

Article

# 11 $\beta$ ,20 $\beta$ -Epoxybriaranes from the Gorgonian Coral *Junceella fragilis* (Ellisellidae)

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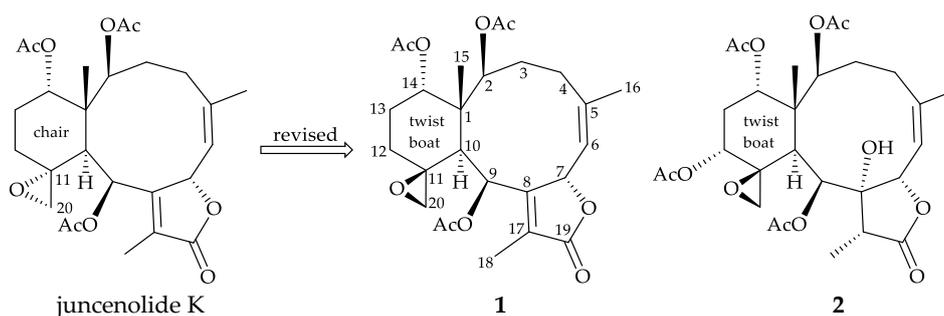
**Abstract:** Two 11,20-epoxybriaranes, including a known compound, juncenolide K (**1**), as well as a new metabolite, fragilide X (**2**), have been isolated from gorgonian *Junceella fragilis* collected off the waters of Taiwan. The absolute configuration of juncenolide K (**1**) was determined by single-crystal X-ray diffraction analysis for the first time in this study and the structure, including the absolute configuration of briarane **2** was established on the basis of spectroscopic analysis and compared with that of model compound **1**. One aspect of the stereochemistry of the known compound **1** was

revised. Briarane **2** was found to enhance the generation of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) release from RAW 264.7 cells.

**Keywords:** *Junceella fragilis*; fragilide; briarane; juncenolide; X-ray; iNOS; COX-2

## 1. Introduction

Gorgonian corals of the genus *Junceella* (family Ellisellidae) [1–3] were proven to be the most important flagship species to produce 11,20-epoxybriarane diterpenoids, a chemical marker for the octocorals belonging to the family Ellisellidae [4,5] and the compounds of this type demonstrate a wide spectrum of biological properties, such as anti-inflammatory activity [6–20], immunomodulatory activity [21], insecticidal activity [22], cytotoxicity [23–32], anti-viral activity [6,33], anti-fouling activity [34–37], antifeedant [35], and anti-microbial activity [28,29,32,38–40]. From the specimens of *J. fragilis* (Ridley 1884) collected off the waters of Taiwan, an area with high biodiversity at the intersection of the Kuroshio current and the South China Sea surface current, we have isolated two briaranes, including a known compound juncenolide K (**1**) [13], along with a new briarane–fragilide X (**2**), featuring an 11,20-epoxy moiety in their structures (Figure 1). A pro-inflammatory assay was employed to assess the activity of these isolates on the release of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) from RAW 264.7 macrophage cells.



