Cladielloides C and D: Novel Eunicellin-Based Diterpenoids from an Indonesian Octocoral *Cladiella* sp.

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Chemical investigation on an Indonesian octocoral identified as *Cladiella* sp. has led to the isolation of two novel eunicellin-based diterpenoids, cladielloides C (**1**) and D (**2**). The structures of **1** and **2** were established by spectroscopic methods. Compound **1** exhibited significant cytotoxicity toward CCRF-CEM tumor cells and metabolites **1** and **2** displayed moderate inhibitory effects on superoxide anion generation by human neutrophils.

Previous investigations on the chemical constituents of octocorals belonging to the genus *Cladiella* have resulted in a series of interesting eunicellin-based (2,11-cyclized cembranoid) diterpenoids.[1–12] The compounds of this type were reported from various octocorals including the genera *Acalycigorgia*,[13] *Astrogorgia*,[14] *Briareum* (=*Solenopodium*),[15,16] *Eleutherobia*,[17] *Eunicella*,[18,19] *Klyxum* (=*Alcyonium*),[20–25] *Litophyton*,[26] *Muricella*,[27] *Pachyclavularia*,[28–30] *Sclerophytum*,[31] and *Sinularia*.[32] Most eunicellins were found to possess complex structures and various interesting bioactivities.[1,33,34] In continuation of our search for bioactive substances from the marine invertebrates distributed in the tropical West Pacific Ocean, an Indonesian octocoral identified as *Cladiella* sp. was studied, and its extract exhibited cytotoxicity toward the tumor cell lines DLD-1 (human colorectal adenocarcinoma), HL-60 (human promyelocytic leukemia), and P388D1 (macrophage-like murine tumor cells) with $IC_{50} = 2.7$, 8.9, and $7.2 \,\mu g \, mL^{-1}$, respectively. In our previous studies, seven eunicellin-based diterpenoids, including cladielloides A and B and cladieunicellins A–E, were obtained from this organism.[11,12] Our further investigation on the natural products from this soft coral has led to the isolation of two novel eunicellins, cladielloides C (**1**) and D (**2**) (Chart 1). In this paper, we report the isolation, structure determination, and bioactivity of the above new diterpenoids **1** and **2**.

