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(+)-12-*epi*-FRAGILIDE G, A NEW CHLORINATED BRIARANE FROM THE SEA WHIP GORGONIAN CORAL *JUNCEELLA FRAGILIS*

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Abstract – Chemical investigation on the EtOAc-soluble fraction from the MeOH/DCM extract of a gorgonian coral *Junceella fragilis* afforded a new chlorinated briarane-type diterpenoid, (+)-12-*epi*-fragilide G (**1**) and this compound was found to be the enantiomer of (–)-12-*epi*-fragilide G (**2**). The structure of **1** was established on the basis of spectroscopic methods. Briarane **1** was found to inhibit the protein expression of pro-inflammatory iNOS in a murine macrophage-like cell line, RAW 264.7, stimulated with LPS and briarane **2** displayed inhibitory an effect on the generation of superoxide anion by human neutrophils.

The Formosan sea whip gorgonian coral *Junceella fragilis* (family Ellisellidae) (Ridley 1834)[1–3] comprises large quantities of briarane analogues, which often have complex structures and bioactivities.[4–21] Our current studies on the constituents of *J. fragilis*, a new chlorinated briarane diterpenoid, (+)-12-*epi*-fragilide G (**1**) (Chart 1), was isolated. In this report, we isolate and determine the structure of new briarane **1**, in addition to study its anti-inflammatory activity.

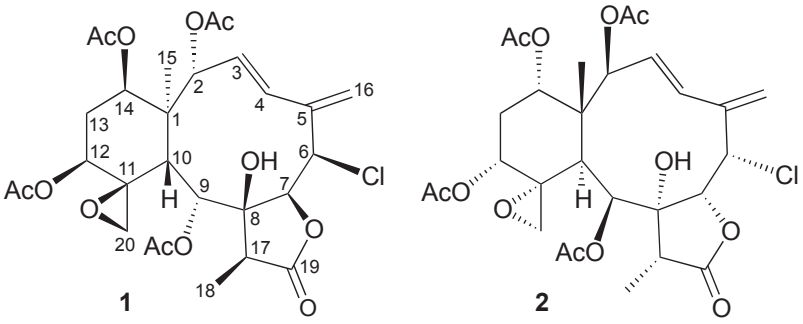

Chart 1. Structures of (+)-12-*epi*-fragilide G (**1**) and (–)-12-*epi*-fragilide G (**2**)

(+)-12-*epi*-Fragilide G (**1**) was obtained as an amorphous powder. The HRESIMS of **1** exhibited two *pseudo*-molecular peaks at *m/z* 621.17079 $(M + Na)^+$ and 623.16759 $(M + 2 + Na)^+$, accounted for a chlorine atom in the molecular formula, $C_{28}H_{35}ClO_{12}$, indicating 11 degrees of unsaturation. IR spectrum analysis showed that **1** had absorption peaks at 3551, 1775, and 1737 cm^{-1}, suggesting that the structure of **1** included hydroxy, γ-lactone, and ester groups. From the ^{13}C and ^1H NMR data of **1** (Table 1), a disubstituted olefin and an exocyclic carbon-carbon double bond were deduced from the signals of four carbons at δ_C 130.3 (CH), 132.7 (CH), 142.0 (C), and 115.1 (CH_2); and further supported by four olefin proton signals at δ_H 6.87 (1H, d, J = 16.0 Hz), 6.00 (1H, d, J = 16.0, 9.6 Hz), 5.33 (1H, s), and 5.25 (1H, s). Moreover, five carbonyl resonances at δ_C 174.7, 170.3, 170.0, 169.9, and 169.6, confirmed the presence of a γ-lactone and four other esters in **1**. In the ^1H NMR spectrum of **1**, four acetate methyls (δ_H 2.10, 2.09, 2.04, and 2.01, each 3H × s) were observed. An exocyclic epoxy group was confirmed from the signals of two oxygenated carbons at δ_C 57.2 (C) and 49.2 (CH_2). The chemical shifts of methylene protons at δ_H 2.77 (1H, dd, J = 3.2, 1.2 Hz) and 2.63 (1H, d, J = 3.2 Hz) confirmed the presence of this group.

