

Article

Briarenolides F and G, New Briarane Diterpenoids from a *Briareum* sp. Octocoral

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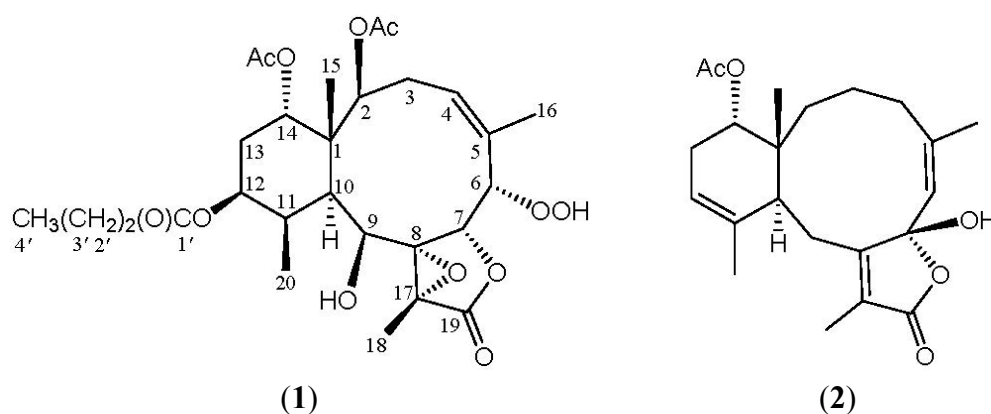
Abstract: Two new briarane diterpenoids, briarenolides, F (**1**) and G (**2**), were isolated from an octocoral identified as *Briareum* sp. The structures of briaranes **1** and **2** were established by spectroscopic methods and by comparison of the spectroscopic data with those of known briarane analogues. Briarenolide F was proven to be the first 6-hydroperoxybriarane derivative and this compound displayed a significant inhibitory effect on the generation of superoxide anion by human neutrophils.

Keywords: briarenolide; briarane; *Briareum*; superoxide anion

1. Introduction

Among the diterpenoids isolated from octocorals, the briarane-type metabolites (3,8-cyclized cembranes) are a major group of compounds [1–3]. The compounds of this type were suggested to be of marine origin and the octocorals belonging to the genus *Briareum* have been proven to be the most important source of briarane-type compounds [4–7]. In previous studies, a series of interesting terpenoid derivatives, including briarane [8–35], cembrane [36] and carotenoid [37], had been isolated from the octocorals belonging to the genus *Briareum* that were distributed in the waters off Taiwan, at the intersection point of the Kuroshio current and the South China Sea surface current. In a continuation of our search for new substances from the Formosan marine invertebrates, the chemical constituents of a specimen octocoral identified as *Briareum* sp. (Briareidae) were studied. A fraction of its organic extract (fraction H, see Experimental Section) displayed inhibitory effects on the generation of superoxide anion (inhibition rate 36.8%) and the release of elastase (inhibition rate 90.3%) at a concentration of 10 $\mu\text{g/mL}$. We further isolated two new briarane-type diterpenoids, briarenolides, F (**1**) and G (**2**) (Figure 1), from the octocoral *Briareum* sp. In this paper, we report the isolation, structure determination and bioactivity of briaranes **1** and **2**.

Figure 1. The structures of briarenolides F (**1**) and G (**2**).



