Cembranoids from the Soft Corals *Sinularia granosa* and *Sinularia querciformis*

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Two new cembranoids, namely granosolides C (1) and D (2), along with one known cembranoid 4, were isolated from the soft coral *Sinularia granosa*. Chemical investigation of *Sinularia querciformis* also afforded one new cembranoid, querciformolide E (3), along with four known cembranoids 4—7. The structures of these compounds were elucidated on the basis of their spectroscopic data. Both 4 and 5 were shown to significantly inhibit the accumulation of the pro-inflammatory inducible nitric oxide synthase protein in lipopolysaccharide-stimulated RAW264.7 macrophage cells.

Key words cembranoid; soft coral; anti-inflammatory activity; *Sinularia granosa*

During the course of our search for bioactive metabolites from marine invertebrates inhabiting Taiwanese waters, several cembrene-type diterpenoids[1—3] and norcembranoid-based metabolites[4—6] have been isolated from soft corals of the genus *Sinularia*. We recently reported six new cembranoids querciformolides A—D and granosolides A and B, along with two known metabolites from the soft coral *Sinularia querciformis* and *S. granosa*.[7] In continuation of our search for bioactive metabolites from the above two soft corals, we have further isolated eight metabolites including three new cembranoids, granosolides C and D (**1**, **2**) and querciformolide E (**3**), along with four known metabolites, flexibilisolide A (**4**),[8] flexilarin (**5**),[9] sinulariolide (**6**)[10] and sinulaflexiolide E (**7**).[11] The structures of compounds **1**—**3** have been established by extensive spectroscopic analysis, including 2D NMR (^{1}H–^{1}H correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond connectivity (HMBC), and nuclear Overhauser effect spectroscopy (NOESY)) experiments. The cytotoxicity of compounds **1**—**7** against human medulloblastoma (Daoy), human breast carcinoma (MCF-7), human cervical epitheloid (HeLa), and human laryngeal (HEp 2) carcinoma cells was studied, and the ability of **1**—**7** 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 was also evaluated.

Granosolide C (**1**) exhibited a $[M+Na]^+$ peak at *m/z* 447.2357 (Calcd for $C_{23}H_{36}O_7Na$ 447.2359) and established the molecular formula $C_{23}H_{36}O_6$ in the HR-electrospray ionization (ESI)-MS. Thus, **1** has six degrees of unsaturation. The IR spectrum showed the presence of hydroxy (3460 cm^{-1}) and carbonyl (1716 cm^{-1}) groups. Furthermore, the similar ^{1}H- and ^{13}C-NMR spectroscopic data (Tables 1, 2) of **1** and **4** revealed that both compounds have the similar 14-membered rings. However, an additional methoxy group (δ_C 52.4, CH_3; δ_H 3.76, s) was observed in **1**. In addition, the methoxy group positioned at C-16 was confirmed by the HMBC correlation between the methoxy protons (δ_H 3.76) and the carbonyl carbon (δ_C 165.9, qC, C-16). In order to confirm the structure, including the stereochemistry of **1**, a base-catalyzed hydrolysis of **4** was performed and the reaction was found to afford **1**.[8] Thus, the relative structure of **1** was established.

Granosolide D (**2**) showed a pseudomolecular ion peak $[M+Na]^+$ at *m/z* 431.2406 in the HR-ESI-MS, corresponding to the molecular formula $C_{23}H_{36}O_6$ and six degrees of unsaturation. The complete assignment of the ^{1}H- and ^{13}C-NMR spectroscopic data (Tables 1, 2) for compound **2** was achieved by a combination of distortionless enhancement by polarization transfer (DEPT), COSY, HMBC, and HMQC data (Fig. 1). Analysis of the ^{1}H-NMR spectrum suggested that one of the four methyl groups is due to an acetoxy group resonating at δ_H 2.13 (s). The planar structure of **2** was proposed by the assistance of extensive 2D NMR study (Fig. 1). In order to confirm the structure, including the stereochemistry of **2**, a base-catalyzed hydrolysis of **6** was performed and the reaction was found to afford a known compound flexibilisin B (**8**).[8] We further observed that acetylation of **8**

