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FRAGILIDES M–O, NEW TRIACETOXYBRIARANES FROM THE GORGONIAN CORAL *JUNCEELLA FRAGILIS* (ELLISELLIDAE)

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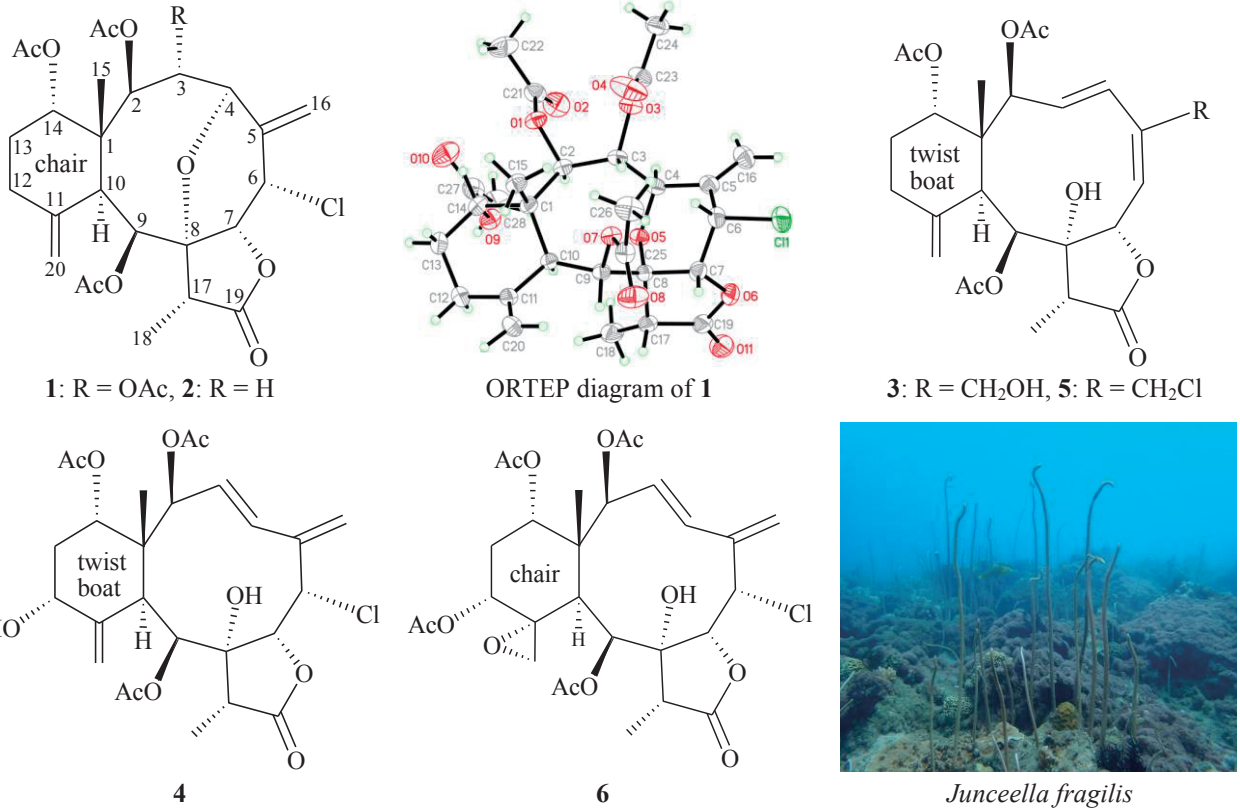
Abstract – Chemical investigation of the ethyl acetate-soluble fraction from the methanol/dichloromethane extract of a sea whip gorgonian coral *Junceella fragilis* afforded four polyacetoxybriaranes, including a known metabolite, junceellin (**1**), along with three new analogues, fragilides M–O (**2**–**4**). The absolute configuration of **1** was determined by X-ray analysis and the structures of **2**–**4** were elucidated on the basis of spectroscopic methods.