2. Results and Discussion

Briarenolide F (**1**) was isolated as a white powder. The molecular formula of **1** was established as $\text{C}_{28}\text{H}_{40}\text{O}_{12}$ (nine degrees of unsaturation) from a sodium adduct at m/z 591 in the ESIMS spectrum and further supported by HRESIMS ($\text{C}_{28}\text{H}_{40}\text{O}_{12}\text{Na}$, m/z 591.2420, alculated 591.2417). The IR spectrum of **1** showed bands at 3498, 1789 and 1743 cm^{-1} , consistent with the presence of hydroxy, γ -lactone and ester carbonyl groups. The ^{13}C NMR and DEPT spectra of **1** showed that this compound had 28 carbons (Table 1), including seven methyls, four sp^3 methylenes, eight sp^3 methines, three sp^3 quaternary carbons, an sp^2 methine and five sp^2 quaternary carbons. From the ^1H and ^{13}C NMR spectra (Table 1), **1** was found to possess two acetoxy groups (δ_{H} 1.99, 2.01, each $3\text{H} \times \text{s}$; δ_{C} 170.6, $2 \times \text{qC}$; 21.3, $2 \times \text{CH}_3$), an *n*-butyrate group (δ_{H} 0.94, 3H , t, $J = 7.2\text{ Hz}$; 1.63, 2H , sext, $J = 7.2\text{ Hz}$; 2.27, 2H ,

t, $J = 7.2$ Hz; δ_C 13.7, CH₃; 18.4, CH₂; 36.3, CH₂; 173.1, qC), a γ -lactone moiety (δ_C 171.0, qC-19) and a trisubstituted olefin (δ_H 5.65, 1H, br d, $J = 13.6$ Hz, H-4; δ_C 130.3, CH-4; 128.8, qC-5). The presence of a tetrasubstituted epoxide containing a methyl substituent was established from the signals of two quaternary oxygenated carbons at δ_C 68.8 (qC-8) and 58.4 (qC-17) and further confirmed by the proton signal of a methyl singlet at δ_H 1.49 (3H, s, H₃-18). Thus, from the above NMR data, five degrees of unsaturation were accounted for and **1** was identified as a tetracyclic compound.

Table 1. ¹H (400 MHz, CDCl₃) and ¹³C (100 MHz, CDCl₃) NMR data, ¹H–¹H COSY and HMBC correlations for briarane **1**.

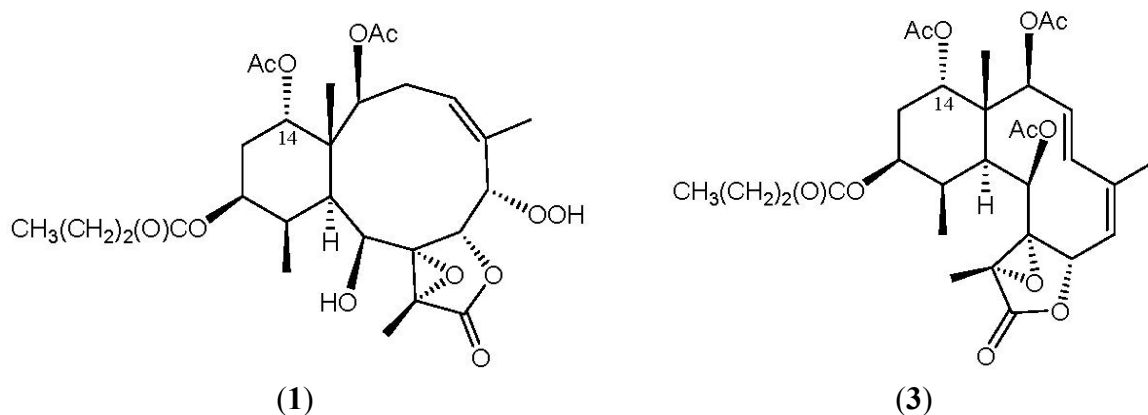
C/H	δ (J in Hz)	δ_C , Mult.	¹ H– ¹ H COSY	HMBC (H→C)
1		44.9, qC		
2	5.22 d (8.0)	75.4, CH	H ₂ -3	C-1, -4, -10, -15, acetate carbonyl
3 α	1.89 m	33.3, CH ₂	H-2, H-3 β , H-4	N.O.
β	3.81 dd (17.2, 13.6)		H-2, H-3 α , H-4	C-4, -5
4	5.65 br d (13.6)	130.3, CH	H ₂ -3, H ₃ -16	N.O.
5		128.8, qC		
6	4.65 d (2.4)	84.9, CH	H-7	C-4, -5, -7, -8, -16
7	5.33 d (2.4)	77.2, CH	H-6	C-5, -6
8		68.8, qC		
9	4.59 d (4.8)	75.1, CH	OH-9	C-1, -8, -10, -11, -17
10	2.06 d (4.0)	39.4, CH	H-11	C-1, -8, -9, -15, -20
11	2.28 m	40.2, CH	H-10, H-12, H ₃ -20	C-1, -10, -12
12	4.98 ddd (12.4, 5.2, 5.2)	70.7, CH	H-11, H ₂ -13	C-20, <i>n</i> -butyrate carbonyl
13	1.87–1.97 m	26.5, CH ₂	H-12, H-14	C-12
14	4.88 dd (3.2, 2.4)	74.9, CH	H ₂ -13	N.O.
15	1.37 s	15.9, CH ₃		C-1, -2, -10, -14
16	1.77 br s	25.4, CH ₃	H-4	C-4, -5, -6
17		58.4, qC		
18	1.49 s	8.8, CH ₃		C-8, -17, -19
19		171.0, qC		
20	1.22 d (7.2)	10.6, CH ₃	H-11	C-10, -11, -12
2-OAc		170.6, qC		
	1.99 s	21.3, CH ₃		Acetate carbonyl
6-OOH	8.71 br s			N.O.
9-OH	2.95 d (4.8)		H-9	N.O.
12-OC(O)Pr		173.1, qC		
	2.27 t (7.2)	36.3, CH ₂	H ₂ -3'	C-1', -3', -4'
	1.63 sext (7.2)	18.4, CH ₂	H ₂ -2', H ₃ -4'	C-1', -2', -4'
	0.94 t (7.2)	13.7, CH ₃	H ₂ -3'	C-2', -3'
14-OAc		170.6, qC		
	2.01 s	21.3, CH ₃		Acetate carbonyl