Results and Discussion

Cladielloide C (**1**) was isolated as a colorless oil that gave a molecular ion $[M + Na]^+$ at *m/z* 485.2516 in the HR-ESI-MS, indicating the molecular formula $C_{26}H_{38}O_7$ (calcd for $C_{26}H_{38}O_7 + Na$, 485.2515) and implying eight degrees of unsaturation. The IR spectrum of **1** showed bands at 3449 and $1745 \, cm^{-1}$, consistent with the presence of hydroxy and ester groups. From the ^1H and ^{13}C NMR spectra (Table 1), **1** was found to possess a trisubstituted olefin (δ_H 5.44, 1H, m, H-12; δ_C 132.5, s, C-11; 121.5, d, C-12), an exocyclic carbon–carbon double bond (δ_H 5.03, 1H, s, H-16a; 5.39, 1H, s, H-16b; δ_C 149.1, s, C-7; 114.9, t, C-16), and a 2-acetoxybutanoate (δ_H 2.12, 3H, s; δ_C 20.5, q; 170.7, s; δ_H 1.00, 3H, t, $J = 7.6 \, Hz$; 1.90, 2H, m; 4.91, 1H, dd, $J = 7.2$, 5.2 Hz; δ_C 9.4, q; 24.3, t; 73.5, d; 170.1, s) group. In the ^1H NMR spectrum of **1**, two doublets at δ_H 0.98 and 0.80 (each 3H, d, $J = 6.8 \, Hz$, H_3-19 and H_3-20) were deduced to be from two methyls of an isopropyl group. A singlet of the tertiary methyl bonded to an oxygenated quaternary carbon was due to the resonance of a signal at δ_H 1.29 (3H, s, H_3-15). In addition, a suite of resonances of proton signals at δ_H 2.46 (1H, m, H-1), 2.81 (1H, br s, H-10), 4.07 (1H, d, $J = 2.4 \, Hz$, H-2), 4.04 (1H, m, H-9), and carbon signals at δ_C 42.0 (d, C-1), 46.2 (d, C-10), 83.6 (d, C-2), and 81.8 (d, C-9), indicated the presence of a tetrahydrofuran structural unit. Thus, from the above data, four
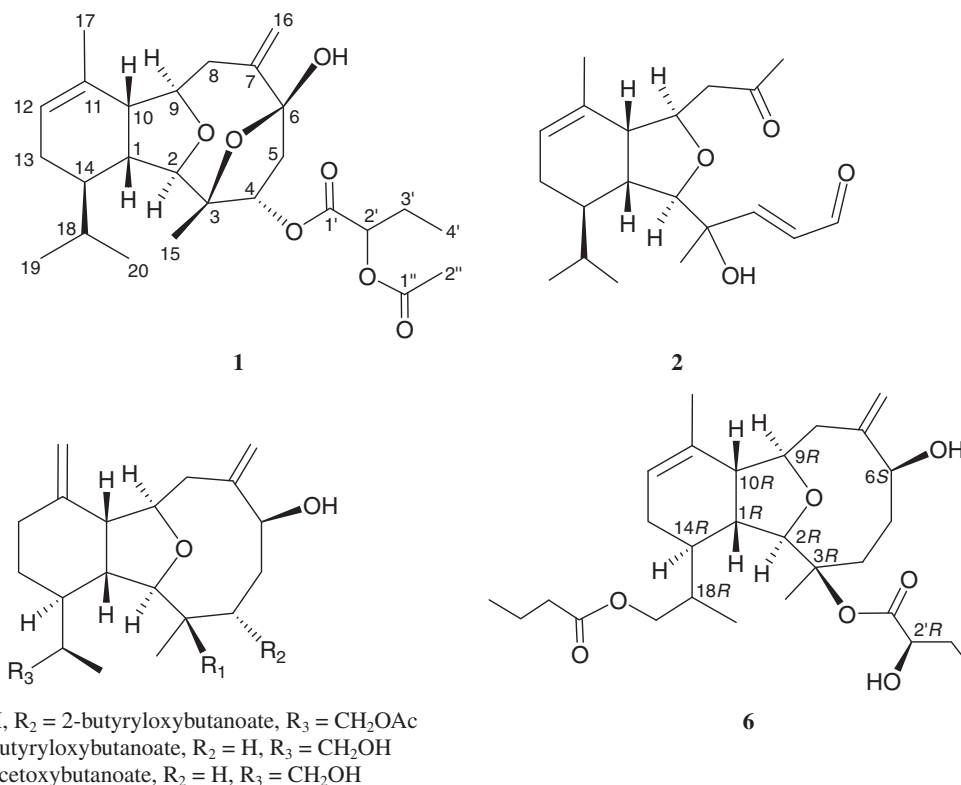
3 : R$_1$ = OH, R$_2$ = 2-butyryloxybutanoate, R$_3$ = CH$_2$OAc
4 : R$_1$ = 2-butyryloxybutanoate, R$_2$ = H, R$_3$ = CH$_2$OH
5 : R$_1$ = 2-acetoxybutanoate, R$_2$ = H, R$_3$ = CH$_2$OH

Chart 1.

degrees of unsaturation were accounted for, and the proposed skeleton of **1** was suggested to be a eunicellin-based metabolite with four rings.