The sea whip gorgonian corals belonging to the genus *Junceella* (family Ellisellidae) are proven to be a rich source of diterpenoids with a briarane carbon skeleton (3,8-cyclized cembranoid) that often have

complex structures and bioactivities.[1] Recently, in our ongoing study of the chemical constituents of *J. fragilis* (Ridley, 1884) has resulted in the isolation of four polyacetoxybriaranes including a known metabolite, junceellin (**1**),[2–10] as well as three new triacetoxybriaranes, fragilides M–O (**2**–**4**) (Chart 1). The isolated compounds were evaluated for anti-inflammatory activity using the inhibition of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) in an *in vitro* pro-inflammatory model.



1: R = OAc, **2**: R = H

ORTEP diagram of **1**

3: R = CH_2OH, **5**: R = CH_2Cl

4

6

Junceella fragilis

Chart 1. Structures of junceellin (**1**), fragilides M–O (**2**–**4**), junceellolide P (**5**), 12-*epi*-fragilide G (**6**), the computer-generated ORTEP diagram of **1** and a picture of *Junceella fragilis*

Junceellin (**1**) was first isolated from a Chinese gorgonian coral *Junceella squamata*;[2] and the structure of this compound was elucidated by spectroscopic methods[2,4,5,8] and X-ray analysis.[3] The absolute configuration of this compound was established in this study by a single-crystal X-ray diffraction analysis (Flack parameter x = 0.057(4)) and the ORTEP diagram (Chart 1) showed that the configurations of stereogenic carbons are 1*R*,2*R*,3*S*,4*R*,6*S*,7*R*,8*R*,9*S*,10*S*,14*S*, and17*R*.

Fragilide M (**2**) was obtained as an amorphous powder. (+)-HRESIMS showed a pseudomolecular peak at *m/z* 547.17060, which established the molecular formula $C_{26}H_{33}ClO_9$ (Calcd for $C_{26}H_{33}{}^{35}ClO_9$ + Na, 547.17053), indicating ten degrees of unsaturation. Absorption peaks at 1790 and 1734 cm^{-1} in the IR spectrum indicated a structure containing γ-lactone and ester groups. The presence of two exocyclic

olefins were deduced from the signals of carbons at δ_C 147.8 (C-11), 137.0 (C-5), 116.6 (CH_2-16), and 111.6 (CH_2-20), and supported by four olefin proton signals at δ_H 5.48 (1H, d, J = 2.0 Hz, H-16b), 5.35 (1H, d, J = 2.0 Hz, H-16a), 5.07 (1H, s, H-20a), and 4.73 (1H, s, H-20b) in the ^{1}H NMR spectrum (Table 2). In addition, four carbonyl resonances at δ_C 174.4 (C-19), 170.8, 170.1, and 169.6 (3 × ester carbonyls), confirmed the presence of a γ-lactone and three ester groups; three acetate methyls (δ_H 2.24, 2.07, and 2.02, each 3H × s; δ_C 21.5, 21.1, 21.0, CH_3 × 3) were observed. Based on the ^{13}C NMR and numbers of unsaturation, **2** was established as a tetracyclic briarane-type diterpenoid.

Table 1. ^{13}C NMR data for briaranes **2**–**4**

C	**2**[a]	**3**[a]	**4**[b]
1	47.4, C[c]	48.9, C	47.4, C
2	73.7, CH	75.6, CH	75.9, CH
3	34.9, CH_2	136.8, CH	132.6, CH
4	76.8, CH	126.7, CH	129.6, CH
5	137.0, C	144.0, C	142.1, C
6	54.3, CH	123.1, CH	64.4, CH
7	79.3, CH	81.3, CH	80.5, CH
8	81.4, C	80.5, C	84.4, C
9	77.9, CH	75.1, CH	74.8, CH
10	43.8, CH	43.3, CH	40.3, CH
11	147.8, C	149.0, C	148.6, C
12	32.7, CH_2	30.5, CH_2	68.3, CH
13	27.6, CH_2	27.6, CH_2	36.2, CH_2
14	74.5, CH	78.3, CH	74.2, CH
15	14.8, CH_3	15.8, CH_3	15.8, CH_3
16	116.6, CH_2	65.3, CH_2	115.9, CH_2
17	50.0, CH	45.4, CH	49.2, CH
18	7.0, CH_3	8.8, CH_3	7.1, CH_3
19	174.4, C	176.0, C	174.1, C
20	111.6, CH_2	111.5, CH_2	112.4, CH_2
Acetate methyls	21.5, CH_3	21.6, CH_3	21.6, CH_3
	21.1, CH_3	21.4, CH_3	21.3, CH_3
	21.0, CH_3	20.9, CH_3	21.0, CH_3
Acetate carbonyls	170.8, C	170.4, C	170.1, C
	170.1, C	170.1, C	170.1, C
	169.6, C	169.8, C	169.9, C