N.O. = Not observed.

¹H–¹H couplings in the COSY spectrum of **1** enabled identification of the C-2/-3/-4, C-6/-7, C-10/-11/-12/-13/-14, C-4/-16 (by allylic coupling) and C-11/-20 units (Table 1), which were

assembled with the assistance of an HMBC experiment. The HMBC correlations between protons and quaternary carbons of **1**, such as H-2, H-9, H-10, H-11, H₃-15/C-1; H-3 β , H-6, H-7, H₃-16/C-5; H-6, H-9, H-10, H₃-18/C-8; H-9, H₃-18/C-17; and H₃-18/C-19, permitted the elucidation of the carbon skeleton (Table 1). The vinyl methyl at C-5 was confirmed by the allylic coupling between H-4/H₃-16 in the ¹H–¹H COSY spectrum and by the HMBC correlations between H₃-16/C-4, -5, -6 and H-6/C-16. The ring junction C-15 methyl group was positioned at C-1 from the HMBC correlations between H₃-15/C-1, -2, -10, -14; H-2/C-15; and H-10/C-15. In addition, the carbon signal at δ_C 173.1 (qC) was correlated with the signal of the methylene protons at δ_H 2.27 in the HMBC spectrum and was consequently assigned as the carbon atom of the *n*-butyrate carbonyl. Additionally, the *n*-butyrate positioned at C-12 was confirmed by the connectivity between H-12 (δ_H 4.98) and the carbonyl carbon (δ_C 173.1, qC) of the *n*-butyrate. Furthermore, an acetate ester at C-2 was established by a correlation between H-2 (δ_H 5.22) and the acetate carbonyl (δ_C 170.6, qC) observed in the HMBC spectrum of **1**. The presence of a hydroxy group at C-9 was deduced from the ¹H–¹H COSY correlation between a hydroxy proton (δ_H 2.95) and H-9 (δ_H 4.59). The presence of a hydroperoxy group in **1** was supported by a hydroperoxy proton signal at δ_H 8.71 as a broad singlet [22,32,38]. Due to absence of HMBC correlations for H-14 (δ_H 4.88) and the hydroperoxy proton (δ_H 8.71), the positions for the remaining acetoxy and hydroperoxy groups could not be determined by this method. By comparison the ¹H and ¹³C NMR data of C-14 oxymethine for **1** (δ_H 4.88; δ_C 74.9) with those of a known briarane analogue, excavatolide F (**3**) (δ_H 4.94; δ_C 74.1) (Figure 2) [10], which possesses a similar cyclohexane moiety as that of **1**, the remaining acetoxy group in **1** was placed at C-14. Thus, the hydroperoxy group is positioned at C-6, an oxymethine at δ_C 84.9 (CH), by analysis of the ¹H–¹H COSY correlations and characteristic NMR signal analysis.