**Figure 1.** The structures of juncenolide K and its revised structure (**1**) and fragilide X (**2**).

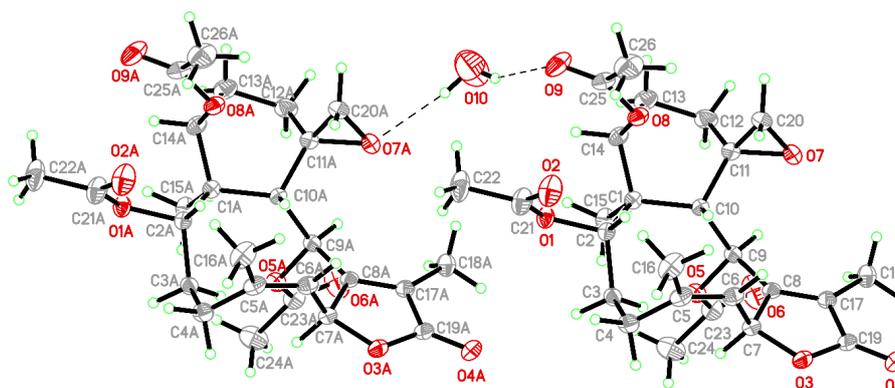
## 2. Results and Discussion

Compound **1** was isolated as a colorless prism that showed a sodiated adduct ion  $[M + Na]^+$  at  $m/z$  513.20949 in the (+)-high-resolution electrospray ionization mass spectrum (HRESIMS) analysis. The result revealed that **1** had a molecular formula of  $C_{26}H_{34}O_9$  (calculated for  $C_{26}H_{34}O_9 + Na$ , 513.20950) (unsaturation degrees = 10). The NMR chemical shifts for **1** and its proton coupling data are identical to those reported for juncenolide K [13] (Table 1). Juncenolide K was initially assigned possessing an  $11\alpha,20\alpha$ -epoxy configuration, and the cyclohexane ring was reported to exist with a chair conformation, but on the basis of our study of juncenolide K by a single-crystal X-ray diffraction analysis (Figure 2) and spectroscopic analysis (Table 1 and Figure 3) (Supplementary Materials, Figures S1–S14), it appears that the 11,20-epoxy group in **1** was found to be  $11\beta,20\beta$ -oriented and **1** possesses a cyclohexane ring in twist-boat form. The X-ray structure shows the twist-boat conformation of the cyclohexane ring in **1** and the Oak Ridge Thermal Ellipsoid Plot (ORTEP) diagram (Figure 2) showed that the absolute configurations of the stereogenic centers of **1** are  $1S,2S,7S,9S,10S,11S$  and  $14S$  (Flack parameter  $x = 0.07(5)$ ).

**Table 1.**  $^1\text{H}$  and  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ) data for juncenolide K and briarane 1.

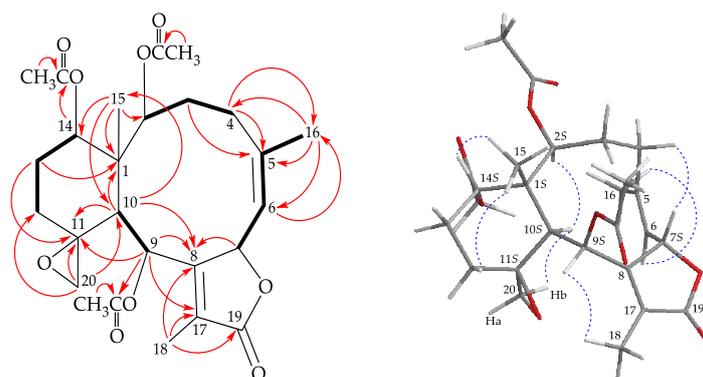
Position	Juncenolide K <sup>a</sup>		1	
	$\delta_{\text{H}}$ (J in Hz) <sup>b</sup>	$\delta_{\text{C}}$ , <sup>c</sup> type	$\delta_{\text{H}}$ (J in Hz) <sup>d</sup>	$\delta_{\text{C}}$ , <sup>e</sup> type
1		46.0, C		46.0, C
2	4.74 br s	74.1, CH	4.72 br s	74.1, CH
3	2.53–2.59 m; 1.74–1.80 m	31.3, CH <sub>2</sub>	2.56 m; 1.74 m	31.3, CH <sub>2</sub>
4	2.60–2.66 m; 2.20–2.26 m	29.1, CH <sub>2</sub>	2.60 m; 2.22 m	29.1, CH <sub>2</sub>
5		143.0, C		143.0, C
6	5.01 d (8.5)	124.7, CH	4.99 d (8.4)	124.8, CH
7	5.51 d (8.5)	77.2, CH	5.50 d (8.4)	77.1, CH
8		155.8, C		155.8, C
9	6.54 d (7.0)	66.5, CH	6.52 d (7.2)	66.6, CH
10	2.63–2.68 m	40.5, CH	2.64 br d (7.2)	40.5, CH
11		59.7, C		59.7, C
12	2.30–2.36 m; 1.10–1.16 m	22.9, CH <sub>2</sub>	2.31 m; 1.12 m	22.9, CH <sub>2</sub>
13	2.11–2.17 m; 1.80–1.86 m	23.8, CH <sub>2</sub>	2.10 m; 1.82 m	23.8, CH <sub>2</sub>
14	4.80 d (3.5)	73.9, CH	4.79 d (3.6)	73.9, CH
15	1.16 s	15.8, CH <sub>3</sub>	1.15 s	15.9, CH <sub>3</sub>
16	2.01 s	27.0, CH <sub>3</sub>	1.99 s	27.0, CH <sub>3</sub>
17		127.5, C		127.4, C
18	2.01 s	9.3, CH <sub>3</sub>	1.99 s	9.3, CH <sub>3</sub>
19		173.6, C		173.6, C
20a/b	2.60–2.66 m; 2.46–2.52 m	58.2, CH <sub>2</sub>	2.62 br s; 2.48 br s	58.2, CH <sub>2</sub>
OAc-2		170.7, C		170.7, C
	2.01 s	21.0, CH <sub>3</sub>	2.00 s	21.0, CH <sub>3</sub>
OAc-9		168.9, C		168.9, C
	2.12 s	21.6, CH <sub>3</sub>	2.11 s	21.7, CH <sub>3</sub>
OAc-14		169.8, C		169.8, C
	1.96 s	20.9, CH <sub>3</sub>	1.95 s	20.9, CH <sub>3</sub>