The ^1H–^1H COSY spectrum of **1** revealed ^1H NMR coupling information showing the existence of H-2/H-3/H-4, H-6/H-7, H-9/H-10, H-12/H_2-13/H-14, and H-17/H_3-18 units, which were established with the assistance of an HMBC experiment (Table 1). Additionally, this experiment enabled clarification of the correlations between protons and quaternary carbons of **1**, such as H-2, H-3, H-9, H-10, H-13α, H-14, H_3-15/C-1; H-3, H-4, H-6, H_2-16/C-5; H-6, H-9, H-10, H-17, H_3-18/C-8; H-9, H-10, H-13α, H_2-20/C-11; and H-17, H_3-18/C-19, which provided conformation of the carbon skeleton. An exocyclic double bond at

C-5 was confirmed by the HMBC correlations between H_2-16/C-4, C-5, C-6; H-4/C-16; H-6/C-16; and further confirmed by the ^1H–^1H COSY correlations between H-4/H_2-16 and H-6/H_2-16 (by allylic coupling). The epoxy group positioned at C-11/20 was confirmed by the connectivity between H_2-20/C-11, C-12. The C-15 methyl group positioned at C-1 from the HMBC correlations between H_3-15/C-1, C-2, C-10, C-14; and H-2, H-10/C-15. In addition, the HMBC correlations also revealed that the acetoxy groups should attach at C-2, C-9, and C-14, respectively (Table 1). The hydroxy proton signal at δ_H 3.13 was revealed by its HMBC correlations to C-7, C-8, and C-9, indicating its attachment to C-8, an oxygenated quaternary carbon at δ_C 82.8. The methine unit at δ_C 65.0 was correlated with the methine proton at δ_H 5.06 in the HSQC spectrum; this proton also showed a ^3J-correlation with H-7 (δ_H 4.15) in the ^1H–^1H COSY spectrum, proving the attachment of a chlorine atom at C-6. Thus, the remaining acetoxy group should be positioned at C-12, as indicated by analysis of the ^1H–^1H COSY correlations and characteristic NMR signals analysis. These data together with the HMBC correlations between H-17/C-8, C-9, C-18, C-19, unambiguously established the molecular framework of **1**.

Table 1. ^1H and ^{13}C NMR data, ^1H–^1H COSY and HMBC correlations for briarane **1**

C/H	δ_H[a] (*J* in Hz)	δ_C, Mult.[b]	^1H–^1H COSY	HMBC (H→C)
1		49.1, C		
2	5.69 d (9.6)	75.7, CH	H-3	C-1, C-3, C-4, C-10, C-14, C-15, acetate carbonyl
3	6.00 dd (16.0, 9.6)	130.3, CH	H-2, H-4	C-1, C-2, C-5
4	6.87 d (16.0)	132.7, CH	H-3, H_2-16	C-2, C-3, C-5, C-6, C-16
5		142.0, C		
6	5.06 d (3.6)	65.0, CH	H-7, H_2-16	C-4, C-5, C-7, C-8, C-16
7	4.15 d (3.6)	80.6, CH	H-6	C-6, C-9
8		82.8, C		
9	5.17 d (2.0)	72.1, CH	H-10	C-1, C-8, C-10, C-11, C-17, acetate carbonyl
10	3.83 br s	33.7, CH	H-9	C-1, C-8, C-9, C-11, C-15
11		57.2, C		
12	4.51 dd (2.8, 2.8)	73.7, CH	H_2-13	n. o.[c]
13α	2.29 ddd (16.8, 2.8, 2.8)	29.0, CH_2	H-12, H-13β, H-14	C-1, C-11
β	2.00 ddd (16.8, 2.8, 2.8)		H-12, H-13α, H-14	C-12
14	4.97 dd (2.8, 2.8)	73.0, CH	H_2-13	C-1, C-10, C-12, acetate carbonyl
15	1.16 s	14.4, CH_3		C-1, C-2, C-10, C-14
16a/b	5.33 s; 5.25 s	115.1, CH_2	H-4, H-6	C-4, C-5, C-6
17	2.84 q (6.8)	50.0, CH	H_3-18	C-8, C-9, C-18, C-19
18	1.24 d (6.8)	6.8, CH_3	H-17	C-8, C-17, C-19
19		174.7, C		
20a/b	2.77 dd (3.2, 1.2); 2.63 d (3.2)	49.2, CH_2		C-11, C-12
2-OAc		169.9, C		
	2.01 s	21.1, CH_3		acetate carbonyl
9-OAc		170.3, C		
	2.10 s	21.4, CH_3		acetate carbonyl
12-OAc		169.6, C		
	2.04 s	20.9, CH_3		acetate carbonyl
14-OAc		170.0, C		
	2.09 s	21.1, CH_3		acetate carbonyl
8-OH	3.13 s			C-7, C-8, C-9

