Two New Cembranoids from the Soft Coral *Lobophytum crassum*

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Two new cembranoids, locrassolides A (**1**) and B (**2**), along with three known metabolites **3**–**5** were isolated from the soft coral *Lobophytum crassum*. None of these compounds were found to be cytotoxic toward a limited panel of cancer cell lines (CCRF-CEM and DLD-1). Metabolite **3** was shown to significantly inhibit the accumulation of the pro-inflammatory iNOS and COX-2 proteins in LPS-stimulated RAW264.7 macrophage cells.

In previous studies a series of novel secondary metabolites, including cembranoids[1,2] and glycolipids[3] have been isolated from Taiwanese soft corals *Lobophytum crassum*. As our interest in the chemistry of biologically active natural products, the continuing investigation on the chemical constituents of the soft coral *L. crassum* was carried out and resulted in the isolation of two new cembranoids, locrassolides A (**1**) and B (**2**), along with three known metabolites cembranolide B (**3**),[4] crassocolide D (**4**),[5] and crassocolide A (**5**)[5] (Chart 1). The structures of these compounds were established by detailed spectroscopic analysis and by comparison with the physical and spectroscopic data of related known compounds. The cytotoxicity of metabolites **1**–**5** against a limited panel of human tumor cell lines including T cell acute lymphoblastic leukemia (CCRF-CEM) and colon adenocarcinoma (DLD-1) was studied, and the ability of **1**–**5** to inhibit the accumulation 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 examined.

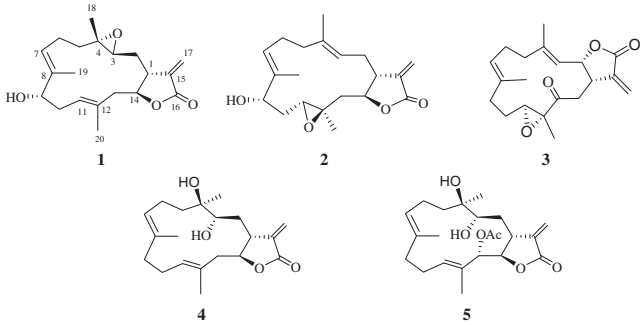

Chart 1.

Locrassolide A (**1**) was isolated as a colorless oil. The molecular formula $C_{20}H_{28}O_4$ was established by HR-ESI-MS, ^{13}C NMR, and DEPT spectroscopic data, requiring seven degrees of unsaturation. IR absorptions were observed at 3422, 1755, and 1645 cm^{-1}, indicative of the presence of hydroxy and α-methylene-γ-lactone functionalities.[1,2] The ^{1}H NMR spectrum of **1** (Table 1) indicated the presence of four olefinic protons [δ 5.08 (dd, $J = 8.0$, 7.0 Hz), 5.35 (dd, $J = 7.0$, 7.0 Hz), 5.64 (d, $J = 2.0$ Hz), and 6.31 (d, $J = 3.0$ Hz)]; three oxymethines [δ 2.64 (dd, $J = 6.5$, 4.5 Hz), 4.01 (dd, $J = 9.0$, 2.0 Hz), and 4.50 (ddd, $J = 8.5$, 4.0, 4.0 Hz) and three methyls [δ 1.31 (s), 1.67 (s), and 1.68 (s)]. The ^{13}C NMR and DEPT spectroscopic data (Table 1) indicated the presence of three methyls, five sp^3 methylenes, one sp^2 methylene, four sp^3 methines (including three oxymethines), two sp^2 methines, one sp^3 quaternary carbon, and four sp^2 quaternary carbons (including one ester carbonyl). The gross structure of **1** was established by 2D NMR experiments, especially by analysis of ^{1}H–^{1}H COSY and HMBC correlations (Figure 1). The ^{1}H–^{1}H COSY experiment assigned three isolated consecutive proton spin systems. Further analysis of the HMBC correlations was employed successfully to establish the gross structure of **1**.