<sup>a</sup> Data were reported by Wang et al., see ref. [13]. <sup>b</sup> 500 MHz, <sup>c</sup> 125 MHz, <sup>d</sup> 400 MHz, <sup>e</sup> 100 MHz.

**Figure 2.** The Oak Ridge Thermal Ellipsoid Plot (ORTEP) of 1.

Fragilide X (**2**) was isolated as an amorphous powder and displayed a sodiated adduct ion  $[\text{M} + \text{Na}]^+$  at  $m/z$  589.22576 in the (+)-HRESIMS, indicating a molecular formula  $\text{C}_{28}\text{H}_{38}\text{O}_{12}$  (calculated for  $\text{C}_{28}\text{H}_{38}\text{O}_{12} + \text{Na}$ , 589.22555) (unsaturation degrees = 10). Absorption peaks at  $3333\text{ cm}^{-1}$ ,  $1773\text{ cm}^{-1}$ , and  $1742\text{ cm}^{-1}$  in the IR spectrum indicate hydroxy,  $\gamma$ -lactone, and ester groups, respectively. Analysis of the  $^1\text{H}$ ,  $^{13}\text{C}$  NMR, and distortionless enhancement by polarization transfer (DEPT) spectra, together with the molecular formula, suggested that there must be an exchangeable proton. The  $^{13}\text{C}$  NMR spectrum (Table 2), in combination with DEPT, HSQC, and HMBSC spectra, revealed the presence of five esters including four acetoxy groups ( $\delta_{\text{C}}$  21.6, 21.0, 20.9, 20.7,  $4 \times \text{CH}_3$ ;  $\delta_{\text{C}}$  170.6, 169.8, 169.4, 169.2,  $4 \times \text{C}$ ) and a lactone moiety ( $\delta_{\text{C}}$  176.2), and a trisubstituted olefin ( $\delta_{\text{C}}$  143.8, C-5; 120.8, CH-6). Based on the  $^{13}\text{C}$  NMR data and numbers of unsaturation, **2** was established as a tetracyclic diterpenoid. The

presence of an exocyclic epoxy group was confirmed from the signals of an oxygenated quaternary carbon at  $\delta_C$  62.3 (C-11) and an oxymethylene at  $\delta_C$  53.9 (CH<sub>2</sub>-20). The chemical shifts of oxymethylene protons at  $\delta_H$  3.20 (1H, d,  $J = 4.4$  Hz, H-20a) and 2.90 (1H, d,  $J = 4.4$  Hz, H-20b) further supported the presence of this group. Moreover, a methyl singlet, two methyl doublets (including a vinyl methyl), three pairs of sp<sup>3</sup> methylene protons, two sp<sup>3</sup> methine protons, five oxymethine protons, an sp<sup>2</sup> methine proton, four acetate methyls, and a hydroxy proton were observed in the <sup>1</sup>H NMR spectrum (Table 2).



