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Bioactive Diterpenoid Containing a Reversible "Spring-Loaded" (*E,Z*)-Dieneone Michael Acceptor

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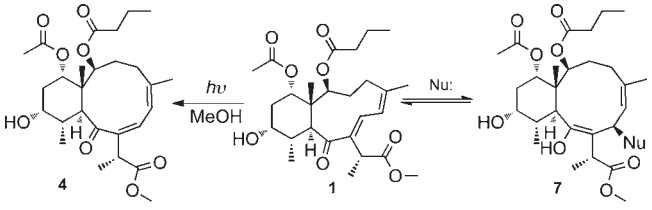
ABSTRACT



Three new briarane diterpenoids, briareolate esters L–N (1–3), have been isolated from a gorgonian *Briareum asbestinum*. Briareolate esters L (1) and M (2) are the first natural products possessing a 10-membered macrocyclic ring with a (*E,Z*)-dieneone and exhibit growth inhibition activity against both human embryonic stem cells (BG02) and a pancreatic cancer cell line (BxPC-3). Briareolate ester L (1) was found to contain a "spring-loaded" (*E,Z*)-dieneone Michael acceptor group that can form a reversible covalent bond to model sulfur-based nucleophiles.

The marine gorgonian of the genus *Briareum* is an abundant source of highly oxygenated diterpenoids belonging to the eunicellin, asbestinane, cembrane, and briarane classes and exhibit numerous biological activities (e.g., cytotoxicity, antimicrobial, anti-inflammatory, antiviral, immunomodulatory, antifouling, and ichthotoxicity).[1] The briareolate esters are a small group of unusual briarane diterpenoids isolated from *Briareum asbestinum* off the coast of Tobago that contain a C-19 methyl ester instead of the γ-lactone ring.[2−4] In the course of screening prefractionated and semipurified extracts of marine invertebrates to discover compounds that impact human embryonic stem cell (hESC) growth using a 96-well plate real-time cell electronic sensing (RT-CES) system, activity was found for the extract of the gorgonian *Briareum asbestinum*. Further purification of the active fractions led to the isolation of three new briarane diterpenoids, briareolate ester L–N (**1**–**3**), together with three known briareolate esters B (**5**), C (**6**), and G (**4**). In this paper we report the isolation, biological activity, and possible mechanism of bioactivity.

Briareolate ester L (**1**) was isolated as a colorless oil. The molecular formula of briareolate ester L (**1**), $C_{27}H_{40}O_8$, that was determined from the HRESIMS of the $[M + Na]^+$ ion at m/z 515.2596 required 8 degrees of unsaturation. An initial analysis of the ^{13}C NMR data revealed four carbonyl carbons (δ_C 201.4, 176.5, 175.5, 173.2), and two C–C double bonds (δ_C 148.1, 147.0, 141.7, 118.5) (see Table S1,

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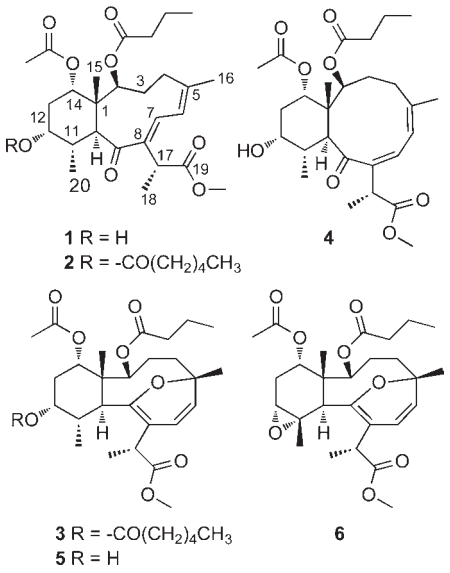
Supporting Information). These data accounted for six of the eight double bond equivalents and indicated that **1** was



bicyclic. The presence of a ketone conjugated with two double bonds (α,*β*,*γ*,*δ*-unsaturated ketone) was indicated from a carbonyl carbon with a chemical shift of δ_C 201.4 (C-9) and the C−C double bond carbons at δ_C 148.1, 147.0, 141.7, and 118.5. The observation of UV absorption maxima at λ_{max} = 284 and 232 nm and a C=O stretching band at 1650 cm^{-1} in the IR spectrum were consistent with this assignment.

