Abstract
Chemical examination of *Sinularia querciformis* afforded one new cembranoid, querciformolide F (1), along with four known cembranoids, sinulariolone (2), granosolide A (3), querciformolide A (4), and sinulariolide (5). The structures of these compounds were determined by extensive spectroscopic (IR, ESIMS, 1H NMR, and 13C NMR) analysis and by comparison with those previously reported in the literature. Compounds 2 to 4 were found to exhibit significant anti-inflammatory activity in lipopolysaccharide (LPS)-induced RAW264.7 mouse macrophages through attenuating the expression of the inducible nitric oxide synthase (iNOS) proteins.

Keywords
*Sinularia querciformis*, cembrane, diterpenoid, soft coral, anti-inflammatory

Results and Discussion
Querciformolide F (1) was isolated as a colorless oil that showed a sodiated adduct ion peak in its HRESIMS at *m/z* 389.19354 (M + Na)\(^+\), accounted for the molecular formula, C\(_{20}\)H\(_{30}\)O\(_6\) (Calcd for C\(_{20}\)H\(_{30}\)O\(_6\) + Na, 389.19346), indicating six degrees of unsaturation in the compound. The IR spectrum revealed absorptions for hydroxyl (\(\nu_{max} 3391\) cm\(^{-1}\)) and \(\epsilon\)-lactone (\(\nu_{max} 1710\) cm\(^{-1}\)) groups. The 13C-NMR spectrum of 1 (Table 1), showed signals of 20 carbons, which were further identified by the assistance of the distortionless enhancement by polarization transfer (DEPT) spectrum as three methyls, six sp\(^3\) methylenes, three sp\(^3\) methines (including two oxymethines), one sp\(^2\) methylene, two sp\(^2\) methines, three sp\(^3\) quaternary carbons and two sp\(^2\) quaternary carbons (including one ester carbonyl). The NMR signals observed at \(\delta\)C 169.4 (C), 144.3 (C), 124.7 (CH\(_2\)), 88.7 (C), 35.7 (CH), 33.4 (CH\(_2\)), 32.2 (CH\(_2\)) and \(\delta\)H 6.31 (s), 5.47 (s) showed the presence of an \(\alpha\)-methylene-\(\epsilon\)-lactone ring by comparing the very similar
NMR data of the cembranoids with the same seven-membered lactone ring. Signals resonating at $\delta^C 61.1$ (C), $61.3$ (CH) and $H 2.89$ (1H, dd, $J = 10.4, 3.2$ Hz) revealed the presence of a trisubstituted epoxide. The NMR signals at $\delta^C 83.6$ (C) and $H 7.34$ (1H, brs) showed the presence of a hydroperoxy group at a methine carbon. One, 1,2-disubstituted double bond was also identified from NMR signals appearing at $\delta^C 125.9$ (CH), $134.4$ (CH), and $H 5.44$ (1H, d, $J = 16.0$ Hz) and $5.68$ (1H, ddd, $J = 16.0, 10.0, 4.8$ Hz).

From the $^1H$$-^1H$ COSY spectrum of 1, the separate spin systems of $H_2$ to 13/$H_2$ to 14/$H-1/H_2$ to 2/$H-3$, $H_2$ to 5/$H-6/H-7$ and $H_2$ to 9/$H_2$ to 10/$H-11$ enabled the identification of three different structural units, which were assembled with the assistance of an HMBC experiment. These data, together with the key HMBC correlations of $H_1$ to 18 to C-3, C-4 and C-5, $H_2$ to 19 to C-7, C-8 and C-9, $H_3$ to 20 to C-11, C-12 and C-13 and $H_2$ to 17 to C-1, C-15 and C-16 permitted the establishment of the carbon skeleton. The relative configurations of the six chiral centers at C-1, C-3, C-4, C-8, C-11, and C-12 in 1 were elucidated by detailed analysis of the nuclear Overhauser effect (NOE) correlations (Figure 2). $H-1$ ($\delta^H 2.68$, dd, $J = 12.0, 7.2$ Hz) showed an NOE interaction with $H-11$ ($\delta^H 3.48$, d, $J = 10.0$ Hz), and $H_3$ to 18 ($\delta^H 1.29$, s); therefore, assuming the $\alpha$-orientation of $H-1$, $H-11$ and $H_3$ to 18 this should also be positioned on the $\alpha$-face. One of the methylene protons at C-10 ($\delta^H 1.92$, m) exhibited NOE correlation with $H-11$ and was characterized as H-10$\alpha$, while the other ($\delta^H 1.61$, m) was assigned as H-10$\beta$. NOE correlations observed between $H_3$ to 18 and $H-6$ ($\delta^H 5.68$, ddd, $J = 16.0, 10.0, 4.8$ Hz), H-6 and H-5$\alpha$ ($\delta^H 2.74$, dd, $J = 12.0, 4.8$ Hz), reflected the $\alpha$-orientation of H-1, H-5$\alpha$, H-6, H-10$\alpha$, H-11, and H$_3$ to 18. The $J$ values for both H-6 and H-7 (16.0 Hz) further confirmed the E-configuration of the 6,7-double bond. Also, the NOE correlations observed for H-7 ($\delta^H 5.44$, d, $J = 16.0$ Hz) with $H_3$ to 19 ($\delta^H 1.40$, s), and $H_3$ to 20 ($\delta^H 1.43$, s), $H_3$ to 19, and H-10$\beta$, reflected the $\beta$-orientation of H-7, H-10$\beta$, $H_3$ to 19, and $H_3$ to 20. From the above observations and further analysis of other NOE interactions, the 1$R^*$, 3$S^*$, 4$S^*$, 8$R^*$, 11$R^*$ and 12$R^*$ relative configurations of 1 were established (Supplemental Materials, Figures S1–S10).