[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] n. o. = not observed.

In a previous study, the ^{13}C chemical shifts of exocyclic 11,20-epoxy groups in briarane analogues were summarized, that while the ^{13}C NMR data for C-11 and C-20 were appeared at δ_C 55–61 and 47–52 ppm, respectively, the cyclohexane ring should be existed in chair conformation.[10] Based on the above observations, the configuration of cyclohexane ring in **1** was existed in a chair conformation. The relative stereochemistry of **1** was elucidated mainly from the interactions observed in a NOESY experiment (Figure 1) and by the vicinal ^1H–^1H coupling constants. As per convention while analyzing the stereochemistry of **1**, H_3-15 and H-10 were assigned to the α and β face, anchoring the stereochemical analysis because no correlation was observed between H_3-15 and H-10. In the NOESY experiment of **1**, H-10 gave correlations to H-2, H-9, OH-8, and H_3-18, suggesting that these protons were located on the same face and assigned as β protons, since C-15 methyl is the α-substituent at C-1. H-14 was found to exhibit responses with H-2, H-13α/β, and H_3-15, but not with H-10, revealing the α-orientation of this proton. In addition, H-12 was found to correlate with H-13α/β and one proton of C-20 methylene (δ_H 2.77, H-20a), indicating the C-12 acetoxy group was β-oriented. H-7 exhibited correlations with H-6 and H-17, suggesting that these protons were positioned on α face in **1**. The *trans* geometry of C-3/4 double bond was indicated by a 16.0 Hz coupling constant between H-3 (δ_H 6.00) and H-4 (δ_H 6.87). Moreover, the olefin proton H-3 showed a correlation with H_3-15 and one proton of C-16 methylene (δ_H 5.33, H-16a), but not with H-2; and H-4 showed responses with H-2 and OH-8, demonstrating the *E*-configuration of $\Delta^{3,4}$ and established the conjugated *s-cis* diene moiety in **1**. Based on the above findings, the structure of **1** was established, and the configurations of all stereogenic centers of **1** are assigned as 1*S**, 2*R**, 6*R**, 7*S**, 8*S**, 9*R**, 10*R**, 11*S**, 12*S**, 14*R**, and 17*S**.

