

Bioactive Cembranoids from the Dongsha Atoll Soft Coral *Lobophytum crassum*

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Two new metabolites, crassumolides H and I (**2** and **3**), and crassumolide G (**1**) which was discovered for the first time from natural sources, were isolated from the ethyl acetate extract of a Taiwanese soft coral *Lobophytum crassum*, along with three known metabolites **4**–**6**. The structures of metabolites **1**–**3** were elucidated by extensive spectroscopic analysis and comparison of the NMR data with those of known compounds. Compounds **1**–**3** were found to inhibit the accumulation of the pro-inflammatory iNOS and COX-2 proteins at 10 μM in LPS-stimulated RAW264.7 marcophage cells.

Soft corals of the *Lobophytum* genus are rich sources of cembrane-type diterpenoids and steroids, of which some have been reported to possess HIV-inhibitory,[1] cytotoxic,[2–6] and anti-inflammatory activities.[7–14] In continuation of our search of bioactive cembranoids from soft corals,[6,8–11,13] we carried out a study to investigate the chemical constituents of the soft coral *Lobophytum crassum*, collected from Taiwanese waters. We initially isolated cembranoids, crassumolides A–F, from *L. crassum* living off the coast of Kenting, Taiwan. As four of the above compounds were shown to exhibit anti-inflammatory activity,[11] we then investigated the metabolites from the same species of soft coral collected from Dongsha Atoll. This study further led to the isolation of two new metabolites crassumolides H and I (**2** and **3**) and crassumolide G (**1**) (Chart 1) which was discovered for the first time from natural sources, along with three known compounds, durumolide B (**4**),[14] sinularolide B (**5**),[15] and durumolide C (**6**).[14] The structures of these compounds have been established by extensive spectroscopic analysis. The ability of **1**–**3** to inhibit the expression of pro-inflammatory proteins iNOS (inducible nitric oxide synthase) and COX-2 (cyclooxygenase-2) in LPS (lipopolysaccharide)-stimulated RAW264.7 macrophage cells was evaluated in order to discover anti-inflammatory compounds. Compounds **1**–**3** were found to display significant in vitro anti-inflammatory activity in LPS-stimulated RAW264.7 macrophage cells by inhibiting the expression of the iNOS protein. Also, **1**–**3** exhibited moderate activity to reduce the expression of COX-2. Furthermore, the absolute configuration of **4** has been confirmed as shown in formula **4**[14] by Mosher's method. On the basis that structure-related metabolites **1**–**6** were isolated from the same organism and should be synthesized from a shared biosynthetic pathway, they might possess the same absolute configuration at C-1 of the lactone ring.

The sliced bodies of the soft coral *L. crassum* were extracted exhaustively with EtOAc. The combined EtOAc extract was concentrated under reduced pressure and the residue was fractionated by column chromatography on silica gel, and the eluted fractions were further separated utilizing normal phase HPLC to yield compounds **1**–**3**.

The HRESIMS spectrum of **1** exhibited a molecular ion peak at m/z 455.2043 $[M + Na]^+$, consisting of the molecular