The relative structure of **1** was elucidated by the analysis of NOE correlations, as shown in Figure 2. The NOE correlations observed between H-7 and H-9, and H_2-10 and H_3-20 reflected the *E*-geometry of double bonds at C-7 and C-11. In addition, H-1 showed correlations with H-13a (δ 2.30), while H-14 showed response to H-13b (δ 2.51), suggesting a *trans*-fused γ-lactone ring in **1**. Moreover, it was found that H-14 showed NOE interactions with H-3 and H_3-20. Thus, assuming the α-orientation of H-14,[2,5] H-3 should be positioned on the α face. Also, H_3-18 was found to interact with H_2-2, but not with H-3, revealing the *trans*-geometry of the trisubstituted epoxide. Furthermore, the NOE correlations found between the hydroxymethine proton H-9 and H-11 and between H-11 and H-1 assigned the α-orientation of the hydroxy group. On the basis of the above findings and other detailed NOE correlations and coupling constants (Figure 2), the relative structure of locrassolide A (**1**) was determined.

HR-ESI-MS and NMR spectroscopic data revealed that locrassolide B (**2**) has the same molecular formula, $C_{20}H_{28}O_4$, as that of **1**. Comparison of the ^{1}H and ^{13}C NMR data of **2** with those of **1** (Table 1) showed that both compounds possess similar structures. The planar structure of **2** was proposed by the assistance of extensive 2D NMR spectra (Figure 1). Careful

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Table 1. ^{1}H and ^{13}C NMR Data of Compounds **1** and **2**

C/H	**1**		**2**	
	^{1}H[a)]	^{13}C[b)]	H[c)]	^{13}C[d)]
1	2.83 m	40.9 (CH)[f)]	2.80 m	44.0 (CH)
2	1.81 m	33.1 (CH_2)	2.48 m, 2.45 m	26.7 (CH_2)
3	2.64 dd (6.5, 4.5)[e)]	60.1 (CH)	4.87 d (10.0)	120.8 (CH)
4		60.2 (C)		135.0 (C)
5	2.05 m, 1.43 m	36.7 (CH_2)	2.30 m, 2.02 m	36.5 (CH_2)
6	2.23 m, 2.08 m	23.1 (CH_2)	2.50 m, 1.32 m	24.0 (CH_2)
7	5.35 dd (7.0, 7.0)	126.6 (CH)	5.25 dd (10.0, 4.0)	121.7 (CH)
8		137.1 (C)		140.3 (C)
9	4.01 dd (9.0, 2.0)	77.0 (CH)	4.17 dd (9.6, 4.4)	77.7 (CH)
10	2.47 m, 2.37 m	33.4 (CH_2)	2.50 m, 2.32 m	32.6 (CH_2)
11	5.08 dd (8.0, 7.0)	124.8 (CH)	2.54 dd (10.0, 2.8)	61.4 (CH)
12		132.0 (C)		60.5 (C)
13	2.51 m, 2.30 m	44.4 (CH_2)	2.26 m, 1.32 m	44.8 (CH_2)
14	4.50 ddd (8.5, 4.0, 4.0)	81.7 (CH)	4.15 m	80.0 (CH)
15		138.5 (C)		137.3 (C)
16		169.5 (C)		169.9 (C)
17	6.31 d (3.0), 5.64 d (2.0)	122.8 (CH_2)	6.39 d (3.2), 5.62 d (2.8)	121.9 (CH_2)
18	1.31 s	17.7 (CH_3)	1.70 s	14.9 (CH_3)
19	1.67 s	11.3 (CH_3)	1.54 s	10.6 (CH_3)
20	1.68 s	17.2 (CH_3)	1.42 s	17.3 (CH_3)

a) Spectra recorded at 500 MHz in $CDCl_3$. b) 125 MHz in $CDCl_3$. c) 400 MHz in $CDCl_3$. d) 100 MHz in $CDCl_3$. e) J values (in Hz) in parentheses. f) Attached protons determined by DEPT experiments.

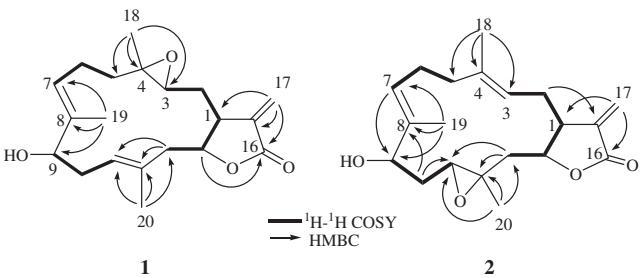

Figure 1. The COSY and HMBC correlations of **1** and **2**.