[a] Spectra recorded at 100 MHz in $CDCl_3$ at 25 °C.
[b] Spectra recorded at 150 MHz in $CDCl_3$ at 25 °C.
[c] Multiplicity deduced by DEPT.

The gross structure of **2** was verified by 2D NMR studies. ^{1}H NMR coupling information in the COSY spectrum enabled identification of C2-C3-C4, C6-C7, C12-C13-C14, and C17-C18 units (Figure 1), which were assembled with the assistance of an HMBC experiment (Figure 1). The HMBC between protons and quaternary carbons, such as H-2, H_2-3, H-9, H-10, H_3-15/C-1; H-3β, H-4, H-7, H-16b/C-5; H-4, H-6, H-9, H-10, H_3-18/C-8; H-9, H-10, H_2-12, H-20b/C-11; and H-17, H_3-18/C-19, permitted elucidation of the carbon skeleton. The ring junction C-15 methyl group was positioned at C-1 from the

HMBC between H_3-15/C-1, C-2, C-10, C-14 and H-2, H-10/C-15. The presence of acetoxy groups at C-2 and C-9 was confirmed by the HMBC from δ_H 5.21 (H-2), 5.96 (H-9) to the acetate carbonyl carbons at δ_C 170.8 (C) and 169.6 (C), respectively. The remaining acetoxy group was positioned at C-14, an oxymethine, as indicated by analysis of the COSY correlations and characteristic NMR signals (δ_H 4.97, 1H, dd, J = 2.8, 2.8 Hz; δ_C 74.5, CH-14), although no HMBC was observed between H-14 and the acetate carbonyl carbon.

Table 2. ^{1}H NMR chemical shifts for briaranes **2**–**4**

H	**2**[a]	**3**[a]	**4**[b]
2	5.21 d (7.6)[c]	5.53 d (5.6)	5.46 d (9.6)
3α/β	1.44 dd (15.6, 4.8); 3.02 ddd (15.6, 12.4, 7.6)	6.15 dd (16.4, 5.6)	5.96 dd (15.6, 9.6)
4	4.90 dd (12.4, 4.8)	6.28 d (16.4)	6.73 d (15.6)
6	4.91 d (3.2)	5.53 d (3.2)	5.13 d (3.6)
7	4.47 d (3.2)	5.72 d (3.2)	4.20 d (3.6)
9	5.96 s	5.89 d (5.2)	5.48 d (6.0)
10	3.03 br s	3.26 d (5.2)	3.74 d (6.0)
12	2.39–2.20 m (2H)	2.27–2.14 m (2H)	4.45 br s
13α/β	1.82 m; 1.69 dddd (14.4, 14.4, 4.2, 2.8)	1.89 m; 1.82 m	1.71 br d (15.6); 2.40 m
14	4.97 dd (2.8, 2.8)	4.84 dd (2.8, 2.8)	4.80 br s
15	1.10 s	0.96 s	1.10 s
16a/b	5.35 d (2.0); 5.48 d (2.0)	4.24 s (2H)	5.40 s; 5.27 s
17	2.75 q (6.8)	2.61 q (7.2)	2.71 q (7.2)
18	1.25 d (6.8)	1.24 d (7.2)	1.13 d (7.2)
20a/b	5.07 s; 4.73 s	5.11 s; 5.08 s	5.33 s; 5.21 s
acetate methyls	2.24 s	2.23 s	2.10 s
	2.07 s	2.13 s	2.04 s
	2.02 s	2.09 s	2.04 s
OH-8		2.48 br s	3.63 br s
OH-12			3.49 br s