From the ^{1}H–^{1}H COSY spectrum of **1** (Table 1), it was possible to identify the separate spin systems among H-1/H-2; H-4/H$_2$-5; H$_2$-8/H-9/H-10/H-1; H-12/H$_2$-13/H-14/H-1; H-14/H-18/H$_3$-19 (H$_3$-20), which were assembled with the assistance of an HMBC experiment (Table 1). The key HMBC correlations between the protons and quaternary carbons of **1**, such as H-5, H$_3$-15/C-3; H$_2$-5, H-8, H$_2$-16/C-6; H$_2$-8, H-9, H-16b/C-7; and H-9, H-10, H$_3$-17/C-11, permitted elucidation of the carbon skeleton. The location of the 2-acetoxybutanoate group in **1** was confirmed by an HMBC correlation between H-4 (δ_H 5.31) and the 2-acetoxybutanoate carbonyl (δ_C 170.1, s, C-1′) and further supported by the HMBC correlations between H-2′ (δ_H 4.91) and the 2-acetoxybutanoate carbonyl at 170.1 (s, C-1′) and acetate carbonyl at δ_C 170.7 (s, C-1″). Thus, the remaining hydroxy group should be positioned at C-6, an oxygenated quaternary carbon. The C-6 hydroxy group was concluded to be a part of a hemiketal constellation on the basis of a characteristic carbon signal at δ_C 104.4 (s, C-6). The HMBC correlations between H$_2$-5 (δ_H 2.98 and 2.49) and each of the two oxygenated low-field quaternary carbons at δ_C 104.4 (s, C-6) and 86.5 (s, C-3) suggested the presence of a C-3/6 ether linkage. The ether bridge between C-2 and C-9 was also supported by the HMBC correlations between H-2/C-9 and H-9/C-2. The vinyl methyl at C-11 was confirmed by the HMBC correlations between H$_3$-17/C-10, -11, -12 and further supported by the allylic coupling between the olefin proton H-12 and the vinyl methyl Me-17 in the ^{1}H–^{1}H COSY spectrum.
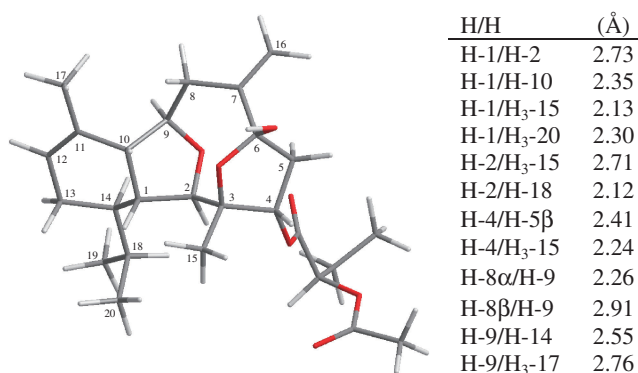
The relative configuration of **1** elucidated mainly by NOESY spectrum was compatible with those of **1** offered by computer modeling (Figure 1), in which the close contacts of atoms calculated in space were consistent with the NOESY correlations. In the NOESY experiment, H-1 correlated with H-10, H$_3$-15, and H$_3$-20, indicating that H-1, H-10, and H$_3$-15, and the isopropyl group were situated on the same face; they were assigned as β protons, as H-14 was α-oriented. H-2 showed correlations with H-1, H$_3$-15, and H-18; and a small coupling constant was found between H-1 and H-2 ($J = 2.4$ Hz), indicating that both the chiral centers C-2 and C-3 should be assigned as R^* form by modeling analysis. H-4 correlated with H$_3$-15 and one proton of C-5 methylene (δ_H 2.49), reflecting the α-orientation of 2-acetoxybutanoate at C-4. Furthermore, H-9 correlated with H$_2$-8, H-14, and H$_3$-17. From consideration of molecular models, H-9 was found to be reasonably close to H$_2$-8, H-14, and H$_3$-17, when H-9 was α-oriented in **1**. Based on the above findings, the structure, including the relative stereochemistry of **1** was established, and the chiral centers for the carbon skeleton of **1** were assigned as 1R^*, 2R^*, 3R^*, 4S^*, 6S^*, 9R^*, 10R^*, and 14R^*. By detailed analysis, the partial structure in the ten-membered ring of **1** was found to be similar with those of known eunicellin derivatives, hirsutalins B–D (**3–5**) (Chart 1), which were isolated from an octocoral, *Cladiella hirsuta*.[10] However, the stereochemistry of the acetoxy group in the 2-acetoxybutanoate moiety has not been determined at this stage.

Our present study also has led to the isolation of a new eunicellin **2** (cladielloide D). IR absorptions at 3423, 1715, and 1691 cm^{-1}, suggested the presence of hydroxy, ketone, and α,β-unsaturated aldehyde groups in **2**. The molecular formula

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Table 1. ^{1}H and ^{13}C NMR Data, ^{1}H–^{1}H COSY, and HMBC Correlations for **1**