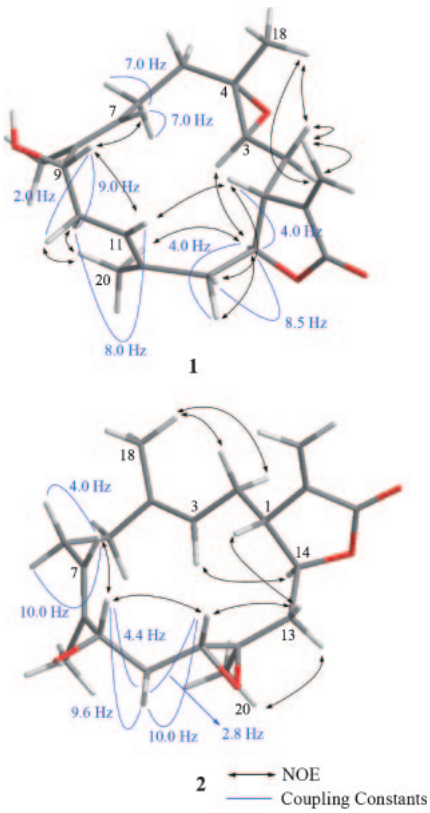

Figure 2. Selective NOESY correlations and coupling constants (J) of **1** and **2**.

analysis of the NOESY spectrum of **2**, in comparison with those of **1**, allowed determination of the relative stereochemistry of **2** as shown in Figure 2. Thus, the structure of **2** was established.

Cytotoxicity study of diterpenoids **1**–**5** against the growth of CCRF-CEM and DLD-1 cancer cell lines showed that none of these compounds possesses cytotoxicity against cell growth ($IC_{50} > 20\,\mu g\,mL^{-1}$). In vitro anti-inflammatory action of compounds **1**–**5** was tested. In this assay, the inhibition of LPS-induced upregulation of the pro-inflammatory iNOS and COX-2 proteins in LPS-stimulated RAW264.7 macrophage cells was evaluated using immunoblot analysis. At a concentration of 10 µM, **3** was found to significantly reduce the levels of iNOS and COX-2 proteins to $9.4 \pm 2.3\%$ and $27.5 \pm 4.7\%$, respectively, relative to the control cells stimulated with LPS only (Figure 3).

Experimental

General Experimental Procedures. Optical rotation values were measured using a JASCO P-1010 digital polarimeter. IR spectra were obtained on a VARIAN DIGLAB FTS 1000 FT-IR spectrophotometer. NMR spectra were recorded on a Varian 400MR FT-NMR (or Varian Unity INOVA 500 FT-NMR) instrument at 400 MHz (or 500 MHz) for ^{1}H and