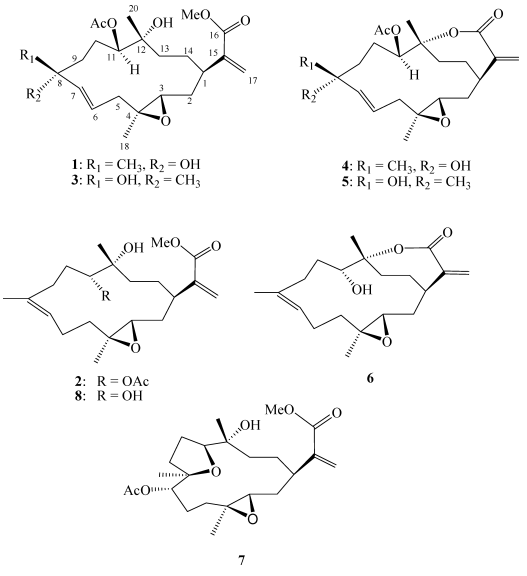

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Table 1. ^{1}H-NMR Data for Compounds **1**—**3**

	1[a)]	**2**[b)]	**3**[b)]
1	2.84 m	2.69 m	2.90 m
2	2.01 m; 1.42 m	1.84 m; 1.56 m	2.04m; 1.37 m
3	2.84 m	2.93 dd (8.0, 5.0)	2.95 dd (10.0, 4.0)
5	2.61 dd (14.4, 6.8)[c)] 2.18 dd (14.4, 8.0)	2.02 m	2.48 d (7.0)
6	5.62 ddd (16.0, 8.0, 6.8)	1.43 m	5.50 dt (15.5, 7.0)
7	5.42 d (16.0)	5.14 dd (7.0, 7.0)	5.46 d (15.5)
9	1.52 m	2.15 m; 1.78 m	1.70 m; 1.31 m
10	1.83 m; 1.64 m	1.81 m	1.54 m; 1.48 m
11	4.95 d (10.0)	5.05 d (8.0)	4.86 d (10.0)
13	1.46 m; 1.43 m	1.53 m	1.50 m; 1.34 m
14	1.52 m	1.70 m; 1.53 m	1.81 m; 1.50 m
17	6.28 s; 5.53 s	6.29 s; 5.60 s	6.30 s; 5.54 s
18	1.40 s	1.31 s	1.35 s
19	1.26 s	1.65 s	1.28 s
20	1.14 s	1.14 s	1.15 s
OMe	3.76 s	3.76 s	3.76 s
OAc	2.11 s	2.13 s	2.11 s

a) Spectra recorded at 400 MHz in $CDCl_3$. *b*) Spectra recorded at 500 MHz in $CDCl_3$. *c*) *J* values (Hz) in parentheses.

Table 2. ^{13}C-NMR Data for Compounds **1**—**3**

	1[a)]	**2**[b)]	**3**[b)]
1	37.1 (CH)[c)]	38.2 (CH)	35.4 (CH)
2	31.6 (CH_2)	34.2 (CH_2)	29.3 (CH_2)
3	59.8 (CH)	60.9 (CH)	58.8 (CH)
4	61.4 (C)	59.7 (C)	61.2 (C)
5	42.1 (CH_2)	37.7 (CH_2)	39.7 (CH_2)
6	123.6 (CH)	23.2 (CH_2)	123.3 (CH)
7	138.3 (CH)	123.8 (CH)	140.1 (CH)
8	73.5 (C)	135.4 (C)	73.3 (C)
9	38.3 (CH_2)	33.6 (CH_2)	38.0 (CH_2)
10	24.7 (CH_2)	26.4 (CH_2)	24.4 (CH_2)
11	79.3 (CH)	76.3 (CH)	79.8 (CH)
12	74.2 (C)	74.3 (C)	74.3 (C)
13	36.1 (CH_2)	37.2 (CH_2)	34.4 (CH_2)
14	26.1 (CH_2)	26.2 (CH_2)	23.2 (CH_2)
15	141.4 (C)	143.1 (C)	142.1 (C)
16	165.9 (C)	167.4 (C)	167.4 (C)
17	123.7 (CH_2)	124.5 (CH_2)	124.6 (CH_2)
18	18.7 (CH_3)	17.0 (CH_3)	18.3 (CH_3)
19	28.9 (CH_3)	17.8 (CH_3)	30.2 (CH_3)
20	24.2 (CH_3)	25.1 (CH_3)	23.3 (CH_3)
OMe	52.4 (CH_3)	52.0 (CH_3)	52.0 (CH_3)
OAc	22.0 (CH_3)	21.0 (CH_3)	21.1 (CH_3)
	170.6 (C)	170.7 (C)	172.1 (C)

a) Spectra recorded at 100 MHz in $CDCl_3$. *b*) Spectra recorded at 125 MHz in $CDCl_3$. *c*) Deduced from DEPT.

gave a product which was found to be identical with **2** by comparison of the physical and spectroscopic data. Thus, the relative structure of **2** was determined.