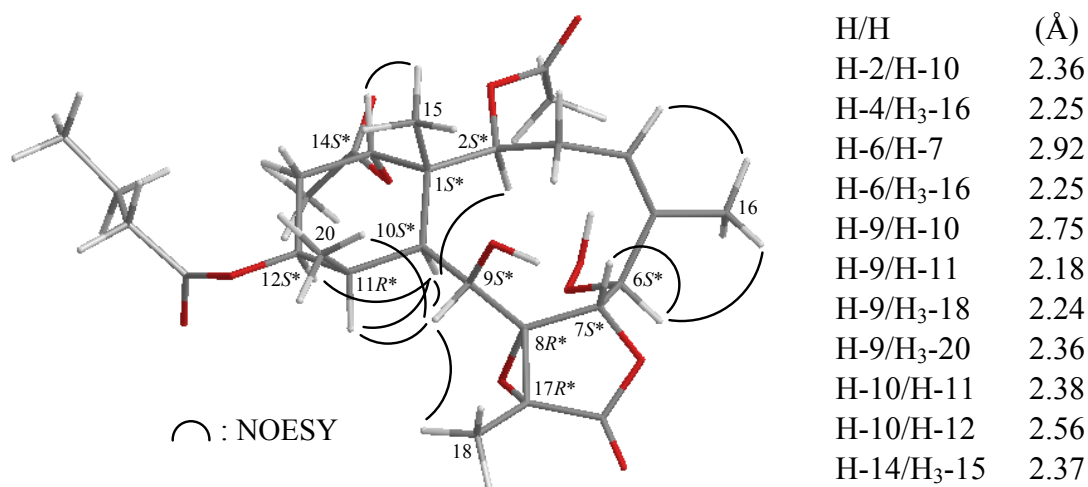
Figure 2. The structures of briarenolide F (**1**) and excavatolide F (**3**).



In all naturally-occurring briaranes, H-10 is *trans* to the C-15 methyl group, and these two groups are assigned as α - and β -oriented in most briarane derivatives [4–7]. The relative configuration of **1** was elucidated from the interactions observed in a NOESY experiment and was found to be compatible with that of **1** offered by computer modeling (Figure 3) [39] and that obtained from vicinal proton coupling constant analysis. In the NOESY experiment of **1**, the correlations of H-10 with H-2, H-9, H-11 and H-12, but not with H₃-15 and H₃-20, indicated that these protons (H-2, H-9, H-10, H-11 and H-12) were situated on the same face, and these were assigned as α protons, since the C-15 and

C-20 methyls are β -substituents at C-1 and C-11, respectively. H-14 was found to exhibit an interaction with H₃-15, but not with H-10, revealing the β -orientation of this proton. The configuration at C-9 is worthy of comment. H-9 was found to exhibit correlations with H-10, H-11, H₃-18 and H₃-20. From a consideration of molecular models, H-9 was found to be reasonably close to H-10, H-11, H₃-18 and H₃-20, while it was placed on the α face in **1**. The C-16 vinyl methyl showed correlations with H-4 and H-6, demonstrating the *Z* configuration of $\Delta^{4,5}$ and the hydroperoxy group at C-6 was α -oriented. The *cis* relationship between H-6 and H-7 was established by a correlation between H-6 and H-7 and a small coupling constant ($J = 2.4$ Hz) between these two protons. Moreover, an acetyl methyl (δ_{H} 2.01) exhibited correlations with H-12 and H-2, further supporting an acetoxy group was positioned on the α -position at C-14 in **1**. Based on the above findings, the configurations of all chiral carbons of **1** were assigned as 1*S**, 2*S**, 6*S**, 7*S**, 8*R**, 9*S**, 10*S**, 11*R**, 12*S**, 14*S**, 17*R**, and the structure of **1** was established unambiguously. To the best of our knowledge, briarane derivatives possessing a hydroperoxy group are rarely found [22,32,38] and briarenolide F (**1**) is the first briarane derivative possessing a 6-hydroperoxy group. A double bond positioned at C-4(5) in briarane-type metabolites is also rarely found [31,40–42].