[a] Spectra recorded at 400 MHz in $CDCl_3$ at 25 °C.
[b] Spectra recorded at 600 MHz in $CDCl_3$ at 25 °C.
[c] J values (in Hz) in parentheses.

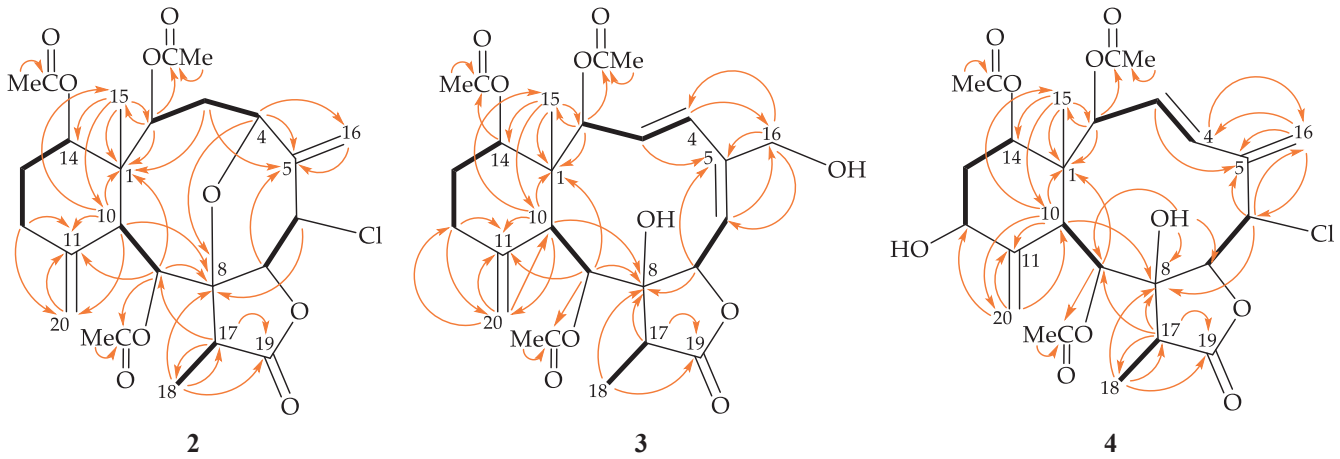


Figure 1. The COSY (▬) correlations and selective HMBC (⌒) of **2**–**4**

The stereochemistry of **2** was established by a NOESY experiment and further supported by MM2 force field analysis,[11] demonstrating that a stable conformer was as shown in Figure 2. In the NOESY spectrum, H-10 was associated with H-2, H-9, and H_3-18, while no associations were seen with Me-15, which suggested that these protons were positioned on the α face, due to the fact that the C-15 methyl is a β-substituent at C-1 and H_3-15 did not show correlation with H-10. The oxymethine proton H-14 was found to exhibit response with H_3-15 but not with H-10, revealing H-14 was β-oriented at C-14. H-7 exhibited correlations with H-6 and H-17, indicating that these protons were on the β-face. Furthermore, H-4 showed a correlation with H-2, indicating that H-4 has an α-orientation at C-4. It was found that the NMR signals of **2** were similar to those of junceellin (**1**), except that the signals corresponding to the acetoxy group at C-3 in **1** were replaced by signals for a proton in **2**. As **2** was isolated along with **1** from the same organism, it is reasonable on biogenetic grounds to assume that **2** has the same absolute configuration as **1**. Therefore, based on above findings, the configurations of the stereogenic centers of **2** were assigned as 1*R*,2*S*,4*S*,6*S*,7*R*,8*R*,9*S*,10*S*,14*S*, and 17*R* and this compound was found to be the 3-deacetoxy derivative of **1**.