Querciformolide E (**3**) was isolated as a white powder and showed a $[M+Na]^+$ ion peak in the HR-ESI-MS corresponding to the molecular formula $C_{23}H_{36}O_7$, the same as that of **1**. Furthermore, it was found that the NMR data of **3** were very similar to those of **1** (Tables 1, 2). By analysis of 2D NMR (^{1}H–^{1}H COSY, HMQC, and HMBC) correlations, compound **3** was shown to possess the same molecular framework as that of **1**. The *J* values for both H-6 and H-7 (15.5 Hz) further confirmed the *E*-configuration of the 6,7-double bond. The relative configurations of the six chiral centers at C-1, C-3, C-4, C-8, C-11, and C-12 in **3** were elucidated by detailed

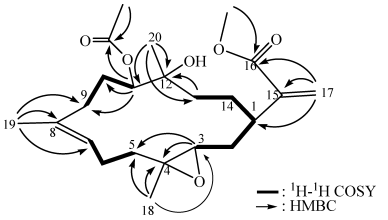

Fig. 1. Key ^{1}H–^{1}H COSY and HMBC Correlations for **2**

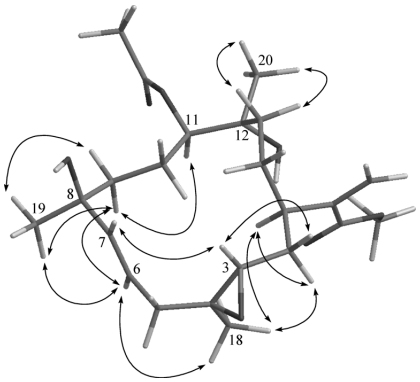

Fig. 2. Selective NOESY Correlations of **3**

analysis of NOE correlations, as shown in Fig. 2. It was found that H-1 (δ_H 2.90, m) showed an NOE interaction with H_3-18 (δ_H 1.35, s), and H-6 (δ_H 5.50, dd, J=15.5, 7.0 Hz) showed NOE interactions with all of H-9α (δ_H 1.31, m), H_3-18, and H_3-19 (δ 1.28, s), and H-9α also showed NOE interactions with H_3-19 and H-11 (δ_H 4.86, d, J=10.0 Hz), while H-7 (δ_H 5.46, d, J=15.5 Hz) was NOE correlated with H-3 (δ_H 2.95, dd, J=10.0, 4.0 Hz). Therefore, H-1, H_3-18, H_3-19, and H-11 are situated on the α-face, and in contrast H-3 and the acetoxy group at C-11 should be positioned on the β-face. Furthermore, the NOE interactions found between H_3-20 and both H-13a and H-13b, but not between H_3-20 and H-11, assigned the α-orientation of the hydroxy group of C-12. From the above observations and further analysis of other NOE interactions (Fig. 2), the 1*R**, 3*S**, 4*S** and 8*S** relative configurations of **3** were established.

In order to explore the biological activities of the isolated compounds, the cytotoxicity and anti-inflammatory activities of these compounds were evaluated. The cytotoxicity of compounds **1**—**7** was tested against the proliferation of a limited panel of cancer cell lines, including Daoy, MCF-7, HeLa and HEp2 cells. The results showed that all of the compounds were not cytotoxic toward the above cancer cells (IC_{50}'s $>20\,\mu$g/ml). The inhibiting up-regulation of pro-inflammatory iNOS and COX-2 proteins of LPS-stimulated RAW264.7 macrophage cells was further evaluated using immunoblot analysis. At a concentration of 10 μM, both **4** and **5** were found to significantly reduce the levels of iNOS protein to 19.4±4.5% and 13.8±2.1%, respectively, relative to the control cells stimulated with LPS only. At the same concentration, metabolites **1**—**7** did not inhibit the COX-2 expression by LPS treatment (Fig. 3).

