0 and 25.4 min, respectively, suggesting the presence of a L-fucose residue in **2**–**5**.

3.6. 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 MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method [18]. Freshly trypsinized cell suspensions were seeded in 96-well microtiter plates at densities of 5000–10000 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 IC_{50} is the concentration of agent that reduced cell growth by 50% under the experimental conditions.

3.7. In Vitro Anti-Inflammatory Assay

Macrophage (RAW264.7) cell line 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 [19,20].

4. Conclusions

Prior investigation of the genus sinularia reported some steroidal glycosides; however, all of them were found to possess a 24-methylene substituted side chain [21,22]. **5** is the first example of steroidal glycoside with a 23,24-dimethyl substitited side chain from soft coral of the genus *sinularia*. Our biological data revealed that 2′-O-acetyl–L-fucose functionality play an important role toward the

inhibition of the pro-inflammatory iNOS. Steroidal glycoside **2** could be useful anti-inflammatory agents, while steroids **1** and **4** have shown their inhibitory activity toward the selected human liver cancer cells.

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