Position	δ_H[a)]	δ_C[b)]	^{1}H–^{1}H COSY	HMBC (H → C)
1	2.46 m	42.0 (d)[d)]	H-2, H-10, H-14	n.o.[e)]
2	4.07 d (2.4)[c)]	83.6 (d)	H-1	C-9, -14
3		86.5 (s)		
4	5.31 t (9.2)	79.1 (d)	H$_2$-5	C-2, -15, -1′
5α	2.98 dd (12.8, 9.2)	43.4 (t)	H-4, H-5β	C-4, -6
β	2.49 dd (12.8, 9.2)		H-4, H-5α	C-3, -4, -6
6		104.4 (s)		
7		149.1 (s)		
8α	2.88 dd (14.4, 4.4)	41.9 (t)	H-8β, H-9	C-7, -9, -16
β	2.67 dd (14.4, 5.2)		H-8α, H-9	C-6, -7, -16
9	4.04 m	81.8 (d)	H$_2$-8, H-10	C-2, -7, -11
10	2.81 br s	46.2 (d)	H-1, H-9	C-1, -8, -9, -11
11		132.5 (s)		
12	5.44 m	121.5 (d)	H$_2$-13, H$_3$-17	n.o.
13α	2.03 m	22.7 (t)	H-12, H-13β, H-14	n.o.
β	1.85 m		H-12, H-13α, H-14	n.o.
14	1.34 m	38.8 (d)	H-1, H$_2$-13, H-18	n.o.
15	1.29 s	22.9 (q)		C-2, -3, -4
16a	5.03 s	114.9 (t)	H-16b	C-6, -8
b	5.39 s		H-16a	C-6, -7, -8
17	1.64 br s	22.3 (q)	H-12	C-10, -11, -12
18	1.82 m	28.2 (d)	H-14, H$_3$-19, H$_3$-20	C-19, -20
19	0.98 d (6.8)	21.7 (q)	H-18	C-14, -18, -20
20	0.80 d (6.8)	17.3 (q)	H-18	C-14, -18, -19
1′		170.1 (s)		
2′	4.91 dd (7.2, 5.2)	73.5 (d)	H$_2$-3′	C-1′, -3′, -4′, C-1″
3′	1.90 m	24.3 (t)	H-2′, H$_3$-4′	C-1′, -2′, -4′
4′	1.00 t (7.6)	9.4 (q)	H$_2$-3′	C-2′, -3′
1″		170.7 (s)		
2″	2.12 s	20.5 (q)		C-1″

a) Spectra measured at 400 MHz in CDCl$_3$ at 25 °C. b) Spectra measured at 100 MHz in CDCl$_3$ at 25 °C. c) J values (in hertz) in parentheses. d) Attached protons were deduced by DEPT and HMQC experiments. e) n.o.: not observed.

H/H	(Å)
H-1/H-2	2.73
H-1/H-10	2.35
H-1/H$_3$-15	2.13
H-1/H$_3$-20	2.30
H-2/H$_3$-15	2.71
H-2/H-18	2.12
H-4/H-5β	2.41
H-4/H$_3$-15	2.24
H-8α/H-9	2.26
H-8β/H-9	2.91
H-9/H-14	2.55
H-9/H$_3$-17	2.76

Figure 1. Key NOESY correlations and computer-generated perspective model using MM2 force field calculations for **1**.

$C_{20}H_{30}O_4$ was deduced from HR-ESI-MS at m/z 357.2044 (calcd for $C_{20}H_{30}O_4$ + Na, 357.2042). Inspection of the NMR data (Table 2) by the assistance of DEPT and HMQC spectra revealed the presence of five methyls, two sp^3 methylenes, six sp^3 methines (including two oxymethines), an sp^3 oxygenated quaternary carbon, a trisubstituted olefin, a 1,2-disubstituted double bond, and two carbonyls. The ^{1}H NMR spectrum also showed the presence of five methyls including a methyl attached to an oxygenated quaternary carbon (δ_H 1.32, 3H, s, H$_3$-15), a vinyl methyl (δ_H 1.67, 3H, d, J = 1.2 Hz, H$_3$-17), a methyl attached to a carbonyl carbon (δ_H 2.20, 3H, s, H$_3$-16), and two methyls of an isopropyl group (δ_H 0.98, 3H, d, J = 6.8 Hz; 0.82, 3H, d, J = 6.8 Hz, H$_3$-19 and H$_3$-20). Three proton signals at δ_H 6.35 (1H, dd, J = 15.6, 8.0 Hz, H-5), 6.89 (1H, d, J = 15.6 Hz, H-4), and 9.58 (1H, d, J = 8.0 Hz, H-6) were assigned as the α,β-olefinic protons and the aldehyde proton of the α,β-unsaturated aldehyde group containing a trans-disubstituted carbon–carbon double bond. By comparison of the ^{1}H and ^{13}C NMR data of **2** with those of **1**, it was found that resonances at δ_H 3.91 (H-2) and 4.00 (H-9) were attributed to the protons of two oxymethines in the tetrahydrofuran unit. The ^{13}C NMR spectrum showed signals at δ_C 207.8 (s, C-7), 193.7 (d, C-6), 162.4 (d, C-4), and 130.2 (d, C-5) further supporting the presence of a normal ketone and an α,β-unsaturated aldehyde. The chemical shifts of two methine protons, located at two ring-junction carbons of the six-membered ring and an ether ring, δ_H 2.50 (H-10) and 2.38 (H-1), were assigned based on the results of ^{1}H–^{1}H COSY and HMQC experiments. Based on the above observations and by analysis of ^{1}H–^{1}H COSY and HMBC spectral data as shown in Table 2, the molecular framework of **2** was established.

