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11β,20β-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).](image)

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 11α,20α-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 11β,20β-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 15S,25S,9S,10S,11S and 14S (Flack parameter *x* = 0.07(5)).
Table 1. $^1$H and $^{13}$C NMR (CDCl$_3$) data for juncenolide K and briarane 1.

| Position | $\delta_H$ (J in Hz) $^b$ | $\delta_C$ $^c$, type | $\delta_H$ (J in Hz) $^d$ | $\delta_C$, $^e$, type |
|----------|--------------------------|----------------------|--------------------------|----------------------|
| 1        | 46.0, C                  | 46.0, C              | 74.1, CH                 | 74.1, CH             |
| 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$_2$         | 2.56 m; 1.74 m           | 31.3, CH$_2$         |
| 4        | 2.60–2.66 m; 2.20–2.26 m | 29.1, CH$_2$         | 2.60 m; 2.22 m           | 29.1, CH$_2$         |
| 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$_2$         | 2.31 m; 1.12 m           | 22.9, CH$_2$         |
| 13       | 2.11–2.17 m; 1.80–1.86 m | 23.8, CH$_2$         | 2.10 m; 1.82 m           | 23.8, CH$_2$         |
| 14       | 4.80 d (3.5)             | 73.9, CH             | 4.79 d (3.6)             | 73.9, CH             |
| 15       | 1.16 s                   | 15.8, CH$_3$         | 1.15 s                   | 15.9, CH$_3$         |
| 16       | 2.01 s                   | 27.0, CH$_3$         | 1.99 s                   | 27.0, CH$_3$         |
| 17       | 127.5, C                 | 127.4, C             |                         |                      |
| 18       | 2.01 s                   | 9.3, CH$_3$          | 1.99 s                   | 9.3, CH$_3$          |
| 19       | 173.6, C                 | 173.6, C             |                         |                      |
| 20a/b    | 2.60–2.66 m; 2.46–2.52 m | 58.2, CH$_2$         | 2.62 br s; 2.48 br s     | 58.2, CH$_2$         |
| OAc-2    | 170.7, C                 | 170.7, C             |                         |                      |
| OAc-9    | 2.01 s                   | 21.0, CH$_3$         | 2.00 s                   | 21.0, CH$_3$         |
| OAc-14   | 168.9, C                 | 168.9, C             |                         |                      |

$^a$ Data were reported by Wang et al., see ref. [13]. $^b$ 500 MHz, $^c$ 125 MHz, $^d$ 400 MHz, $^e$ 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 [M + Na]$^+$ at m/z 589.22576 in the (+)-HRESIMS, indicating a molecular formula C$_{26}$H$_{34}$O$_{9}$ (calculated for C$_{26}$H$_{34}$O$_{9}$ + Na, 589.22555) (unsaturation degrees  = 10). Absorption peaks at 3333 cm$^{-1}$, 1773 cm$^{-1}$, and 1742 cm$^{-1}$ in the IR spectrum indicate hydroxy, γ-lactone, and ester groups, respectively. Analysis of the $^1$H, $^{13}$C NMR, and distortionless enhancement by polarization transfer (DEPT) spectra, together with the molecular formula, suggested that there must be an exchangeable proton. The $^{13}$C NMR spectrum (Table 2), in combination with DEPT, HSQC, and HMBC spectra, revealed the presence of five esters including four acetoxy groups ($\delta_C$ 21.6, 21.0, 20.9, 20.7, 4 $\times$ CH$_3$; $\delta_C$ 170.6, 169.8, 169.4, 169.2, 4 $\times$ C) and a lactone moiety ($\delta_C$ 176.2), and a trisubstituted olefin ($\delta_C$ 143.8, C-5; 120.8, CH-6). Based on the $^{13}$C NMR data and numbers of unsaturation, 2 was established as a tetracyclic diterpenoid.
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 (CH2-20). 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\(^3\) methylene protons, two sp\(^2\) methine protons, five oxymethine protons, an sp\(^2\) methine proton, four acetate methyls, and a hydroxy proton were observed in the \(^1\)H NMR spectrum (Table 2).