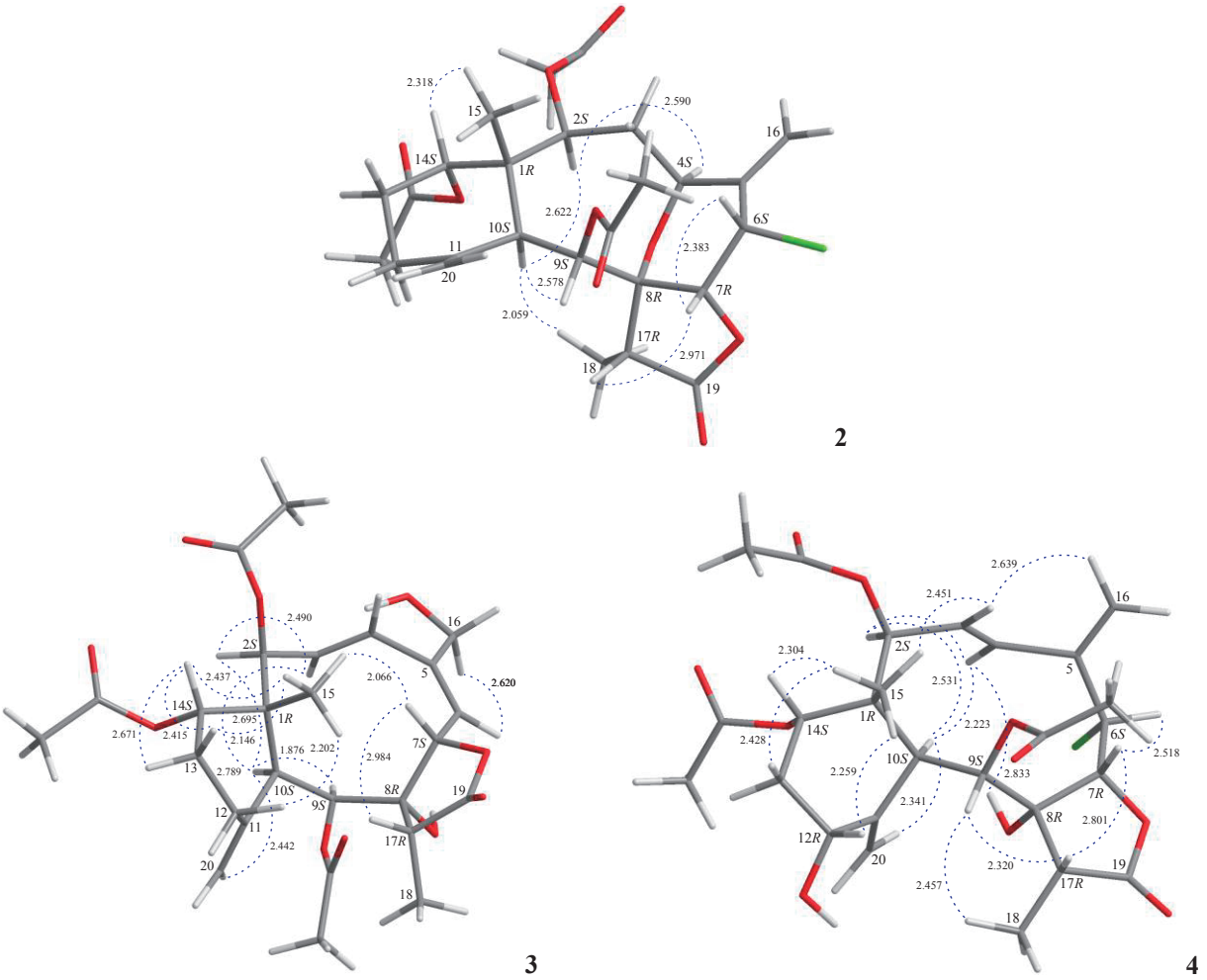


Figure 2. The stereoview of **2**–**4** (generated from computer modeling) and the calculated distances (Å) between selected protons with key NOESY ( ) correlations