Table 2. ^1H and ^{13}C NMR Data, ^1H–^1H COSY, and HMBC Correlations for **2**

Position	δ_H[a]	δ_C[b]	^1H–^1H COSY	HMBC (H → C)
1	2.38 ddd (9.6, 8.0, 4.0)[c]	40.4 (d)[d]	H-2, H-10, H-14	n.o.[e]
2	3.91 d (4.0)	87.0 (d)	H-1	C-14
3		74.8 (s)		
4	6.89 d (15.6)	162.4 (d)	H-5	C-3, -6
5	6.35 dd (15.6, 8.0)	130.2 (d)	H-4, H-6	C-3
6	9.58 d (8.0)	193.7 (d)	H-5	C-5
7		207.8 (s)		
8a	2.75 dd (16.0, 6.8)	48.0 (t)	H-8b, H-9	C-7, -9
b	2.87 dd (16.0, 3.6)		H-8a, H-9	C-7, -9
9	4.00 ddd (8.0, 6.8, 3.6)	79.0 (d)	H$_2$-8, H-10	n.o.
10	2.50 br t (8.0)	47.4 (d)	H-1, H-9	C-8, -9, -11, -12, -14
11		129.9 (s)		
12	5.53 m	123.7 (d)	H$_2$-13, H$_3$-17	n.o.
13α	2.02 m	23.3 (t)	H-12, H-13β, H-14	n.o.
β	1.91 m		H-12, H-13α, H-14	C-14
14	1.46 m	38.9 (d)	H-1, H$_2$-13, H-18	C-13
15	1.32 s	23.7 (q)		C-2, -3, -4
16	2.20 s	30.9 (q)		C-7, -8
17	1.67 d (1.2)	22.7 (q)	H-12	C-10, -11, -12
18	1.76 m	27.6 (d)	H-14, H$_3$-19, H$_3$-20	C-19, -20
19	0.98 d (6.8)	21.8 (q)	H-18	C-14, -18, -20
20	0.82 d (6.8)	17.4 (q)	H-18	C-14, -18, -19
3-OH	3.66 s			C-15

a) Spectra measured at 400 MHz in CDCl$_3$ at 25 °C. b) Spectra measured at 100 MHz in CDCl$_3$ at 25 °C. c) *J* values (in hertz) in parentheses. d) Attached protons were deduced by DEPT and HMQC experiments. e) n.o.: not observed.

H/H	(Å)
H-1/H-10	2.63
H-2/H-14	2.71
H-2/H$_3$-15	2.35
H-8α/H-9	2.43
H-8β/H-9	2.36
H-9/H-14	2.75
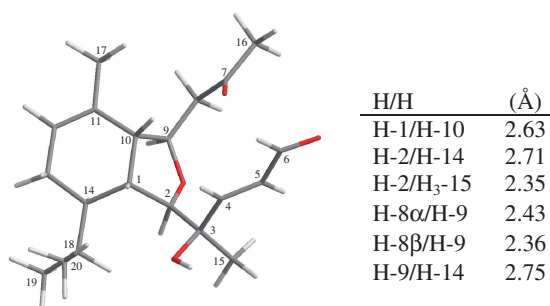
Figure 2. Key NOESY correlations and computer-generated perspective model using MM2 force field calculations for **2**.