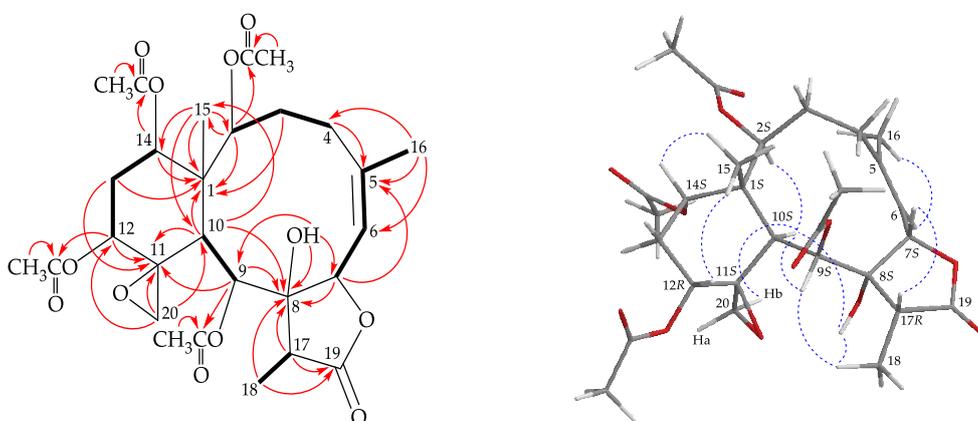
**Figure 3.** The COSY (—) correlations, selective HMBC correlations (↪), and selective protons with key NOESY (⋯) correlations of **1**.

**Table 2.** <sup>1</sup>H and <sup>13</sup>C NMR (CDCl<sub>3</sub>) data for **2**.

Position	$\delta_H$ <sup>a</sup> (J in Hz)	$\delta_C$ , <sup>b</sup> Type
1		46.8, C
2	4.74 d (4.8)	74.4, CH
3 $\alpha$ / $\beta$	1.67 m; 2.43 ddd (16.0, 16.0, 4.4)	32.1, CH <sub>2</sub>
4 $\alpha$ / $\beta$	2.52 br d (16.0); 2.07 m	28.7, CH <sub>2</sub>
5		143.8, C
6	5.61 br d (10.0)	120.8, CH
7	5.14 dd (10.0, 1.2)	77.7, CH
8		80.2, C
9	5.67 d (5.6)	67.3, CH
10	2.58 d (5.6)	39.9, CH
11		62.3, C
12	5.40 dd (8.4, 8.4)	62.0, CH
13 $\alpha$ / $\beta$	1.58 m; 2.70 m	32.6, CH <sub>2</sub>
14	4.85 d (4.4)	73.6, CH
15	1.16 s	15.3, CH <sub>3</sub>
16	2.03 d (1.2)	28.1, CH <sub>3</sub>
17	2.35 q (7.2)	42.3, CH
18	1.15 d (7.2)	6.7, CH <sub>3</sub>
19		176.2, C
20a/b	3.20 d (4.4); 2.90 d (4.4)	53.9, CH <sub>2</sub>
OH-8	4.57 d (1.2)	
OAc-2		170.6, C
	2.01 s	21.0, CH <sub>3</sub> <sup>c</sup>
OAc-9		169.2, C
	2.22 s	21.6, CH <sub>3</sub>
OAc-12		169.4, C
	1.98 s	20.7, CH <sub>3</sub> <sup>c</sup>
OAc-14		169.8, C
	2.02 s	20.9, CH <sub>3</sub> <sup>c</sup>

<sup>a</sup> 400 MHz, <sup>b</sup> 100 MHz, <sup>c</sup> Data exchangeable.