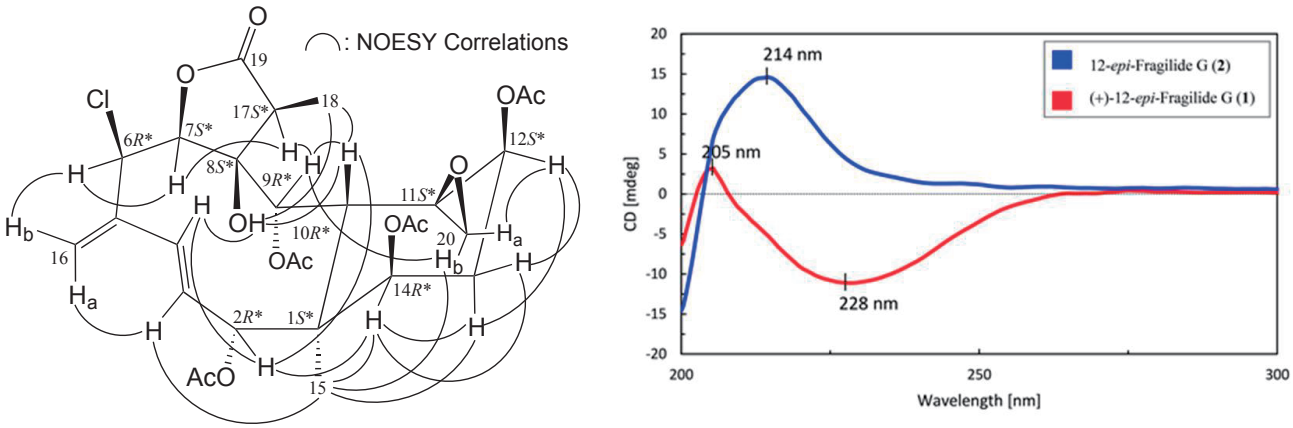

Figure 1. Selected protons with key NOESY correlations of **1** and the CD spectra of **1** and **2**

The spectral (^1H, ^{13}C NMR, and IR spectra) and physical (melting point) data of **1** were full agreement with those of a known briarane diterpenoid, 12-*epi*-fragilide G (**2**), which was isolated from the gorgonian coral *Ellisella robusta*.[22] However, the optical rotation value of **1** ($[\alpha]_D^{22}$ +10, *c* 0.8, $CHCl_3$) was

substantially different from that of **2** ($[\alpha]_D^{22}$ –28, *c* 0.02, $CHCl_3$);[22] indicating that **1** is an enantiomer of **2**. The CD spectrum of (+)-12-*epi*-fragilide G (**1**) in methanol displayed a negative Cotton effect at 228 nm. 12-*epi*-Fragilide G (**2**) displayed a positive Cotton effect at 214 nm. This highlights confirmed the different configurations of briaranes **1** and **2** (Figure 1). On the basis of these facts, compound **1** must be the enantiomer of 12-*epi*-fragilide G, and thus we assigned **1** as (+)-12-*epi*-fragilide G. Additional example of antipodal isolation within this class of compounds include junceellolide A, which was isolated in both enantiomeric forms from *J. fragilis*.[23,24]

Anti-inflammatory activity assays using an *in vitro* cell culture model were performed in this study, and western blot analysis was employed to evaluate the changes in pro-inflammatory inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) proteins in a lipopolysaccharide (LPS)-stimulated pro-inflammatory response in a murine macrophage RAW264.7 cell line. As compared with cells stimulated with LPS alone, macrophages treated with a concentration of 10 μM, briarane **1** reduced level of iNOS to 54.7% (Table 2 and Figure 2). Using trypan blue staining to measure the cytotoxic effect of

Table 2. Effects of briarane **1** on LPS-induced iNOS and COX-2 protein expressions in macrophages

Compounds	iNOS	COX-2	β-Actin
	Expression (% of LPS group)	Expression (% of LPS group)	Expression (% of LPS group)
Control	1.0 ± 0.2	8.6 ± 1.7	86.1 ± 8.8
LPS	100.0 ± 11.3	100.0 ± 3.2	100.0 ± 0.1
1	54.7 ± 4.9	106.2 ± 3.7	77.0 ± 16.8
Dexamethasone[a]	30.8 ± 6.7	9.1 ± 0.7	100.9 ± 3.1

[a] Dexamethasone (10 μM) was used as a positive control.