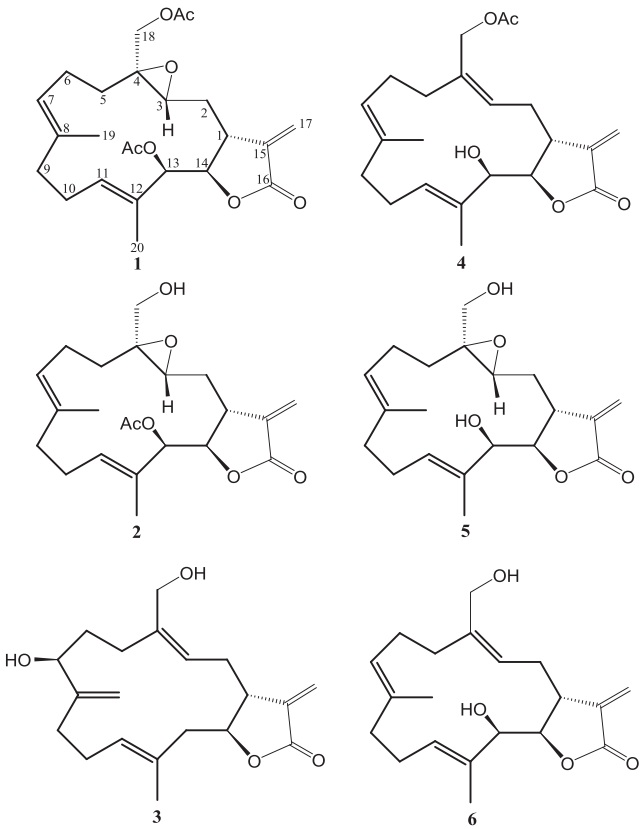

Chart 1.

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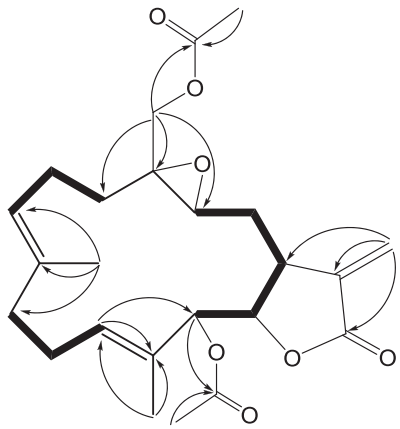
Table 1. ^{1}H and ^{13}C NMR Chemical Shifts for Compounds **1**–**3**

	1		**2**		**3**	
C/H	^{1}H[a)]	^{13}C[b)]	^{1}H[a)]	^{13}C[b)]	^{1}H[a)]	^{13}C[b)]
1	2.85 m	42.1 (CH)[d)]	2.84 m	42.2 (CH)	2.82 m	45.1 (CH)
2	1.85 dt (15.0, 3.0)	31.6 (CH_2)	1.86 m	31.5 (CH_2)	2.25 m	29.8 (CH_2)
	1.58 m		1.66 m			
3	2.88 dd (6.5, 3.0)	62.1 (CH)	2.89 dd (6.5, 4.0)	62.9 (CH)	5.29 t (7.0)	128.5 (CH)
4		60.4 (C)		62.8 (C)		140.4 (C)
5	2.35 m	32.4 (CH_2)	2.37 m	32.8 (CH_2)	2.34 m	29.3 (CH_2)
	1.28 m		1.31 m			
6	2.23 m	23.5 (CH_2)	2.26 m	23.5 (CH_2)	1.88 m	33.6 (CH_2)
	2.17 m		2.12 m		1.58 m	
7	5.04 dd (7.0, 6.5)	124.2 (CH)	5.04 t (6.5)[c)]	124.3 (CH)	4.07 dd (9.5, 2.5)	70.5 (CH)
8		135.1 (C)		134.9 (C)		153.9 (C)
9	2.35 m	38.3 (CH_2)	2.33 m	38.3 (CH_2)	2.42 m	33.0 (CH_2)
	2.10 m		2.12 m		2.30 m	
10	2.44 m	24.7 (CH_2)	2.44 m	24.7 (CH_2)	2.32 m	33.3 (CH_2)
	2.20 m		2.18 m		2.02 m	
11	5.53 dd (9.0, 5.0)	133.5 (CH)	5.52 dd (9.0, 4.5)	133.6 (CH)	5.35 dd (8.5, 6.5)	124.7 (CH)
12		129.1 (C)		129.0 (C)		131.1 (C)
13	5.21 d (8.0)	80.2 (CH)	5.20 d (9.0)	80.3 (CH)	2.47 m	45.1 (CH_2)
					2.06 m	
14	4.19 dd (8.0, 7.0)	79.6 (CH)	4.22 dd (9.0, 7.0)	79.6 (CH)	4.30 m	81.5 (CH)
15		138.1 (C)		138.3 (C)		139.4 (C)
16		168.9 (C)		169.1 (C)		170.0 (C)
17	6.33 d (2.5)	124.2 (CH_2)	6.33 d (3.0)	123.9 (CH_2)	6.29 d (2.0)	122.6 (CH_2)
	6.01 d (2.5)		6.01 d (3.0)		5.68 d (2.0)	
18	4.36 d (12.0)	64.0 (CH_2)	3.83 d (12.0)	61.9 (CH_2)	4.32 d (12.0)	61.0 (CH_2)
	3.88 d (12.0)		3.60 d (12.0)		4.05 d (12.0)	
19	1.63 s	15.9 (CH_3)	1.63 s	15.9 (CH_3)	5.12 s	109.8 (CH_2)
					4.96 s	
20	1.70 s	13.2 (CH_3)	1.69 s	13.1 (CH_3)	1.68 s	17.6 (CH_3)
13-OAc	2.11 s	169.6 (C)	2.10 s	169.6 (C)		
		21.1 (CH_3)		21.0 (CH_3)		
18-OAc	2.14 s	170.7 (C)				
		20.8 (CH_3)				