The  $^1\text{H}$  NMR coupling information in the correlation spectroscopy analysis enabled the determination of five different spin systems, H-2/H<sub>2</sub>-3/H<sub>2</sub>-4, H-6/H-7, H-9/H-10, H-12/H<sub>2</sub>-13/H-14, and H-17/H<sub>3</sub>-18, which were assembled with the assistance of an HMBC experiment (Figure 4). The HMBC correlations between protons and quaternary carbons, such as H-2, H-3 $\beta$ , H-10, H-13 $\alpha$ , H-14, H<sub>3</sub>-15 to C-1; H<sub>2</sub>-4, H-7, H<sub>3</sub>-16 to C-5; H-7, H-9, H-10, H-17, H<sub>3</sub>-18 to C-8; H-9, H-10, H-12, H<sub>2</sub>-20, H<sub>2</sub>-13 to C-11; and H-17, H<sub>3</sub>-18 to C-19, respectively, permitted elucidation of the carbon skeleton of 2. A methyl at C-5 was confirmed by the HMBC correlations between H<sub>3</sub>-16 to C-4, C-5, and C-6; and further confirmed by an allylic coupling between H-6/H<sub>3</sub>-16 ( $J = 1.2$  Hz). The methyl group Me-15 on C-1 was substantiated by the HMBC correlations from H<sub>3</sub>-15 to C-1, C-2, C-10, C-14; and H-2, H-10 to C-15, respectively. The epoxy group at C-11/20 was confirmed by the HMBC correlations between H<sub>2</sub>-20 to C-10, C-11, C-12. The hydroxy group at C-8 was deduced from the HMBC correlations of a hydroxy proton at  $\delta_{\text{H}}$  4.57 to C-7, C-8, and C-9. Moreover, HMBC correlations from the oxymethine protons at  $\delta_{\text{H}}$  4.74 (H-2), 5.67 (H-9), 5.40 (H-12), and 4.85 (H-14) to the acetate carbonyls at  $\delta_{\text{C}}$  170.6, 169.2, 169.4, and 169.8, placed the acetate groups on C-2, C-9, C-12, and C-14, respectively.



**Figure 4.** The COSY (—) correlations, selective HMBC correlations (↔), and selective protons with key NOESY (⋯) correlations of 2.

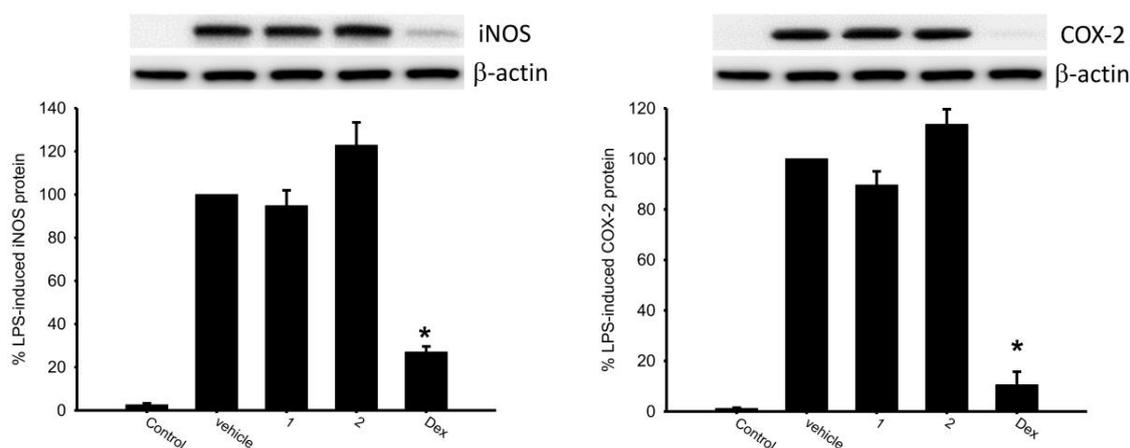
The stereochemistry of 2 was determined by NOE correlations observed in a NOESY experiment (Figure 4) and possible biogenetic considerations. The NOE correlations of H-10/H-2, H-10/OH-8, H-10/H-9, and H-10/H-20b indicated that these protons are situated on the same face of the structure and were assigned as the  $\alpha$  protons since the C-15 methyl is the  $\beta$ -substituent at C-1. Meanwhile, correlations of H<sub>3</sub>-15/H-12 and H<sub>3</sub>-15/H-14 indicated that H-12 and H-14 were  $\beta$ -oriented, and the cyclohexane ring may exhibit a twist-boat conformation. The NOESY spectrum showed a correlation from H-6 to H<sub>3</sub>-16, revealing the *Z* geometry of the C-5/6 double bond. H<sub>3</sub>-18 exhibited correlations to OH-8 and H-9, suggesting the  $\alpha$ -orientation of Me-18 at C-17. H-7 displayed a correlation with H-17, which confirmed that these two protons were  $\beta$ -oriented at C-7 and C-17, respectively. As briarane 2 was isolated along with 1 from the same organism, it is reasonable on biogenetic grounds to assume that 2 possessed the same absolute configuration as that of 1. Therefore, the configurations of the stereogenic carbons of 2 should be assigned as 1*S*,2*S*,7*S*,8*S*,9*S*,10*S*,11*S*,12*R*,14*S*, and 17*R* (Supplementary Materials, Figures S15–S29).