Figure 3. The stereoview of **1** (generated from computer modeling) and the calculated distances (Å) between selected protons with key NOESY correlations.



Briarenolide G (**2**) was isolated as a white powder whose HRESIMS showed a molecular ion at m/z 397.1989 implying that **2** had the molecular formula C₂₂H₃₀O₅ (C₂₂H₃₀O₅Na, calculated 397.1991). The IR spectrum revealed absorptions for hydroxy (3397 cm⁻¹) and ester carbonyl (1757 and 1734 cm⁻¹) groups. The ¹H NMR data (Table 2) showed resonances due to an acetyl methyl (δ_{H} 2.03, 3H, s), three vinyl methyls (δ_{H} 1.78, 3H, br s, H₃-16; 1.87, 3H, d, $J = 1.6$ Hz, H₃-18; 1.63, 3H, d, $J = 0.8$ Hz, H₃-20), a quaternary methyl (δ_{H} 0.77, 3H, s, H₃-15), two olefinic protons (δ_{H} 5.29, 1H, br s, H-6; 5.12, 1H, m, H-12) and an oxymethine signal (δ_{H} 4.78, 1H, br s, H-14). The ¹³C NMR and DEPT spectra of **2** (Table 2) revealed the presence of a tetrasubstituted (δ_{C} 160.8, qC-8; 125.1, qC-17) and two trisubstituted (δ_{C} 144.4, qC-5; 124.6, CH-6; 136.3, qC-11; 117.8, CH-12) carbon-carbon double bonds, a hemiketal carbon (δ_{C} 106.7, qC-7), an acetate carbonyl (δ_{C} 170.9, qC), an α,β -unsaturated- γ -lactone carbonyl (δ_{C} 171.1, qC-19), a tetrasubstituted carbon atom bearing a carbon substituent (δ_{C} 39.1, qC-1) and an oxymethine (δ_{C} 77.2, CH-14).

Table 2. ^1H (400 MHz, CDCl_3) and ^{13}C (100 MHz, CDCl_3) NMR data, ^1H - ^1H COSY and HMBC correlations for briarane **2**.

C/H	δ_{H} (J in Hz)	δ_{C} , Mult.	^1H - ^1H COSY	HMBC (H \rightarrow C)
1		39.1, qC		
2 α	1.69 m	35.4, CH ₂	H-2 β , H ₂ -3	C-3, -14
β	1.29 m		H-2 α , H ₂ -3	C-1, -3, -10, -14
3	1.72 m	23.7, CH ₂	H ₂ -2, H ₂ -4	C-2
4 α	1.90 m	29.6, CH ₂	H ₂ -3, H-4 β	N.O.
β	3.72 m		H ₂ -3, H-4 α	C-3, -16
5		144.4, qC		
6	5.29 br s	124.6, CH	H ₃ -16	C-4, -7, -16
7		106.7, qC		
8		160.8, qC		
9 α	2.54 br d (15.2)	25.3, CH ₂	H-9 β , H-10	C-8, -10, -11, -17
β	2.37 dd (15.2, 10.8)		H-9 α , H-10	C-7, -8, -10, -11, -17
10	3.76 d (10.8)	35.9, CH	H ₂ -9	N.O.
11		136.3, qC		
12	5.12 m	117.8, CH	H ₂ -13, H ₃ -20	N.O.
13 α	2.06 m	29.3, CH ₂	H-12, H-13 β	C-11, -12, -14
β	2.33 m		H-12, H-13 α	C-11
14	4.78 br s	77.2, CH	H ₂ -13	C-12
15	0.77 s	21.9, CH ₃		C-1, -2, -10, -14
16	1.78 br s	23.9, CH ₃	H-6	C-4, -5, -6
17		125.1, qC		
18	1.87 d (1.6)	9.1, CH ₃		C-8, -17, -19
19		171.7, qC		
20	1.63 d (0.8)	21.9, CH ₃	H-12	C-10, -11, -12
7-OH	3.35 s			C-6, -7, -8
14-OAc		170.9, qC		
	2.03 s	21.7, CH ₃		Acetate carbonyl