Experimental

Melting points were determined using a Fisher-Johns melting point apparatus and were uncorrected. Optical rotations were measured on a JASCO DIP-1000 digital polarimeter. UV spectra were recorded on a JASCO V650

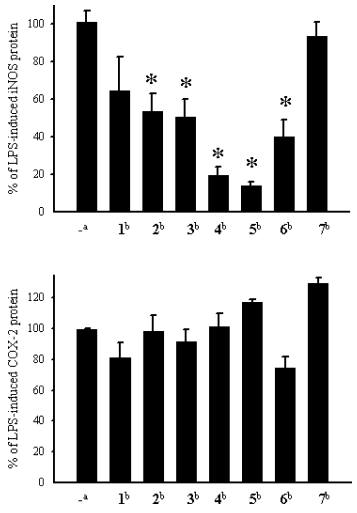

Fig. 3. Effect of Compounds **1**—**7** on iNOS and COX-2 Proteins Expression of RAW264.7 Macrophage Cells by Immunoblot Analysis

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. *b*) Stimulated with LPS in the presence of **1**—**7** (10 μM).

spectrophotometer. IR spectra were recorded on a JASCO FT/IR-4100 infrared spectrophotometer. ESI-MS were obtained with a Bruker APEX II mass spectrometer. The NMR spectra were recorded on a Varian Unity INOVA 500 FT-NMR at 500 MHz for ^{1}H and 125 MHz for ^{13}C or on a Varian 400 MR FT-NMR at 400 MHz for ^{1}H and 100 MHz for ^{13}C, respectively, in $CDCl_3$ using TMS as an internal standard. 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 was performed on a Hitachi L-7100 HPLC apparatus with a Merck Hibar Si-60 column (250×21 mm, 7 μm).

Animal Material *Sinularia querciformis* (specimen no. 20040112-7) and *S. granosa* (specimen no. 20040112-2) were collected by hand by scuba diving off the coast of Pingtung, located on the southernmost tip of Taiwan in January, 2004, at a depth of 5—10 m, and stored in a freezer until extraction. Two voucher samples were deposited at the Department of Marine Biotechnology and Resources, National Sun Yat-sen University.

Extraction and Separation The sliced tissues of the soft coral *Sinularia granosa* (0.8 kg, wet wt) were exhaustively extracted with EtOH (1 l×5). The combined EtOH extract was filtered and concentrated under reduced pressure. The residue was partitioned between EtOAc and H_2O. The EtOAc extract (8.7 g) was chromatographed over silica gel by column chromatography and eluted with EtOAc in *n*-hexane (0—100%, stepwise) then with MeOH in EtOAc (5—50%, stepwise) to yield 26 fractions. Fraction 17, eluted with *n*-hexane–EtOAc (1 : 1), was further separated by normal phase HPLC using *n*-hexane–acetone (6 : 1) to yield **2** (1.8 mg). Fraction 19, eluted with *n*-hexane–EtOAc (1 : 2), was further purified by Si gel column chromatography using *n*-hexane–acetone (6 : 1) to afford **1** (3.0 mg) and **4** (8.9 mg).

Sliced tissues of the soft coral *Sinularia querciformis* (0.9 kg, wet wt) were exhaustively extracted with EtOH (1 l×6). The combined EtOH extract was filtered and concentrated under reduced pressure. The residue was partitioned between EtOAc and H_2O, and the EtOAc extract (9.4 g) subjected to column chromatography on silica gel and eluted with EtOAc in *n*-hexane (0—100%, stepwise) to yield 15 fractions. Fraction 9, eluted with *n*-hexane–EtOAc (1 : 1), was further purified over silica gel using *n*-hexane–acetone (4 : 1) to afford 3 subfractions. Subfraction 2 was also separated by normal phase HPLC using *n*-hexane–acetone (7 : 1) to afford **6** (32.3 mg) and **7** (22.2 mg). Fraction 10, eluted with *n*-hexane–EtOAc (1 : 2), was purified on a silica gel column using *n*-hexane–acetone (2 : 1) followed by normal phase HPLC, using *n*-hexane–acetone (4 : 1) to afford **4** (11.4 mg) and **5** (5.1 mg). Fraction 11, eluted with *n*-hexane–EtOAc (1 : 3), was rechromatographed on a silica gel column using *n*-hexane–acetone (1 : 1) followed by purification by normal phase HPLC, using *n*-hexane–acetone (2 : 1) to afford **3** (1.8 mg).