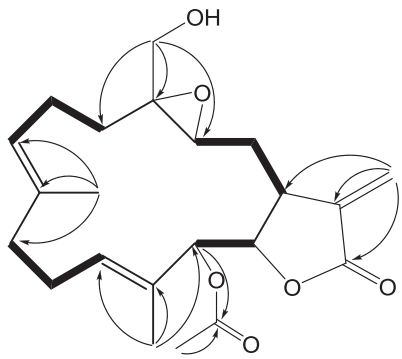
a) Spectra recorded at 500 MHz in $CDCl_3$. b) 125 MHz in $CDCl_3$. c) *J* values (in Hz) parentheses. d) Attached protons determined by DEPT experiments.

formula $C_{24}H_{32}O_7Na$ and implying nine degrees of unsaturation. IR spectrum revealed the absorption of ester ($\nu_{max} = 1769\,cm^{-1}$) functional group. ^{13}C NMR spectral data of **1** (Table 1), measured in $CDCl_3$, showed the presence of twenty-four carbon signals, which were assigned by the assistance of DEPT spectrum to four methyls, six sp^3 methylenes, one sp^2 methylene, four sp^3 methines (including three oxymethines), two sp^2 methines, one sp^3 quaternary carbon, and six sp^2 quaternary carbons. The NMR spectroscopic data of **1** (Table 1) showed the presence of two acetyl moiety [δ_C 169.6 (C), 21.1 (CH_3), and δ_H 2.11, s; δ_C 170.7 (C), 20.8 (CH_3), and δ_H 2.14, s]. Comparison of the NMR data of **1** with those of sinularolide B (**5**)[15,16] revealed that the difference between both compounds was the replacement of the two acetoxy groups at C-13 and C-18 in **1** by the two hydroxy groups in **5**. The gross structure of **1** was further established by 2D NMR studies, particularly by correlations of ^{1}H–^{1}H COSY and HMBC spectra (Figure 1).