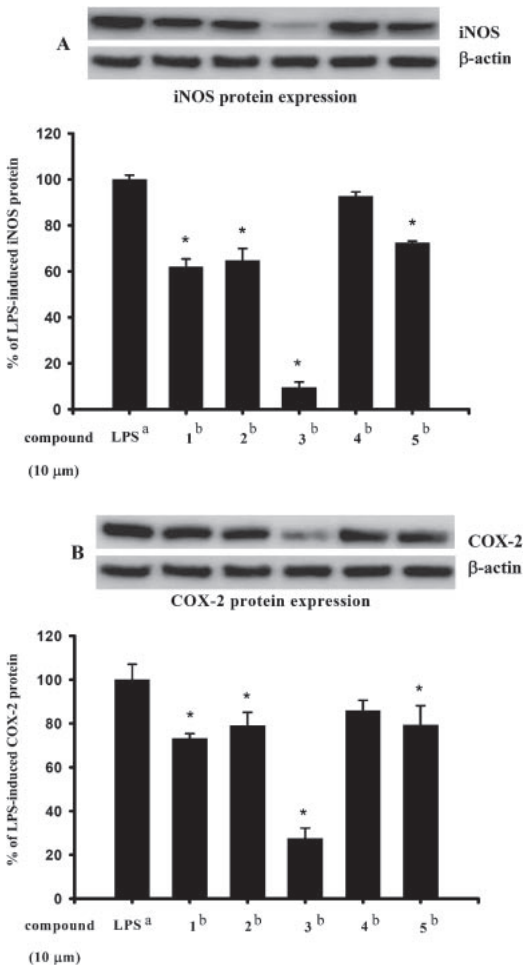

Figure 3. Effect of compounds **1**–**5** at 10 μM on the expression of iNOS and COX-2 proteins of RAW264.7 macrophage cells examined by immunoblot analysis. (**A**) Immunoblots of iNOS and β-actin; (**B**) immunoblots of COX-2 and β-actin. Values represent mean ± SEM ($n = 6$). The relative intensity of the LPS-only-stimulated group was taken as 100%. *Significantly different from the LPS-only-stimulated group ($^*P < 0.05$). [a]Stimulated with LPS; [b]stimulated with LPS in the presence of **1**–**5**.

100 MHz (or 125 MHz) for ^{13}C in $CDCl_3$. ESI-MS and HR-ESI-MS data were recorded on a BRUKER APEX II mass spectrometer. Silica gel 60 (230–400 mesh; Merck, Darmstadt, Germany) was used for column chromatography. Gravity column chromatography was performed on silica gel (230–400 mesh; Merck). TLC was carried out on precoated Kieselgel 60 F254 (0.2 mm; Merck) and spots were visualized by spraying with 10% H_2SO_4 solution followed by heating. High-performance liquid chromatography was performed on a Hitachi L-7100 pump equipped with a Hitachi L-7400 UV detector at 210 nm and a semipreparative reversed-phase column (Merck, Hibar Purospher RP-18e, 5 μm, 250 × 10 mm).

Animal Material. The marine soft coral *Lobophytum crassum* was collected by scuba at a depth of around 10 m off the coast of Gueishan Island, in August 2007, and the sample was frozen immediately after collection.

Extraction and Separation. The frozen bodies of *L. crassum* (1.2 kg, wet wt) were minced, combined, and exhaustively extracted with EtOAc (1 L × 5). The EtOAc extract (12.5 g) was chromatographed over silica gel by column chromatography and eluted with EtOAc in *n*-hexane (0–100%, stepwise) to yield 10 fractions. Fractions 5, eluting with EtOAc–*n*-hexane (1:5), was further separated by RP-18 HPLC using CH_3CN–H_2O (3:2) to yield **3** (5.6 mg). Fraction 8, eluted with EtOAc–*n*-hexane (1:1), was further purified over silica gel using EtOAc–*n*-hexane (1:2) to afford 3 subfractions. Subfraction 1 was separated by RP-18 HPLC using CH_3CN–H_2O (1:1) to afford **4** (3.0 mg) and **5** (2.9 mg). Subfraction 2 was also subjected to RP-18 HPLC (CH_3CN–H_2O, 1:1) to obtain **1** (1.5 mg) and **2** (1.3 mg).

Locrassolide A (1): Colorless oil (1.5 mg); $[\alpha]_D^{25} = +20.0$ (*c* 0.2, $CHCl_3$); IR (neat): ν_{max} 3422, 2956, 2928, 1755, 1645, 1370, 1269 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz), see Table 1; ESI-MS: m/z 355 $[M + Na]^+$; HR-ESI-MS: m/z 355.1884 (calcd for $C_{20}H_{28}O_4Na$, 355.1885).

Locrassolide B (2): Colorless oil (1.3 mg); $[\alpha]_D^{25} = +34.1$ (*c* 0.1, $CHCl_3$); IR (neat): ν_{max} 3422, 2958, 2932, 1750, 1653, 1366 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 1; ESI-MS: m/z 355 $[M + Na]^+$; HR-ESI-MS: m/z 355.1886 (calcd for $C_{20}H_{28}O_4Na$, 355.1885).

Cytotoxicity Testing. Cell lines were purchased from the American Type Culture Collection (ATCC). Cytotoxicity assays of the compounds **1**–**5** were performed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method.[6]

In Vitro Anti-Inflammatory Assay. Assay procedure was as previously reported.[7,8]

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Supporting Information

1D and 2D NMR spectra of compounds **1** and **2**. This material is available free of charge on the web at http://www.csj.jp/journals/bcsj/.

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