The in vitro anti-inflammatory activities of compounds 1 to 5 were measured by examining the inhibition of LPS (lipopolysaccharide)-induced upregulation of iNOS (inducible nitric oxide synthetase) and COX-2 (cyclooxygenase-2) proteins in macrophages using Western blotting analysis. RAW264.7 cells were obtained from the American Type Culture Collection (ATCC TIB-71, Manassas, VA, USA). Compounds 2 to 4 were found to inhibit iNOS release by 82.8, 84.5, and 77.3%, respectively (Figure 3 and Table 2).

Materials and Methods

General Experimental Procedures

A digital polarimeter (model P-1010; JASCO Corp., Tokyo, Japan) was used to determine optical rotations of the samples.
IR spectra were collected using a Nicolet iS5 FT-IR spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). NMR spectra were taken on a Varian NMR Mercury Plus spectrometer operating at 400 MHz for $^1$H and 100 MHz for $^{13}$C in CDCl$_3$ using the residual CHCl$_3$ signal ($\delta_H$ 7.26 ppm) and CDCl$_3$ ($\delta_C$ 77.1 ppm) as the internal standard for $^1$H and $^{13}$C NMR, respectively; coupling constants ($J$) are given in Hz. For ESIMS and HRESIMS, the results were obtained using a SolarX FTMS mass spectrometer (7 Tesla; Bruker, Bremen, Germany). Gravity column chromatography was performed on silica gel (range, 230 to 400 mesh, Merck). TLC was carried out on precoated Kieselgel 60 F$_{254}$ (0.2 mm, Merck) and compounds were visualized by spraying with 10% H$_2$SO$_4$ solution followed by heating. High-performance liquid chromatography (HPLC) was performed using a system comprised of a Hitachi L-7100 pump and a Rheodyne 7725 injection port. A semi-preparative normal phase column (Hibar 250 × 10 mm, Supelco, silica gel 60, 5 μm) was used.

**Animal Material**

Specimens of *S. querciformis* were manually collected by an underwater diver at a depth of 10 to 15 m from the sea around southern Taiwan on third June 2016. A representative sample of the soft coral (voucher no: NMMBA-TW-SC-2015-0603) is stored in the National Museum of Marine Biology and Aquarium, Taiwan.

**Extraction and Isolation**

*S. querciformis* (wet/dry weight = 1870/570 g) was sliced and extracted with a mixture of methanol: dichloromethane (1:1). The extract was partitioned between ethyl acetate (EtOAc) and H$_2$O. The EtOAc layer (7.60 g) was applied to a silica gel column and eluted with a gradient of n-hexane : EtOAc : acetone (from 100% n-hexane to 100% acetone) to yield 13 fractions, A–M. Fraction I was chromatographed using Si gel CC using a mixture of n-hexane : acetone (7:3) to yield querciformolide F (1.7 mg). Fraction L was chromatographed by Si gel CC using