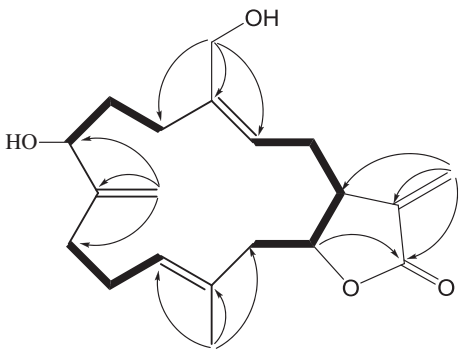
The relative configuration of **1** was determined by NOE correlations observed in a NOESY spectrum and also with the aid of molecular modeling using MM2 force field calculations. The NOESY spectrum showed correlations (Figure 2) between H-1/H-2b (δ 1.85), H-1/H-11, H-1/H-13, H-2b (δ 1.85)/H-3, H-2a (δ 1.58)/H-14, H-2a (δ 1.58)/H-18a (δ 3.88), H-7/H-9b (δ 2.35), and H-11/H-13, thus, H-1, H-3, and H-13 were assumed to be positioned on the β face, and H-14 and H_2-18 were assumed to be positioned on the α face, as shown in Figure 2. From the above observations, the structure of crassumolide G (**1**) was established as (1*R*,3*R*,4*S*,13*R*,14*R*,7*E*,11*E*)-13,18-diacetoxy-3,4-epoxy-cembra-7,11,15(17)-trien-16,14-olide. Literature survey showed that **1** was independently prepared from two C-13 stereochemistry unknown precursors **7** and **8** (Chart 2),[17] and later by modification of sinularolide B (**5**) and 20-acetylsinularolide B (**7**).[16] However, **1** was isolated for the first time from natural sources. Furthermore, our study also confirmed that both **7** and **8** should possess 13*R* configuration, as sinularolide B (**5**) and 20-acetylsinularolide B (**7**) were found to be identical with the earlier reported compounds **7** and **8**, respectively, in comparison with the NMR spectroscopic data.



1



2



3

—— :^{1}H-^{1}H COSY

⟶ :HMBC

Figure 1. The COSY and HMBC correlations for **1**–**3**.

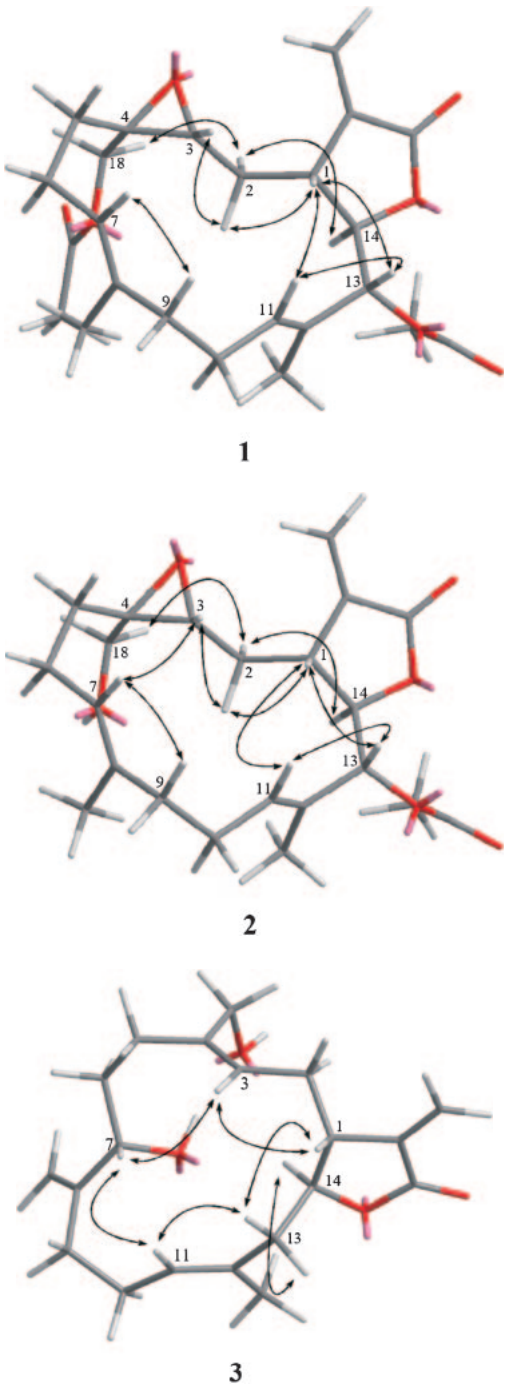
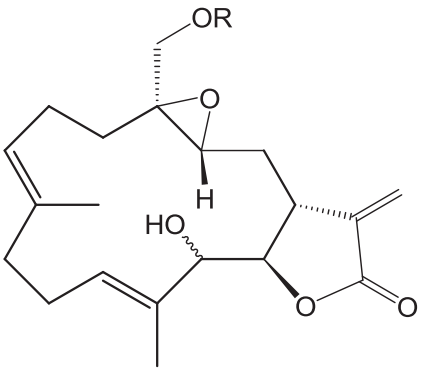


Figure 2. Selective NOE correlations for **1**–**3**.