N.O. = Not observed.

From the ^1H - ^1H COSY experiment of **2** (Table 2), it was possible to establish the separate spin systems that map out the proton sequences from H₂-2/H₂-3/H₂-4 and H₂-9/H-10. These data, together with the HMBC correlations between H₂-2/C-1, -3, -10; H₂-3/C-2; H-4 β /C-3; H-6/C-4, -7; and H₂-9/C-7, -8, -10, established the connectivity from C-1 to C-10 in the ten-membered ring (Table 2). The vinyl methyl at C-5 was confirmed by the HMBC correlations between H₃-16/C-4, -5, -6; H-4 β /C-16; and H-6/C-16, and further supported by the allylic coupling between H-6 and H₃-16. The methylcyclohexene ring, which is fused to the ten-membered ring at C-1 and C-10, was elucidated by the ^1H - ^1H COSY correlations between H-12/H₂-13/H-14 and H-12/H₃-20 (by allylic coupling) and by the HMBC correlations between H₂-2/C-14, H₂-9/C-11 and H₃-20/C-10, -11, -12. The ring junction C-15 methyl group was positioned at C-1 from the HMBC correlations between H₃-15/C-1, -2, -10, -14. In addition, the acetate ester at C-14 was established by a correlation between H-14 (δ_{H} 4.78) and the acetate carbonyl observed in the HMBC spectrum of **2**. The presence of a hydroxy group at C-7 was deduced from the HMBC correlations between the hydroxy proton (δ_{H} 3.35, 1H, s, OH-7) and C-6, C-7,

and C-8. The C-7 hydroxy group was concluded to be a part of hemiketal constellation on the basis of a characteristic carbon signal at δ_C 106.7 (a quaternary hemiketal carbon, qC-7). These data, together with the HMBC correlations between H₃-18/C-8, -17, -19, were used to establish the molecular framework of **2**.

NOESY measurements were carried out in order to deduce the relative stereochemical features of **2** (Figure 4). Thus, H₃-15 gave a correlation with H-14, but not with H-10, indicating that H₃-15 and H-14 are located on the same face (assigned as the β -face) and that H-10 lies on the opposite side, α -face. The NOESY spectrum showed correlations between H-6/H₃-16 and H-12/H₃-20, revealing the *Z* geometry of the C-5/6 and C-11/12 double bonds in **2**. Due to the absence of NOESY correlations for the C-7 hydroxy group, the configuration at that chiral center could not be determined by this method. By comparison of the ¹³C NMR chemical shifts of C-6 (δ_C 124.6), C-7 (δ_C 106.7) and C-8 (δ_C 160.8) for **2** with those of an unnamed known 7 β -hydroxybriarane analogue **4** (δ_C 124.8, C-6; 106.2, C-7, 160.1, C-8), which was obtained from a Caribbean octocoral *Briareum polyanthes* [43] (Figure 5), we deduced that the C-7 hydroxy group was β -oriented and the configuration of all the chiral carbons in **2** were assigned as 1*R**, 7*S**, 10*S**, 14*S**.