The effects of briaranes 1 and 2 on the release of iNOS and COX-2 from lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells were assessed (Table 3 and Figure 5). It is interesting to note that 2 at 10  $\mu\text{M}$  enhanced the release of iNOS and COX-2 to 122.87% and 113.65%, respectively, as compared to results of the cells stimulated with LPS only.

**Table 3.** Effects of briaranes **1** and **2** on lipopolysaccharide (LPS)-induced pro-inflammatory iNOS and COX-2 protein expression in macrophages.

Compound	iNOS	COX-2	$\beta$ -Actin
	Expression (% of LPS)		
Control	2.59 $\pm$ 0.65	1.14 $\pm$ 0.34	100.15 $\pm$ 7.70
LPS	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00
<b>1</b>	94.81 $\pm$ 7.11	89.59 $\pm$ 5.45	101.09 $\pm$ 1.91
<b>2</b>	122.87 $\pm$ 10.53	113.65 $\pm$ 6.00	99.50 $\pm$ 1.64
Dexamethasone	26.99 $\pm$ 2.66	10.52 $\pm$ 5.23	99.02 $\pm$ 1.53

Data were normalized to those of cells treated with LPS alone, and cells treated with dexamethasone were used as a positive control. Data are expressed as the mean  $\pm$  standard error of the mean (SEM) ( $n = 3$ ).



**Figure 5.** Western blotting showed that briarane **2** enhanced the expression of iNOS and COX-2. Data were normalized to the cells treated with LPS only, and cells treated with dexamethasone (Dex) (10  $\mu$ M) were used as a positive control. Data are presented as the mean  $\pm$  SEM ( $n = 3$ ). \* Significantly different from cells treated with LPS ( $p < 0.05$ ).

### 3. Materials and Methods

#### 3.1. General Experimental Procedures

NMR spectra were recorded on a 400 MHz Jeol NMR (model ECZ 400S, Tokyo, Japan) spectrometer using the residual  $\text{CHCl}_3$  signal ( $\delta_{\text{H}}$  7.26 ppm) and  $\text{CDCl}_3$  ( $\delta_{\text{C}}$  77.1 ppm) as internal references for  $^1\text{H}$  and  $^{13}\text{C}$  NMR, respectively. ESIMS and HRESIMS were obtained from a Bruker mass spectrometer with 7 Tesla magnets (model: Solarix FTMS system, Bremen, Germany). Column chromatography, HPLC, IR spectra, and optical rotation values were performed according to our earlier research [19].

#### 3.2. Animal Material

The specimens coral *J. fragilis* were collected in July 2019 by hand, using self-contained underwater breathing apparatus (SCUBA) off the coast of Orchid Island (Lanyu Island), Taiwan. The samples were stored in a  $-20$   $^{\circ}\text{C}$  freezer until extraction. A voucher specimen was deposited in the National Museum of Marine Biology and Aquarium (NMMBA) (voucher no.: NMMBA-TW-GC-2019-017). This organism was identified by comparison with previous descriptions [1–3].