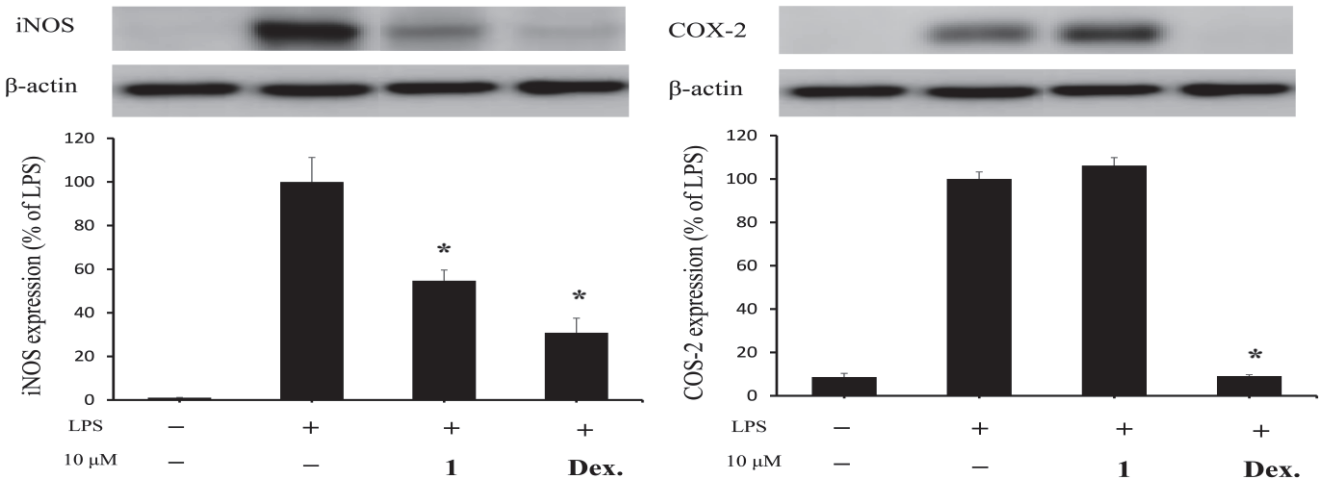

Figure 2. Effects of compound **1** on the expression of pro-inflammatory iNOS and COX-2 proteins in murine RAW264.7 macrophage cell. Using immunoblot analysis, briarane **1** was demonstrated to reduce LPS-induced expressions of iNOS. Data were normalized to those of cells treated with LPS alone, and cells treated with dexamethasone (10 μM) were used as a positive control. Data are expressed as the mean ± SEM ($n = 4$). * Significantly different from cells treated with LPS ($p < 0.05$).

the compound **1**, it was observed that briarane **1** did not induce significant cytotoxicity in RAW264.7 macrophage cells.

Furthermore, briarane **2** showed a 74.5% inhibitory effect on human neutrophils in terms of the generation of superoxide anions at a concentration of 20 μM (Table 3). These results implied that the configurations of briaranes **1** and **2** played important roles in determining the activity of the compounds.

Table 3. Inhibitory effects of briaranes **1** and **2** on superoxide anion generation and elastase release by human neutrophiles in response to fMet-Leu-Phe/Cytochalastin B

Compounds	Superoxide Anions		Elastase Release	
	Inh % (10 μM)[a]	Inh % (20 μM)[a]	Inh % (10 μM)	Inh % (20 μM)
1	3.6 ± 2.3	34.8 ± 4.1 **	4.6 ± 0.9 **	19.4 ± 2.4 **
2	18.7 ± 3.0 **	74.5 ± 3.7 ***	14.0 ± 4.0 *	26.2 ± 5.1 **
Genistein[b]	80.0 ± 5.6 ***		40.3 ± 3.4 ***	

[a] Percentage of inhibition (Inh %) at 10 and 20 μM concentration. Results are presented as meas ± S.E.M. (n = 3 or 4). *p < 0.05, **p < 0.01, ***p < 0.001 compared with the control. (fMLP/CB), basal (solvent), or fMLP. [b] Genistein was used as a positive control.