Granosolide C (**1**): Colorless oil; $[\alpha]_D^{25}$ −11.8 (c=0.53, $CHCl_3$); IR (neat) ν_{max} 3460, 2968, 2929, 2859, 1716, 1626, 1440, 1375 and 1235 cm^{-1}; UV (MeOH) λ_{max} 216 (log ε=3.8); ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz), see Tables 1 and 2; ESI-MS *m/z*: 447 $[M+Na]^+$; HR-ESI-MS *m/z*: 447.2357 $[M+Na]^+$ (Calcd for $C_{23}H_{32}O_6Na$, 447.2359).

Granosolide D (**2**): Colorless oil; $[\alpha]_D^{25}$ −101.7 (c=0.18, $CHCl_3$); IR (neat) ν_{max} 3440, 2966, 1721, 1376 and 1242 cm^{-1}; UV (MeOH) λ_{max} 213 (log ε=3.8); ^{1}H-NMR ($CDCl_3$, 500 MHz) and ^{13}C-NMR ($CDCl_3$, 125 MHz), see Tables 1 and 2; ESI-MS *m/z*: 431 $[M+Na]^+$; HR-ESI-MS *m/z*: 431.2406 $[M+Na]^+$ (Calcd for $C_{23}H_{36}O_6Na$, 431.2409).

Querciformolide E (**3**): White powder; mp 70—72 °C; $[\alpha]_D^{25}$ −17.1 (c=0.14, $CHCl_3$); IR (neat) ν_{max} 3448, 2926, 1738, 1710, 1456, 1375, and 1242 cm^{-1}; UV (MeOH) λ_{max} 211 (log ε=3.9); ^{1}H-NMR ($CDCl_3$, 500 MHz) and ^{13}C-NMR ($CDCl_3$, 125 MHz), see Tables 1 and 2; ESI-MS *m/z*: 447 $[M+Na]^+$; HR-ESI-MS *m/z*: 447.2361 $[M+Na]^+$ (Calcd for $C_{23}H_{36}O_7Na$, 447.2359).

Hydrolysis of 4 A solution of **4** (8.0 mg) was dissolved in 10% methanolic NaOH solution (1.0 ml), and the mixture was stirred at 0 °C for 12 h. The mixture was then neutralized with diluted HCl (0.1 N) and evaporated, and the residue was extracted with $CHCl_3$ (2.0 ml×3). The $CHCl_3$-soluble layers were combined, dried over anhydrous $NaSO_4$ and evaporated. The residue was subjected to column chromatography over silica gel using EtOAc–*n*-hexane (1 : 3) to yield **1** (1.5 mg, 17.3%).

Hydrolysis of 6 By using the same procedure as for the preparation of **8**, the reaction of **6** (5.0 mg) with 10% methanolic NaOH solution (1 ml) afforded a crude product which was subjected to column chromatography over silica gel using EtOAc–*n*-hexane (1 : 1) to yield **8** (1.8 mg, 32.8%).

Acetylation of 8 A solution of **8** (1.8 mg) in pyridine (0.1 ml) was mixed with Ac_2O (0.1 ml), and the mixture was stirred at room temperature for 12 h. After evaporation of excess reagent, the residue was subjected to column chromatography over Si gel using *n*-hexane–acetone (6 : 1) to yield the diacetyl derivative **2** (1.5 mg, 75.0%).

Cytotoxicity Testing Cell lines were purchased from the American Type Culture Collection (ATCC). Cytotoxicity assays of compounds **1**—**7** were performed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method.[12,13]

***In Vitro* Anti-inflammatory Assay** Macrophage (RAW264.7) cell line was purchased from ATCC. *In vitro* anti-inflammatory activities of compounds **1**—**4** and **6**—**8** were measured by examining the inhibition of LPS (lipopolysaccharide)-stimulated upregulation of iNOS (inducible nitric oxide synthetase) and COX-2 (cyclooxygenase-2) proteins in macrophage cells using Western blot analysis.[7,14,15]

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