The new metabolite crassumolide H (**2**) exhibited an ion peak at m/z 413.1942 $[M + Na]^+$ in HRESIMS, appropriate for a molecular formula of $C_{22}H_{30}O_6Na$ and implying eight degrees of unsaturation. The IR spectrum of **2** indicated the presence of hydroxy ($\nu_{max} = 3445\,cm^{-1}$) and α-methylene-γ-lactonic ($\nu_{max} = 1767\,cm^{-1}$) functionalities. The NMR spectroscopic data of **2** (Table 1) were quite similar to **1**. However, resonances for the acetoxy group in **1** were absent from the NMR spectra of **2**. In addition, the acetoxyl containing methylene protons at δ 4.36 and 3.88 (each 1H, d, $J = 12.0$ Hz) in **1** were upfield-shifted to 3.83 and 3.60 (each 1H, d, $J = 12.0$ Hz) in **2**, relative to those of **1**, suggesting that **2** should be the C-18 deacetyl derivative of **1**. By 2D NMR (^{1}H-^{1}H COSY, HMQC, and HMBC) analysis, compound **2** was shown to possess the same molecular framework as that of **1**. Thus, **2** might be the 18-*O*-deacetyl derivative of **1**. Further analysis of other NOE interactions (Figure 2) established **2** as (1*R*,3*R*,4*S*,13*R*,14*R*,7*E*,11*E*)-13-acetoxy-18-hydroxy-3,4-epoxycembra-7,11,15(17)-trien-16,14-olide.



7: R = Ac
8: R = H

Chart 2.

The molecular formula of crassumolide I (**3**) was found to be $C_{20}H_{28}O_4$, as deduced from the HRESIMS and NMR spectroscopic data, implying seven degrees of unsaturation. The IR spectrum of **3** showed the presence of hydroxy ($\nu_{max} = 3394\,cm^{-1}$) and α-methylene-γ-lactonic ($\nu_{max} = 1757\,cm^{-1}$) groups and the ^1H and ^{13}C NMR spectroscopic data of **3** (Table 1) show the presence of four olefinic groups. Protons of CH_2-18 exhibited HMBC connectivities to C-3 (δ_C 128.5) and C-4 (δ_C 140.4); thus, the hydroxymethyl-substituted 3,4-double bond was established. Furthermore, the oxygenated methine proton, H-7 (δ_H 4.07, dd, $J = 9.5$, 2.5 Hz), showed HMBC connectivities with carbons of a 1,1-disubstituted bond (δ_C 153.9 and 109.8, C-8/C-19), and H_3-20 (δ_H 1.68, s) showed connectivities with C-11 (δ_C 124.7), C-12 (δ_C 131.1), and C-13 (δ_C 45.1). Thus, the planar structure of **3**, including the positions of the above-mentioned double bonds and the hydroxy group, was determined. Careful analysis of the NOESY spectrum of **3** allowed the determination of the relative configuration of **3** as shown in Figure 2. NOE correlations between H-1 and H-3, H-1 and H-13β (δ 2.06), H-3 and H-7, H-3 and H-13β (δ 2.06), H-7 and H-11, H-14 and H-13α (δ 2.47), and H-11 and H-13β (δ 2.06) suggested the presence of a *trans*-fused γ-lactone and *E* geometry of the two double bonds at C-3 and C-11. From the above observations and by consideration of the absolute configuration of related compound **1**, compound **3** was established as (1*R*,7*S*,14*S*,3*E*,11*E*)-7,18-dihydroxy-cembra-3,8(19),11,15(17)-tetraen-16,14-olide. It is worthwhile to note here that cembranolides with exo-methylene at C-8 are rare.[18]