The relative stereochemistry of **2** was determined mainly by a NOESY experiment and the results are illustrated in Figure 2. A correlation between H-1 with H-10, suggested that these two protons are on the same side of the molecule and assigned as β-oriented. The oxymethine proton H-2 exhibited a correlation with H-14. Thus, H-2 and H-14 should be positioned on the α face. H-9 showed correlations with H$_2$-8 and H-14 and this proton exhibited coupling with H$_2$-8 (*J* = 6.8, 3.6 Hz) and H-10 (*J* = 8.0 Hz), indicating that H-9 was α-oriented. Based on the above findings, the chiral centers of **2** were assigned as $1R^*$, $2R^*$, $9R^*$, $10R^*$, and $14R^*$. However, due to the free rotation of the carbon–carbon bond between C-2 and C-3, the stereochemistry of C-3 hydroxy group is not determined, although a correlation between H-2 and Me-15 was observed in the

NOESY spectrum of **2**. Because cladiellolides A–D were isolated from the same animal,[11] the stereochemistry at C2–C3 part of cladiellolide D was deduced to be same as that of cladiellolides A–C. Geometric optimization of **2** was performed with Chem3D Pro software. The conformation search suggested that the most stable conformation and the calculated minimum energy for **2** are shown in Figure 2. It was found that the calculated distances between those protons having key NOESY correlations of **2** are all shorter than 3 Å as shown in Figure 2. It is worth noting that the eunicellin metabolites possessing a cleavage bond between C-6/7 are rarely found. Cladielloide D (**2**) is the second 6,7-secoeunicellin ever discovered.[29]

In a previous study, the absolute configuration of a known eunicellin analog, hirsutalin A (**6**) (Chart 1),[10] which was isolated from the octocoral belonging the same genus *Cladiella* as that of eunicellins **1** and **2**, was determined using a modified Mosher's method. Thus, the new eunicellins **1** and **2** are assumed to have the same absolute configuration as **6** because these compounds were all isolated from the same genus collected from the tropical West Pacific Ocean.

The cytotoxicity of eunicellins **1** and **2** toward a limited panel of tumor cell lines, including CCRF-CEM (human T cell acute lymphoblastic leukemia), HL-60, DLD-1, and P388D1 cells was evaluated (Table 3). The results showed that cladielloide C (**1**) exhibited significant cytotoxicity toward CCRF-CEM cells. The in vitro anti-inflammatory effects of metabolites **1** and **2** were tested. Metabolites **1** and **2** displayed moderate inhibitory effects on superoxide anion generation by human neutrophils at 10 µg mL^{-1}, respectively (Table 4).

Table 3. Cytotoxic Data of Eunicellins **1** and **2**

Compound	Cell lines IC$_{50}$/µg mL^{-1} [a]			
	CCRF-CEM	HL-60	DLD-1	P388D1
1	3.6	12.6	8.5	8.3
2	11.6	>40	35.1	>40
Doxorubicin[b]	0.18	0.03	0.09	0.11

a) For significant activity of pure compounds, values of IC$_{50}$ ≤ 4.0 µg mL^{-1} are required. Please see Geran et al.[35] b) Doxorubicin was used as a reference compound.

Table 4. Inhibitory Effects of Eunicellins **1** and **2** on Superoxide Anion Generation and Elastase Release by Human Neutrophils in Response to FMLP/CB

Compound	Superoxide anion IC$_{50}$/µg mL^{-1} [a] or Inh %[b]	Elastase release IC$_{50}$/µg mL^{-1} or Inh %
1	36.7 ± 7.6[b]	27.2 ± 3.6[b]
2	31.4 ± 6.9[b]	10.7 ± 5.6[b]
DPI[c]	0.8 ± 0.2[a]	
Elastatinal[c]		30.8 ± 5.7[a]

a) Concentration necessary for 50% inhibition (IC$_{50}$). b) Percentage of inhibition (Inh %) at 10 µg mL^{-1}. c) DPI (diphenylene indonium) and elastatinal were used as reference compounds.