#### 3.3. Extraction and Isolation

Sliced bodies (wet/dry weight = 1125 g/588 g) of the coral specimen were prepared and extracted with a mixture of methanol (MeOH) and dichloromethane ( $\text{CH}_2\text{Cl}_2$ ) (1:1) to give a crude extract (29.0 g) which was partitioned between ethyl acetate (EtOAc) and  $\text{H}_2\text{O}$ . The EtOAc extract (17.0 g) was then applied to a silica gel column chromatograph (C.C.) (500 g) and eluted with gradients of hexanes/acetone

(stepwise from 50:1 (3000 mL)-30:1 (3000 mL)-20:1 (3000 mL)-10:1 (3000 mL)-5:1 (3000 mL)-4:1 (3000 mL)-3:1 (3000 mL)-2:1 (3000 mL)-1:1 (3000 mL)-1:2 (3000 mL)) to furnish fractions A–J. Fraction F (913.9 mg) was separated on silica gel C.C. and eluted with gradients of hexanes/acetone (stepwise from 20:1 (2400 mL)-15:1 (2400 mL)-10:1 (2400 mL)-8:1 (2400 mL)-6:1 (2400 mL)-4:1 (2400 mL)-2:1 (2400 mL)-1:1 (2400 mL) to furnish fractions F1–F8. Fraction F5 was further separated by silica gel C.C. with a mixture of hexanes/acetone (10:1 to 1:1, stepwise) to afford fractions F5A–F5F. Afterward, fraction F5C was separated by normal-phase HPLC (NP-HPLC) using a mixture of CH<sub>2</sub>Cl<sub>2</sub> and acetone (10:1) to yield fractions F5C1–F5C4. Fraction F5C2 was purified by NP-HPLC using a mixture of *n*-hexane and EtOAc (2:1; at a flow rate = 2.0 mL/min) to afford **1** (32.4 mg). Fraction G was applied to a silica gel C.C. and eluted with a mixture of hexanes/acetone (3:1) to furnish fractions G1–G6. Fraction G4 was separated by silica gel C.C. using a mixture of CH<sub>2</sub>Cl<sub>2</sub> and acetone (20:1) to afford fractions G4A–G4F. Fraction G4E was separated by NP-HPLC using a mixture of *n*-hexane/EtOAc/acetone (5:2:1) to yield fractions G4E1–G4E5. Fraction G4E4 was purified by NP-HPLC using a mixture of CH<sub>2</sub>Cl<sub>2</sub> and acetone (10:1) to afford fractions G4E4A–G4E4C. Fraction G4E4A was separated by reverse-phase HPLC (RP-HPLC) using a mixture of acetonitrile and H<sub>2</sub>O (55:45; at a flow rate = 4.0 mL/min) to obtain **2** (0.7 mg).

Juncenolide K (**1**): Colorless crystals;  $[\alpha]_D^{26} -90$  (*c* 1.62, CHCl<sub>3</sub>) (ref. [13]  $[\alpha]_D -85$  (*c* 0.2, CH<sub>2</sub>Cl<sub>2</sub>)); IR (ATR)  $\nu_{\max}$  2926, 1746, 1728, 1372, 1251, 1216, 759 cm<sup>-1</sup>; <sup>1</sup>H (400 MHz, CDCl<sub>3</sub>) and <sup>13</sup>C (100 MHz, CDCl<sub>3</sub>) NMR data, see Table 1; ESIMS: *m/z* 513 [M + Na]<sup>+</sup>; HRESIMS: *m/z* 513.20949 (calcd. for C<sub>26</sub>H<sub>34</sub>O<sub>9</sub> + Na, 513.20950).

Fragilide X (**2**): Amorphous powder;  $[\alpha]_D^{25} +232$  (*c* 0.23, CHCl<sub>3</sub>); IR (KBr)  $\nu_{\max}$  3333, 2942, 1773, 1742, 1374, 1219, 756 cm<sup>-1</sup>; <sup>1</sup>H (400 MHz, CDCl<sub>3</sub>) and <sup>13</sup>C (100 MHz, CDCl<sub>3</sub>) NMR data, see Table 2; ESIMS: *m/z* 589 [M + Na]<sup>+</sup>; HRESIMS: *m/z* 589.22576 (calcd. for C<sub>28</sub>H<sub>38</sub>O<sub>12</sub> + Na, 589.22555).