Known compounds **4**–**6** have been shown to possess promising anti-inflammatory activity. In order to discover new bioactive substances, we further investigated the inhibitory activity of **1**–**3** toward LPS-induced pro-inflammatory protein (iNOS and COX-2) expression in murine RAW264.7 macrophage cells by Western blot analysis using in vitro anti-inflammatory assay that was modified from the established procedure (Figure 3).[19] At a concentration of 10 μM, compounds **1**–**3** could significantly reduce the levels of iNOS to 20.1 ± 5.8%, 34.9 ± 6.7%, and 12.8 ± 3.1%, respectively. At this concentration, **1**–**3** showed moderate activity to reduce the levels of COX-2 to 57.1 ± 21.0%, 57.5 ± 9.6%, and 53.6 ± 19.0%, respectively, in comparison with those of control cells

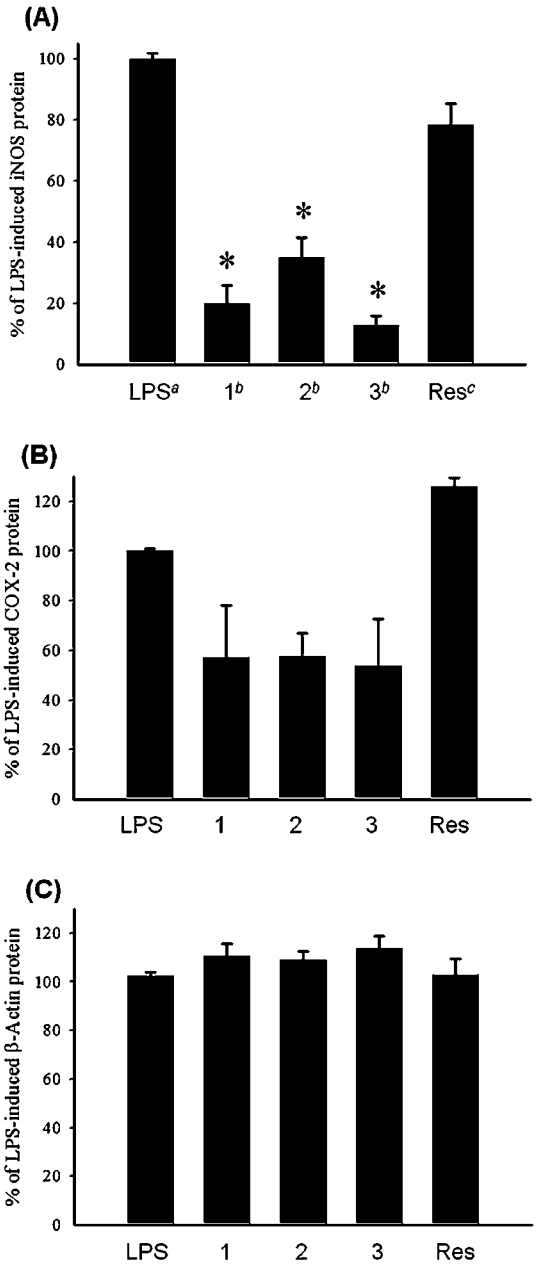


Figure 3. Effect of compounds **1**–**3** at 10 μM on the LPS-induced pro-inflammatory iNOS and on COX-2 protein expression of RAW264.7 macrophage cells by immunoblot analysis. (A) Quantification of immunoblots of iNOS. (B) Quantification of immunoblots of COX-2. (C) Quantification of immunoblots of β-actin. The values are mean ± SEM ($n = 4$). The relative intensity of the LPS alone stimulated group was taken as 100%. [a]Stimulated with LPS. [b]Stimulated with LPS in the presence of **1**–**3** (10 μM). [c]Resveratrol (Res) as positive control.

stimulated with LPS only (100% for both iNOS and COX-2). Also, **1**–**3** did not reduce the level of the house-keeping protein, β-actin, suggesting that these compounds are not cytotoxic against RAW264.7 macrophage cells. At the same concentration, resveratrol, a known anti-inflammatory natural stilbene,[20–22] could reduce the expression of iNOS only to 78.4 ± 6.9% and has no ability to inhibit the expression of COX-2.