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

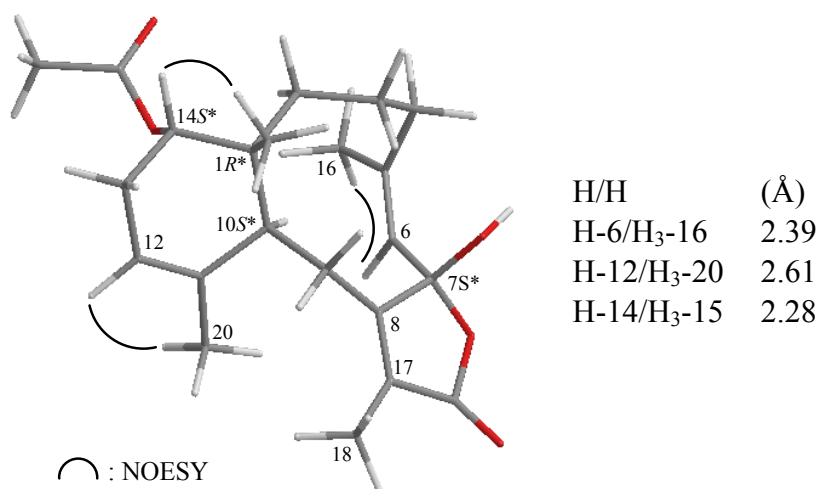
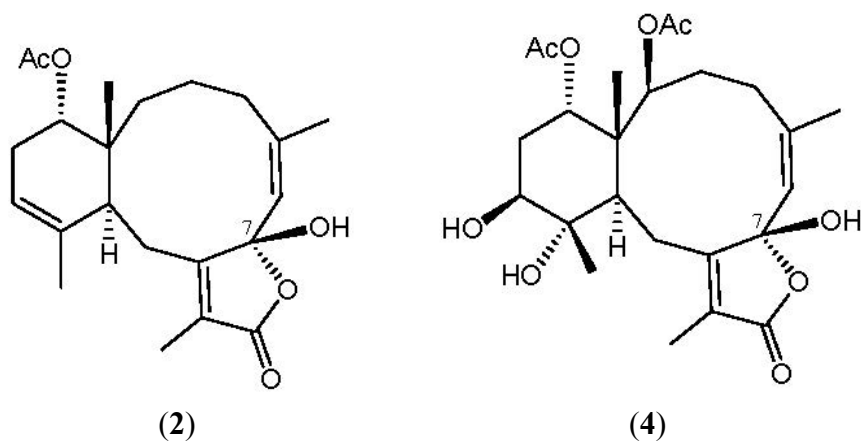


Figure 5. The structures of briarenolide G (**2**) and briarane (**4**).



The *in vitro* anti-inflammatory effects of briaranes **1** and **2** were tested. Briarenolide F (**1**) was found to display a significant inhibitory effect on the generation of superoxide anion by human neutrophils (Table 3).

Table 3. Inhibitory effects of briaranes **1** and **2** on the generation of superoxide anion and the release of elastase by human neutrophils in response to FMLP/CB.

Compounds	Superoxide Anion		Elastase Release	
	IC ₅₀ (µg/mL)	Inh% ^a	IC ₅₀ (µg/mL)	Inh% ^a
1	3.82 ± 0.45	76.65 ± 4.21	>10.0	27.48 ± 6.60
2	>10.0	22.04 ± 3.43	>10.0	12.98 ± 4.68
DPI ^b	0.82 ± 0.31			
Elastatinal ^b			31.82 ± 5.92	

^a Percentage of inhibition (Inh%) at a concentration of 10 µg/mL; ^b DPI (diphenylene indonium) and elastatinal were used as reference compounds.

3. Experimental Section

3.1. General Experimental Procedures

Optical rotations were measured on a Jasco P-1010 digital polarimeter. Infrared spectra were recorded on a Varian Digilab FTS 1000 FT-IR spectrometer; peaks are reported in cm⁻¹. The NMR spectra were recorded on a Varian Mercury Plus 400 NMR spectrometer. Coupling constants (*J*) are given in Hz. ¹H and ¹³C NMR assignments were supported by ¹H–¹H COSY, HMQC, HMBC and NOESY experiments. ESIMS and HRESIMS were recorded on a 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 F₂₅₄ (0.25 mm, Merck), and spots were visualized by spraying with 10% H₂SO₄ solution followed by heating. HPLC was performed using a system comprised of a Hitachi L-7100 pump and a Rheodyne injection port. A normal phase column (Hibar 250 × 10 mm, Merck, silica gel 60, 5 µm) was used for HPLC.