Briarane **3** (Fragilide N) was found to have a molecular formula of $C_{26}H_{34}O_{10}$ based on its (+)-HRESIMS peak at *m/z* 529.20416 (Calcd for $C_{26}H_{34}O_{10}$ + Na, 529.20422). Its absorption peaks in the IR spectrum showed ester carbonyl, γ-lactone, and broad OH stretching at 1736, 1773, and 3470 cm^{-1}, respectively. The ^{13}C NMR spectrum indicated that three esters and a γ-lactone were present, as carbonyl resonances were observed at δ_C 170.4, 170.1, 169.8, and 176.0, respectively (Table 1). The ^{1}H NMR spectrum indicated the presence of three acetate methyls (δ_H 2.23, 2.13, 2.09, each 3H × s) (Table 2). It was found that the ^{1}H and ^{13}C NMR spectra of **3** resembled those of a known analogue, junceellolide P (**5**) (Chart 1), isolated from *Junceella gemmacea*, collected off the South China Sea,[12] except that the signals corresponding to a chlorinated methylene (C-16) in **5** was replaced by an oxygenated methylene in **3**. The locations of the functional groups were further confirmed by other HMBC and COSY correlations (Figure 1), and hence fragilide N was assigned the structure of **3**, with the same stereochemistry as that of **5**, and the configurations of the stereogenic carbons were assigned as 1*R*,2*S*,7*S*,8*R*,9*S*,10*S*,14*S*, and 17*R* (Figure 2).

The proton chemical shifts of briarane-type natural products containing an 11,20-exocyclic carbon-carbon double bond were summarized and the difference between the two olefin protons (H-20a/b) was smaller than 0.2 ppm, whereas the methylidenecyclohexane rings exhibited a twisted boat conformation.[13] Owing to the chemical shifts of the C-20 methylene protons (δ_H 5.11 and 5.08), the conformation of the methylidenecyclohexane ring in **3** was concluded to be a twisted boat conformation and this finding was further supported by the NOESY correlations between H-10 (δ_H 3.26) and H-20b (δ_H 5.08); and H-12 (δ_H 2.27–2.14) and H_3-15 (δ_H 0.96) in the NOESY spectrum (Figure 2).

Fragilide O (**4**) was present as an amorphous powder. From (+)-HRESIMS analysis, the peak at *m/z* 563.16543 suggested the molecular formula to be $C_{26}H_{33}ClO_{10}$ (Calcd for $C_{26}H_{33}ClO_{10}$ + Na, 563.16545). The 1D and 2D NMR of **4** (Tables 1 and 2, Figure 1) were similar to those of a known briarane analogue, 12-*epi*-fragilide G (**6**) (Chart 1),[14] except that the signals related to the 11,20-epoxy and 12-acetoxy groups in **6** were substituted by signals for a methylidene and a hydroxy groups in **4**, respectively. Based on the proton chemical shifts of H_2-20 (δ_H 5.33 and 5.21), the methylidenecyclohexane ring in **4** was found to be existed in a twisted boat conformation and this finding was further supported by the NOESY correlations between H-12 (δ_H 4.45) and H_3-15 (δ_H 1.10); and H-10 (δ_H 3.74) and H-20b (δ_H 5.21), in the NOESY spectrum and the configurations of the stereogenic carbons were assigned as 1*R*,2*S*,6*S*,7*R*,8*R*, 9*S*,10*S*,12*R*,14*S*, and 17*R* (Figure 2).