![Diagram](image)

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

| Position | \( \delta_H \) \(^a\) (in Hz) | \( \delta_C \) \(^b\) Type |
|----------|---------------------|---------------------|
| 1        | 46.8, CH             |                     |
| 2        | 4.74 d (4.8)         | 74.4, CH            |
| 3α/β     | 1.67 m; 2.43 ddd (16.0, 16.0, 4.4) | 32.1, CH\(_2\) |
| 4α/β     | 2.52 br d (16.0); 2.07 m | 28.7, CH\(_2\) |
| 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            |
| 13α/β    | 1.58 m; 2.70 m       | 32.6, CH\(_2\)     |
| 14       | 4.85 d (4.4)         | 73.6, CH            |
| 15       | 1.16 s              | 15.3, CH\(_3\)     |
| 16       | 2.03 d (1.2)         | 28.1, CH\(_3\)     |
| 17       | 2.35 q (7.2)         | 42.3, CH            |
| 18       | 1.15 d (7.2)         | 6.7, CH\(_3\)      |
| 19       |                     | 176.2, C            |
| 20α/b    | 3.20 d (4.4); 2.90 d (4.4) | 53.9, CH\(_2\) |
| OH-8     | 4.57 d (1.2)         |                     |
| OAc-2    |                     | 170.6, C            |
| OAc-9    | 2.01 s              | 21.0, CH\(_3\)\(^c\) |
| OAc-12   | 2.22 s              | 169.2, C            |
| OAc-14   | 1.98 s              | 21.6, CH\(_3\)\(^c\) |
| OAc-14   | 2.02 s              | 169.8, C            |

\(^a\) 400 MHz, \(^b\) 100 MHz, \(^c\) Data exchangeable.
The $^1$H NMR coupling information in the correlation spectroscopy analysis enabled the determination of five different spin systems, H-2/H$_2$-3/H$_2$-4, H-6/H-7, H-9/H-10, H-12/H$_2$-13/H-14, and H-17/H$_2$-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-3β, H-10, H-13α, H-14, H$_3$-15 to C-1; H$_2$-4, H-7, H$_3$-16 to C-5; H-7, H-9, H-10, H-17, H$_3$-18 to C-8; H-9, H-10, H-12, H$_2$-20, H$_2$-13 to C-11; and H-17, H$_3$-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$_3$-16 to C-4, C-5, and C-6; and further confirmed by an allylic coupling between H-6/H$_3$-16 ($J = 1.2$ Hz). The methyl group Me-15 on C-1 was substantiated by the HMBC correlations from H$_3$-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$_2$-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 $\delta_H$ 4.57 to C-7, C-8, and C-9. Moreover, HMBC correlations from the oxymethine protons at $\delta_H$ 4.74 (H-2), 5.67 (H-9), 5.40 (H-12), and 4.85 (H-14) to the acetate carbonyls at $\delta_C$ 170.6, 169.2, 169.4, and 169.8, placed the acetoxy groups on C-2, C-9, C-12, and C-14, respectively.

![Figure 4](image-url)  
**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 α protons since the C-15 methyl is the β-substituent at C-1. Meanwhile, correlations of H$_3$-15/H-12 and H$_3$-15/H-14 indicated that H-12 and H-14 were β-oriented, and the cyclohexane ring may exhibit a twist-boat conformation. The NOESY spectrum showed a correlation from H-6 to H$_3$-16, revealing the Z geometry of the C-5/6 double bond. H$_3$-18 exhibited correlations to OH-8 and H-9, suggesting the α-orientation of Me-18 at C-17. H-7 displayed a correlation with H-17, which confirmed that these two protons were β-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 1S,2S,7S,8S,9S,10S,11S,12R,14S, and 17R (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 μM 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      | β-Actin    |
|---------------------|------------|------------|------------|
| Control             | 2.59 ± 0.65| 1.14 ± 0.34| 100.15 ± 7.70 |
| LPS                 | 100.00 ± 0.00| 100.00 ± 0.00| 100.00 ± 0.00 |
| 1                   | 94.81 ± 7.11| 89.59 ± 5.45| 101.09 ± 1.91 |
| 2                   | 122.87 ± 10.53| 113.65 ± 6.00| 99.50 ± 1.64 |
| Dexamethasone       | 26.99 ± 2.66| 10.52 ± 5.23| 99.02 ± 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 ± 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 μM) were used as a positive control. Data are presented as the mean ± 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 CHCl₃ signal (δH 7.26 ppm) and CDCl₃ (δC 77.1 ppm) as internal references for ¹H and ¹³C NMR, respectively. ESIMS and HRESIMS were obtained from a Bruker mass spectrometer using the residual CHCl₃ as a reference. General Experimental Procedures 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 °C freezer until extraction. A voucher specimen was deposited in the National Museum of Marine Biology and Aquarium (NMMA) (voucher no.: NMMA-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 (CH₂Cl₂) (1:1) to give a crude extract (29.0 g) which was partitioned between ethyl acetate (EtOAc) and H₂O. 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...
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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 mm³) belongs to the orthorhombic system, space group P2₁2₁2₁ (#19), with a = 9.8842(2) Å, b = 15.5702(2) Å, c = 17.0502(3) Å, V = 2624.01(8) Å³, Z = 4, Dcalc = 1.264 Mg/m³, λ (Cu Ka) = 1.54178 Å. Intensity data were measured on a Bruker D8 Venture diffractometer up to θmax 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 BioChemi 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 (1H, 13C 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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