EXPERIMENTAL

General Experimental Procedures. Melting points of the natural products were determined using FARGO apparatus and the values were uncorrected. Optical rotation values were measured using a JASCO P-1010 digital polarimeter. Circular dichroism (CD) spectra were recorded using a JASCO J-815 CD spectrometer. IR spectra were obtained with a THERMO SCIENTIFIC NICOLET iS5 FT-IR spectrophotometer. NMR spectra were recorded on a 400 MHz JEOL ECZ 400S NMR spectrometer using the residual $CHCl_3$ signal (δ_H 7.26 ppm) and $CDCl_3$ (δ_C 77.1 ppm) as the internal standard for ^1H NMR and ^{13}C NMR; coupling constants (J) are presented in Hz. ESIMS and HRESIMS were recorded using a BRUKER 7 Tesla solariX FTMS system. Column chromatography was carried out with silica gel (230–400 mesh, Merck). TLC was performed on plates precoated Kieselgel 60 F_{254} (0.25-mm-thick, Merck) and the plates were sprayed with 10% H_2SO_4 solution followed by heating to visualize the spots. Normal-phase HPLC (NP-HPLC) was performed using a HPLC system equipped with a HITACHI L-7110 pump and a RHEODYNE 7725 injection port. A semi-preparative normal-phase column (Supelco Ascentis Si, Cat #:581515-U, 25 cm × 21.2 mm, 5 μm; Sigma-Aldrich) was used for HPLC.

Animal Material. Specimens of the gorgonian coral *Junceella fragilis* were collected by hand by scuba divers off the coast of Southern Taiwan in June, 2017. The freshly-harvested samples were stored in a –20 °C freezer until used for extraction. A sample was deposited in the National Museum of Marine Biology and Aquarium, Taiwan, as a voucher specimen (NMMBA-TW-GC-2017-017).

Extraction and Isolation. Sliced bodies of *J. fragilis* (wet weight 929 g, dry weight 374 g) were extracted with a mixture of MeOH and CH_2Cl_2 (v:v = 1:1). The extract (20.3 g) was partitioned between

EtOAc and H_2O. The EtOAc layer (8.7 g) was separated on silica gel and eluted with *n*-hexane/EtOAc/MeOH (stepwise, v:v:v = 100:0:0 to 100% MeOH) to yield fourteen subfractions A–N. Fraction I was purified by NP-HPLC using a mixture of *n*-hexane/acetone (v:v = 3:1 at a flow rate of 5.0 mL/min) to yield (+)-12-*epi*-fragilide G (**1**) (26.7 mg).

(+)-12-*epi*-Fragilide G (1): amorphous powder; mp 235–237 °C; $[\alpha]_D^{23}$ +10 (*c* 1.05, $CHCl_3$); IR (neat) ν_{max} 3551, 1775, 1737 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data, see Table 1; ESIMS *m*/*z* 621 $(M + Na)^+$, 623 $(M + 2 + Na)^+$; HRESIMS *m*/*z* 621.17079 (Calcd for $C_{28}H_{35}{}^{35}ClO_{12}$ + Na, 621.17093).

***In vitro* Anti-inflammatory Assay.** RAW264.7 (TIB-71) cells was purchased from the American Type Culture Collection (ATCC, No. TIB-71) (Manassas, VA, USA).[25–28] The *in vitro* anti-inflammatory activity of compound **1** was measured by investigating their inhibition effects on LPS-induced pro-inflammatory iNOS protein expressions in the macrophage cell line using Western blot analysis.[28–30] Briefly, an inflammation response in RAW264.7 cells was induced by incubating cells in medium containing only LPS (10 μM) without test compounds for 16 h. For the anti-inflammatory activity assay, compound **1** was added to the cells 10 min before LPS treatment. After incubation, the cells were lysed and the protein lysates analyzed by Western blotting. The protein expression levels were determined based on the immunoreactivity of proteins to antibodies, and were calculated with respect to the average optical density of the corresponding LPS-stimulated cells. Moreover, the effects of compound **1** on the viability of RAW 264.7 cells were also evaluated by the trypan blue exclusion test. For statistical analysis, the data were analyzed by one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls *post hoc test* for multiple comparisons. A significant difference was defined as a *p*-value of < 0.05.

Superoxide Anion Generation and Elastase Release by Human Neutrophils. Human neutrophils were obtained by means of dextran sedimentation and Ficoll centrifugation. Measurements of superoxide anion generation and elastase release were carried out according to previously described procedures. 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.[31,32]

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