In *in vitro* anti-inflammatory activity tests, upregulation of pro-inflammatory inducible nitric oxide synthase (iNOS) protein expression in LPS-stimulated RAW 264.7 macrophage cells was evaluated using immunoblot analysis. At a concentration of 10 μM, briaranes **1**–**4** were found to be inactive to reduce the

level of COX-2 and iNOS, respectively, in relation to control cells stimulated with LPS only. Using trypan blue staining to measure the cytotoxic effects of the compounds, it was observed that briaranes **1**–**4** did not induce cytotoxicity in RAW 264.7 macrophage cells. Due to the screening platforms are limited and lots of material were consumed in physical and spectral experiments, the other possible bioactivities for the new interesting natural substances will not be assayed at this stage. The possible bioactivity for these compounds will be studied if we can get enough material from the *J. fragilis* in the future.

EXPERIMENTAL

General Experimental Procedures. Melting points were determined using Fargo apparatus and the values were uncorrected. Optical rotation values were measured using a Jasco P-1010 digital polarimeter with 0.3 mL cell. IR spectra were obtained using a spectrophotometer (Nicolet iS5 FT-IR; Thermo Scientific). NMR spectra were recorded on 400 or 600 MHz Jeol ECZ NMR spectrometers 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 and ^{13}C NMR, respectively; coupling constants (*J*) are presented in Hz. ESIMS and HRESIMS were obtained from the Bruker mass spectrometer with 7 Tesla magnets (model: SolariX FTMS system). Column chromatography was carried out with silica gel (230–400 mesh, Merck). TLC was performed on plates precoated with Kieselgel 60 F_{254} (0.25-mm-thick, Merck), then sprayed with 10% H_2SO_4 solution followed by heating to visualize the spots. Normal-phase HPLC (NP-HPLC) was performed using a system comprised of a Hitachi 5110 pump and an injection port (7725i, Rheodyne). A semi-preparative normal-phase column (Supelco Ascentis Si Cat# 581515-U, 25 cm × 21.2 mm 5 μM, Sigma-Aldrich) was used for NP-HPLC. Reverse-phase HPLC (RP-HPLC) was performed using a system comprised of a Hitachi L-2130 pump, a Hitachi L-2455 photodiode array detector, a Rheodyne 7725i injection port, and a 250 mm × 21.2 mm column (5 μM, Luna RP-18e; Phenomenex Inc.).

Animal Material. Specimens of the sea whip gorgonian coral *Junceella fragilis* were collected in June 2017 by hand by SCUBA divers off the coast of Lanyu Island (Orchid Island), Taiwan. The samples were then stored in a –20 °C freezer until extraction. A voucher specimen was deposited in the National Museum of Marine Biology and Aquarium (NMMBA), Taiwan (NMMBA-TW-GC-2017-018). Identification of the species of this organism was performed by comparison as described in previous publications.[15–17]

Extraction and Isolation. Sliced bodies of *J. fragilis* (wet weigt 423 g) were extracted with a mixture of organic solvent (MeOH:CH_2Cl_2 = 1:1; volume ratio). The resulting 5.53 g extract was partitioned between ethyl acetate (EtOAc) and H_2O. The EtOAc layer (2.50 g) was separated on silica gel and eluted with a mixture of *n*-hexane/acetone (stepwise from 50:1 to 1:2; volume ratio) to yield 8 subfractions A–H. Fraction E was purified by NP-HPLC using a mixture of *n*-hexane/EtOAc (4.5:1 of volume ratio at a flow rate of 2.0 mL/min) to yield **2** (7.6 mg) and **1** (17.7 mg), respectively. Fraction G was separated on silica

gel cc and eluted with a mixture of *n*-hexane/acetone (1:1; volume ratio) to afford 9 subfractions G1–G9. Fraction G3 was separated by RP-HPLC using a mixture of MeOH and H_2O (with volume:volume = 65:35; at a flow rate = 4.0 mL/min) to yield **4** (0.5 mg). Fraction G5 was purified by NP-HPLC using a mixture of *n*-hexane/EtOAc (1:1; volume ratio) to yield 8 subfractions G5A–G5H. Fraction G5F was separated by RP-HPLC using a mixture of MeOH and H_2O (with volume:volume = 65:35; at a flow rate = 4.0 mL/min) to yield **3** (4.0 mg).