Experimental

General Experimental Procedures. Optical rotations were measured on a JASCO P-1020 polarimeter. IR spectra were recorded on a JASCO FT/IR-4100 infrared spectrophotometer. 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, respectively, in $CDCl_3$ using TMS as internal standard. LRMS and HRMS were obtained by ESI on a Bruker APEX II mass spectrometer. 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. *Lobophytum crassum* was collected by hand via SCUBA at Dongsha Atoll, located in northeastern South China Sea in March 2008, at a depth of 10 to 15 m, and stored in a freezer until extraction. The species of the soft coral was identified by Prof. Chang-Feng Dai, National Taiwan University. A voucher sample was deposited at the Department of Marine Biotechnology and Resources, National Sun Yat-Sen University (specimen No. 20080329-2).

Extraction and Isolation. The frozen bodies of *L. crassum* (1.5 kg, wet wt) were minced and exhaustively extracted with EtOAc (1 L × 3). The solvent-free EtOAc extract (18.6 g) was subjected to silica gel column chromatography and eluted with *n*-hexane in EtOAc (0–100%, gradient) to yield 22 fractions. Fraction 9 eluted with *n*-hexane–EtOAc (5:1) was chromatographed by normal phase HPLC using *n*-hexane–EtOAc (7:1) to yield **4** (1.5 mg). Fraction 11 eluted with *n*-hexane–EtOAc (2:1) was separated by normal phase HPLC using *n*-hexane–EtOAc (3:1) to yield **1** (1.6 mg), and **2** (1.5 mg). Fraction 12 eluted with *n*-hexane–EtOAc (1:1) was separated by normal phase HPLC using *n*-hexane–EtOAc (2:1) to afford **3** (1.4 mg). Finally, fraction 13 eluted with *n*-hexane–EtOAc (1:2) was separated by normal phase HPLC using *n*-hexane–EtOAc (1:1) to afford **5** (22.4 mg) and **6** (25.6 mg).

Crassumolides G (1): Colorless gum (1.6 mg); $[\alpha]_D^{28} = -86$ (*c* 0.05, $CHCl_3$); IR (neat) ν_{max} 1769, 1732, 1434, 1371, and 1236 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS *m/z* 455 $[M + Na]^+$; HRESIMS *m/z* 455.2043 (calcd for $C_{24}H_{32}O_7Na$ 455.2046).

Crassumolides H (2): Colorless gum (1.2 mg); $[\alpha]_D^{28} = -90$ (*c* 0.06, $CHCl_3$); IR (neat) ν_{max} 3445, 1767, 1745, and 1235 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS *m/z* 413 $[M + Na]^+$; HRESIMS *m/z* 413.1942 (calcd for $C_{22}H_{30}O_6Na$ 413.1940).

Crassumolides I (3): Colorless gum (1.5 mg); $[\alpha]_D^{28} = -24$ (*c* 0.06, $CHCl_3$); IR (neat) ν_{max} 3394, 1757, and 1267 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS *m/z* 355 $[M + Na]^+$; HRESIMS *m/z* 355.1886 (calcd for $C_{20}H_{28}O_4Na$ 355.1885).

In Vitro Anti-Inflammatory Assay. Murine RAW 264.7 macrophages were purchased from the American Type Culture Collection (ATCC, No TIB-71). The anti-inflammatory assay was modified from a known procedure.[19]

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