| Position | $\delta_H$ ($J$ in Hz)$^a$ | $\delta_C$, type$^c$ |
|----------|-------------------------|------------------|
| 1        | 2.68 dd (12.0, 7.2)     | 35.7, CH         |
| 2        | 1.42 m                  | 32.9, CH$_2$     |
| 3        | 2.89 dd (10.4, 3.2)     | 61.3, CH         |
| 4        |                        | 61.1, C          |
| 5/5’     | 2.74 dd (12.0, 4.8); 1.71 dd (12.0, 10.0) | 42.9, CH$_2$ |
| 6        | 5.68 ddd (16.0, 10.0, 4.8) | 125.9, CH |
| 7        | 5.44 d (16.0)           | 134.4, CH        |
| 8        |                        | 83.6, C          |
| 9        | 2.03 m                  | 33.3, CH         |
| 10/10’   | 1.92 m; 1.61 m          | 27.9, CH$_2$     |
| 11       | 3.48 d (10.0)           | 76.7, CH         |
| 12       |                        | 88.7, C          |
| 13       | 1.48 m                  | 33.4, CH$_2$     |
| 14       | 2.31 m                  | 32.2, CH$_2$     |
| 15       |                        | 144.3, C         |
| 16       |                        | 169.4, C         |
| 17/17’   | 6.31 s; 5.47 s          | 124.7, CH$_2$    |
| 18       | 1.29 s                  | 16.4, CH$_3$     |
| 19       | 1.40 s                  | 24.9, CH$_3$     |
| 20       | 1.43 s                  | 27.5, CH$_3$     |
| OOH      | 7.34 br s               |                  |

$^a$Spectra recorded at 400 MHz in CDCl$_3$ at 25 °C.

$^b$Spectra recorded at 100 MHz in CDCl$_3$ at 25 °C.

$^c$Multiplicity deduced by DEPT spectra.

**Table 1.** $^1$H and $^{13}$C NMR Spectroscopic Data for Querciformolide F.

Figure 2. Key COSY (Fx1), HMBC (Fx2), and protons with NOESY (Fx3) correlations of 1.
Fraction L7 was separated by NP-HPLC using a mixture of n-hexane : acetone (3:1) to yield sinulariolone (2) (3.9 mg). Fraction I7 was separated by NP-HPLC using a mixture of n-hexane : acetone (4:1) to yield granosolide A (3) (0.7 mg), and sinulariolide (5) (2.7 mg). Fraction G was chromatographed by Si gel CC using n-hexane : EtOAc : acetone : MeOH (from 100% n-hexane to 100% MeOH) to obtain fractions G1-G15. Fraction G8 was separated by NP-HPLC using a mixture of n-hexane : EtOAc (4:3) to yield querciformolide A (4) (2.9 mg).

**Querciformolide F (1).** Colorless oil; [α]_D −27.7 (c 0.1, CHCl_3); IR ν_max 3391, 2923, 1710, 1260 cm⁻¹; 1H (CDCl_3, 400 MHz) and 13C (CDCl_3, 100 MHz) NMR data, see Table 1; ESIMS m/z 389 (M + Na); HRESIMS m/z 389.19354 (M + Na) (Calcd for C_{20}H_{30}O_{6} + Na, 389.19346).

**Anti-Inflammatory Test**

A macrophage (RAW264.7) cell line was purchased from ATCC. We measured the in vitro anti-inflammatory activities of 1 to 5 by examining the inhibition of LPS-simulated upregulation of the iNOS (inducible nitric oxide synthetase) and COX-2 (cyclooxygenase-2) proteins in macrophages using Western blotting analysis.¹³

**Conclusion**

One new cembranoid, querciformolide F (1), and four known cembranoids were isolated from the soft coral *Sinularia quercifomis*. These characteristic α-methylene-γ-lactone-containing cembranoids were elucidated using spectroscopic methods. Compounds 2 to 4 displayed inhibitory effects on the production of iNOS at a concentration of 10 μM. These results revealed a series of anti-inflammatory lead compounds which could be referred for future medicinal modifications.

**Table 2. Effects of Compounds 1 to 5 on LPS-Induced iNOS and COX-2 Protein Expressions in Macrophages.**

| Compounds (10 μM) | Inos (%) | COX-2 (%) |
|------------------|----------|----------|
| Control          | 0.44 ± 0.07 | 0.93 ± 0.33 |
| Vehicle          | 100.0 ± 3.5  | 100.0 ± 3.0  |
| 1                | 94.2 ± 3.8   | 100.1 ± 2.0  |
| 2                | 82.8 ± 3.1   | 95.7 ± 2.9   |
| 3                | 84.5 ± 2.7   | 98.6 ± 4.3   |
| 4                | 77.3 ± 3.9   | 97.2 ± 4.9   |
| 5                | 102.1 ± 7.4  | 108.5 ± 6.4  |
| DEX*             | 65.6 ± 1.4   | 22.0 ± 4.3   |

*Dexamethasone (DEX, 10 μM) was used as a positive control.

**Ethical Approval**

Our institution does not require ethical approval for reporting individual cases or case series.

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Statement of Human and Animal Rights
This article does not contain any studies with human or animal subjects.

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Trial Registration
Not applicable, because this article does not contain any clinical trials.

Supplemental Material
Supplemental material for this article is available online.

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