Junceellin (1): colorless crystal; mp 272–275 °C (ref.[2] mp 272–274 °C; ref.[5] mp 240–242 °C; ref.[8], mp 271–272 °C); $[\alpha]_D^{25}$ –2 (*c* 0.89, $CHCl_3$) (ref.[5] $[\alpha]_D^{30}$ –13 (*c* 0.95, $CHCl_3$); ref.[8] $[\alpha]_D^{25}$ –10 (*c* 1.8, $CHCl_3$)); IR (ATR) ν_{max} 1795, 1744 cm^{-1}; ^1H and ^{13}C NMR data were found to be in agreement with previous studies;[2,4,5,8] ESIMS: *m/z* 605 $(M + Na)^+$, 607 $(M + 2 + Na)^+$.

Fragilide M (2): amorphous powder; $[\alpha]_D^{25}$ −3 (*c* 0.38, $CHCl_3$); IR (ATR) ν_{max} 1790, 1734 cm^{-1}; ^{13}C NMR ($CDCl_3$, 100 MHz) and ^1H NMR ($CDCl_3$, 400 MHz) data, see Tables 1 and 2; ESIMS *m/z* 547 $(M + Na)^+$, 549 $(M + 2 + Na)^+$; HRESIMS *m/z* 547.17060 (Calcd for $C_{26}H_{33}{}^{35}ClO_9$ + Na, 547.17053).

Fragilide N (3): amorphous powder; $[\alpha]_D^{20}$ −5 (*c* 0.17, $CHCl_3$); IR (ATR) ν_{max} 3470, 1773, 1736 cm^{-1}; ^{13}C NMR ($CDCl_3$, 100 MHz) and ^1H ($CDCl_3$, 400 MHz) NMR data, see Tables 1 and 2; ESIMS *m/z* 529 $(M + Na)^+$; HRESIMS *m/z* 529.20416 (Calcd for $C_{26}H_{34}O_{10}$ + Na, 529.20442).

Fragilide O (4): amorphous powder; $[\alpha]_D^{20}$ +4 (*c* 0.11, $CHCl_3$); IR (ATR) ν_{max} 3441, 1785, 1732 cm^{-1}; ^{13}C NMR ($CDCl_3$, 150 MHz) and ^1H ($CDCl_3$, 600 MHz) NMR data, see Tables 1 and 2; ESIMS *m/z* 563 $(M + Na)^+$, 565 $(M + 2 + Na)^+$; HRESIMS *m/z* 563.16543 (Calcd for $C_{26}H_{33}{}^{35}ClO_{10}$ + Na, 563.16545).

Molecular Mechanics Calculations. The molecular models were generated by implementing the MM2 force field[11] in ChemBio 3D Ultra software (ver. 12.0) which was created by CambridgeSoft (PerkinElmer, Cambridge, MA, USA).

Single-crystal X-Ray Crystallography of Junceellin (1). Suitable colorless prisms of **1** were obtained from a solution of EtOAc. The crystal (0.268 × 0.231 × 0.111 mm^3) belongs to the orthorhombic system, space group $P2_12_12$ (#18), with *a* = 16.7350(4) Å, *b* = 16.7668(4) Å, *c* = 10.2338(2) Å, *V* = 2871.53(11) $Å^3$, *Z* = 4, D_{calcd} = 1.349 Mg/m^3, λ (Cu Kα) = 1.54178 Å. Intensity data were measured on a Bruker D8 Venture diffractometer up to θ_{max} of 75.0°. All 19527 reflections were collected. The structure was solved by direct methods and refined by a full-matrix least-squares procedure. The refined structural model converged to a final R1 = 0.0293; wR2 = 0.0784 for 5894 observed reflections [$I > 2\sigma(I)$] and 371 variable parameters. The absolute configuration was determined by Flack parameter x = 0.057(4).[18,19] Crystallographic data for the structure of junceellin (**1**) have been deposited with the Cambridge Crystallographic Data Center as supplementary publication number CCDC 1906759. These data can be

obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html (or from the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033; e-mail: deposit@ccdc.cam.ac.uk).

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