### 3.4. Single-Crystal X-ray Crystallography of Juncenolide K (**1**)

Suitable colorless prisms of **1** were obtained from a solution of MeOH and petroleum ether. The crystal (0.255 × 0.233 × 0.114 mm<sup>3</sup>) belongs to the orthorhombic system, space group *P*2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> (#19), with *a* = 9.8842(2) Å, *b* = 15.5702(2) Å, *c* = 17.0502(3) Å, *V* = 2624.01(8) Å<sup>3</sup>, *Z* = 4, *D*<sub>calcd</sub> = 1.264 Mg/m<sup>3</sup>, λ (Cu Kα) = 1.54178 Å. Intensity data were measured on a Bruker D8 Venture diffractometer up to  $\theta_{\max}$  of 75.0°. All 12,468 reflections were collected. The structure was solved by direct methods and refined by a full-matrix least-squares procedure [41,42]. The refined structural model converged to a final R1 (the R-value, is the agreement between the calculated and observed models) = 0.0396; wR2 (wR2 is similar to R1, but refers to squared F-values) = 0.1090 for 5385 observed reflections [*I* > 2σ(*I*)] and 335 variable parameters. The absolute configuration was determined by the Flack parameter *x* = 0.07(5) [43,44]. Crystallographic data for the structure of juncenolide K (**1**) were deposited with the Cambridge Crystallographic Data Center (CCDC) as supplementary publication number CCDC 1973681 [45].

### 3.5. In Vitro Inflammatory Assay

Murine RAW 264.7 macrophages were obtained from the American Type Culture Collection (ATCC; No. TIB-71). Inflammation in macrophages was induced by incubating them for 16 h in a medium containing only LPS (0.01 µg/mL) without compounds. For the anti-inflammatory activity assay, compounds (10 µM) were added to the cells 5 min before LPS challenge. The cells were then washed with ice cold phosphate-buffered saline (PBS), lysed in ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 100 µg/mL phenylmethylsulfonyl fluoride, 1 µg/mL aprotinin), and then centrifuged at 20,000× *g* for 30 min at 4 °C. The supernatant was decanted from the pellet and retained for Western blot analysis of pro-inflammation inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein expression. Protein concentrations were determined using the detergent compatible (DC) protein assay kit (Bio-Rad, Hercules, CA, USA). Western blotting was performed according to the method described in a previous study [46]. An equal volume of sample

buffer (2% 2-mercaptoethanol, 2% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue, 10% glycerol, and 50 mM Tris-HCl (pH 7.2)) was added to the samples, and the protein lysates were loaded onto a 10% SDS-polyacrylamide gel. Electrophoresis was carried out at 150 V for 90 min. After electrophoresis, gels were transferred overnight at 4 °C in transfer buffer (380 mM glycine, 50 mM Tris-HCl, 1% SDS and 20% methanol) onto a polyvinylidene difluoride membrane (PVDF; Immobilon-P, Millipore Corp. (0.45 µm pore size)). The PVDF membrane was first blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween (TTBS; 20 mM Tris-HCl, 0.1% Tween 20, and 137 mM NaCl (pH 7.4)) and incubated overnight at 4 °C with the primary antibodies for iNOS, COX-2, and β-actin proteins. Anti-iNOS and anti-COX-2 antibodies were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). A horseradish peroxidase-conjugated secondary antibody was used for detection. It was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). The bound antibodies were detected by chemiluminescence (Millipore Corp.). The images were obtained using the UVP BioChem Imaging System, and the LabWorks 4.0 software (UVP, Upland, CA, USA) was used to quantify the relative densities.

#### 4. Conclusions

*J. fragilis* has been demonstrated to have a wide structural diversity of briarane-type diterpenoids that possess various potential bioactivities. In our continued study on *J. fragilis*, a previously unreported 11,20-epoxybriarane, fragilide X (2), along with a known briarane, juncenolide K (1) were isolated. Revision of the structure and absolute configuration of juncenolide K (1) was confirmed by a single-crystal X-ray diffraction analysis. In the present study, a pro-inflammatory assay was employed to assess the activity of isolates, and fragilide X (2) was found to enhance the release of iNOS and COX-2, respectively.

**Supplementary Materials:** Supplementary Materials are available online at <http://www.mdpi.com/1660-3397/18/4/183/s1>. HRESIMS, IR, 1D (<sup>1</sup>H, <sup>13</sup>C NMR and DEPT spectra), and 2D (HSQC, HMBC, COSY, and NOESY) NMR spectra of juncenolide K (1) and fragilide X (2), and X-ray Crystallography of 1.

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