3.2. Animal Material

Specimens of the octocorals *Briareum* sp. were collected by hand using scuba equipment off the coast of southern Taiwan in July 2011 and stored in a freezer until extraction. A voucher specimen (NMMBA-TW-SC-2011-77) was deposited in the National Museum of Marine Biology and Aquarium. This organism was identified by comparison with previous descriptions [44–47].

3.3. Extraction and Isolation

Sliced bodies of *Briareum* sp. (wet weight 6.32 kg, dry weight 2.78 kg) were extracted with a mixture of methanol (MeOH) and dichloromethane (DCM) (1:1). The extract was partitioned between ethyl acetate (EtOAc) and H₂O. The EtOAc layer was separated on silica gel and eluted using *n*-hexane/EtOAc (stepwise, 100:1–pure EtOAc) to yield 18 fractions A–R. Fraction H was chromatographed on silica gel and eluted using *n*-hexane/acetone (stepwise, 40:1–pure acetone) to

afford 45 fractions H1–H45. Fraction H11 was separated by normal-phase HPLC (NP-HPLC) using a mixture of *n*-hexane and EtOAc (5:2) as the mobile phase to afford compound **2** (0.4 mg). Fraction H16 was further purified by normal-phase HPLC using a mixture of *n*-hexane and acetone as the mobile phase (7:2) to afford compound **1** (2.3 mg).

Briarenolide F (**1**): white powder; mp 141–142 °C; $[\alpha]_D^{25} +32$ (*c* 0.1, CHCl₃); IR (neat) ν_{\max} 3498, 1789, 1743 cm⁻¹; ¹H (CDCl₃, 400 MHz) and ¹³C (CDCl₃, 100 MHz) NMR data, see Table 1; ESIMS: *m/z* 591 [M + Na]⁺; HRESIMS: *m/z* 591.2420 (calcd for C₂₈H₄₀O₁₂Na, 591.2417).

Briarenolide G (**2**): white powder; mp 78–80 °C; $[\alpha]_D^{25} -97$ (*c* 0.02, CHCl₃); IR (neat) ν_{\max} 3397, 1757, 1734 cm⁻¹; ¹H (CDCl₃, 400 MHz) and ¹³C (CDCl₃, 100 MHz) NMR data, see Table 2; ESIMS: *m/z* 397 [M + Na]⁺; HRESIMS: *m/z* 397.1989 (calcd for C₂₂H₃₀O₅Na, 397.1991).

3.4. Molecular Mechanics Calculations

Implementation of the MM2 force field [39] in CHEM3D PRO software from CambridgeSoft Corporation (version 9.0, Cambridge, MA, USA; 2005) was used to calculate the molecular models.

3.5. 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 [48,49]. 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.

4. Conclusions

Briarane-type natural products (3,8-cyclized cembranoid) were found in various marine organisms, particularly with the octocorals belonging to the genus *Briareum* (family Briareidae) [4–7]. It is interesting to note that the briarane-type natural products are major constituents of the extracts of octocorals *Briareum* spp. distributed in the tropical and subtropical Indo-Pacific Ocean. In the past 35 years, over 500 briarane analogues have been obtained and the number is still increasing based on their structural complexity and interesting bioactivities. It is worth noting that only three hydroperoxybriarane analogues have been isolated to date [22,32,38] and that briarenolide F (**1**) is the first 6-hydroperoxybriarane. 7-Hydroxybriarane derivatives are also rarely found [43,50–52]; the new briarane, briarenolide G (**2**) was the first 7-hydroxybriarane derivative isolated from the octocorals collected off the waters of Taiwan. The study material *Briareum* sp. has begun to be transplanted in tanks for the extraction of natural products in order to establish a stable supply of bioactive material.

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