Experimental

General Experimental Procedures. Optical rotation values were measured with a JASCO-P1010 digital polarimeter. Infrared spectra were obtained on a VARIAN DIGLAB FTS 1000 FT-IR spectrometer. NMR spectra were recorded on a VARIAN MERCURY PLUS 400 FT-NMR at 400 MHz for ^{1}H and 100 MHz for ^{13}C in CDCl$_3$ at 25 °C. Proton chemical shifts were referenced to the residual CHCl$_3$ signal (δ_H 7.26). ^{13}C NMR spectra were referenced to the center peak of CDCl$_3$ at δ_C 77.1. ESI-MS and HR-ESI-MS data were recorded on BRUKER APEX II mass spectrometer. Column chromatography was performed on silica gel (230–400 mesh, Merck, Darmstadt, Germany). TLC was carried out on precoated Kieselgel 60 F$_{254}$ (0.25 mm, Merck) and spots were visualized by spraying with 10% H$_2$SO$_4$ solution followed by heating. HPLC was performed using a system comprised of a HITACHI L-7100 pump, a HITACHI L-7455 photodiode array detector, and a RHEODYNE 7725 injection port. A normal phase column (Hibra 250 × 10 mm, Merck, silica gel 60, 5 µm) was used for HPLC.

Animal Material. The octocoral *Cladiella* sp. were collected from Indonesia in 2004 and stored in a freezer until extraction. A voucher specimen was deposited in the National Museum of Marine Biology and Aquarium (NMMBA), Taiwan. This organism was identified by comparison with previous descriptions.[36,37]

Extraction and Isolation. Sliced bodies of *Cladiella* sp. (wet weight 402 g, dry weight 144 g) were extracted with a mixture of MeOH and CH$_2$Cl$_2$ (1:1). The extract was partitioned between EtOAc and H$_2$O. The EtOAc layer was separated on silica gel and eluted using *n*-hexane/EtOAc

(stepwise, 25:1–pure EtOAc) to yield the 19 fractions A–S. Fraction G was repurified by normal-phase HPLC, using the mixtures of *n*-hexane and EtOAc as a mobile phase to afford compound **2** (4:1). Fraction L was separated by normal-phase HPLC, using the mixtures of CH$_2$Cl$_2$ and acetone as a mobile phase to afford compound **1** (27:1).

Cladielloide C (1): Colorless oil (1.2 mg); $[\alpha]_D^{23}$ +220 (*c* 0.02, CHCl$_3$); IR (neat): ν_{max} 3449, 1745 cm^{-1}; ^{1}H (CDCl$_3$, 400 MHz) and ^{13}C (CDCl$_3$, 100 MHz) NMR data, see Table 1; ESI-MS: *m/z* 485 [M + Na]$^{+}$; HR-ESI-MS: *m/z* 485.2516 (calcd for C$_{26}$H$_{38}$O$_7$ + Na, 485.2515).

Cladielloide D (2): Colorless oil (2.1 mg); $[\alpha]_D^{23}$ +4 (*c* 0.11, CHCl$_3$); IR (neat): ν_{max} 3423, 1715, 1691 cm^{-1}; ^{1}H (CDCl$_3$, 400 MHz) and ^{13}C (CDCl$_3$, 100 MHz) NMR data, see Table 2; ESI-MS: *m/z* 357 [M + Na]$^{+}$; HR-ESI-MS: *m/z* 357.2044 (calcd for C$_{20}$H$_{30}$O$_4$ + Na, 357.2042).

Cytotoxicity Testing. The cytotoxicity of compounds **1** and **2** was assayed with a modification of the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method. Cytotoxicity assays were carried out according to procedures described previously.[38,39]

Molecular Mechanics Calculations. Implementation of the MM2 force filed[40] in Chem3D Pro software from Cambridge Soft Corporation, Cambridge, MA, USA (ver 9.0, 2005), was used to calculate molecular models.

Human Neutrophil Superoxide Anion Generation and Elastase Release. Human neutrophils were obtained by means of dextran sedimentation and Ficoll centrifugation. Superoxide generation and elastase release were carried out according to procedures described previously.[41,42] Briefly, superoxide anion production was assayed by monitoring the superoxide dismutase-inhibitable reduction of ferricytochrome *c*. Elastase release experiments were performed using MeO–Suc–Ala–Ala–Pro–Valp–nitroanilide as the elastase substrate.

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