The structure of **1** was determined by a detailed analysis of the NMR data (Figure 1). The HSQC experiment allowed the assignment of all the protons to the corresponding carbon atoms. The $^1H-^1H$ COSY and HMBC experiments allowed the gross structure of **1** to be determined and revealed that **1** had the same planar structure as the C-19 methyl ester briarane diterpenoid briareolate ester G (**4**). However, the signals of **1**, at δ_H 6.21 (br s) and δ_H 7.68 (br s), appeared more downfield when compared with the analogous signals of briareolate ester G **4** [H-6 (δ_H 6.07) and H-7 (δ_H 6.62)][4] suggesting a change in the geometry of the double bonds.

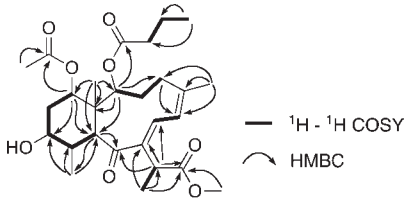


Figure 1. Selected 2D NMR correlations for **1**.

From the 2D ROESY data, it was found that H-7 exhibited NOE correlations with H-2 and H-10 and placed H-7 on the inside of the 10-membered ring and allowed the geometry of the C-7−C-8 double bond to be assigned as *E*. In addition, NOE correlations observed from H-6 to H-17, and H_3-16 established the geometry of the C-5−C-6 double bond as *Z*. The relative configuration of C-1, C-2, C-10, C-11, C-12, and C-14 was determined from coupling patterns in the ^1H NMR spectrum and NOE correlations observed in a ROESY spectrum (see Figure S1, Supporting Information (SI)). The configuration at C-17 could not be definitely determined due to limited NOE data; however a correlation from H-6 to H-17 is consistent with the assignment of the Me-18 being α-orientated as observed in all previously reported briareolate esters.[2−4]

The absolute configuration of **1** was assigned by analogy to the known coisolated compounds briareolate esters G (**4**) and B (**5**), whose structures were assigned on the basis X-ray crystallography of compounds in the series, that were found to have identical NMR spectral data and comparable optical rotation values ($[\alpha]^{25}_D$ −112, lit$[\alpha]_D$ −122 for **4**; and $[\alpha]^{25}_D$ +147 lit$[\alpha]_D$ +150 for **5**).[3,4] Thus the absolute configuration of briareolate ester L (**1**) is therefore defined as 1*S*,2*S*,5*Z*,7*E*,10*S*,11*S*,12*R*,14*S*,17*R*.

Briareolate ester M (**2**) was isolated as a colorless oil. The HRESIMS of briareolate ester M (**2**) showed an $[M + Na]^+$ ion at *m*/*z* 613.3350, corresponding to the molecular formula $C_{33}H_{50}O_9$, 98 mass units higher than that of **1**. The ^1H NMR spectrum of **2** was similar to that of briareolate ester L (**1**), except that H-12 [δ_H 4.82, br q (3.0)] was shifted downfield by 1.05 ppm (Table 1) as compared with that of **1**. In the ^{13}C NMR spectrum, the resonance of C-12 (δ_C 73.0) was shifted downfield by 2.5 ppm and those of C-11 (δ_C 33.2) and C-13 (δ_C 29.1) were shifted upfield by 2.7 and 3.6 ppm, respectively, in comparison with those of **1**. This suggested that the 12-hydroxy group of **1** was replaced by a hexanoate group at C-12 in **2**. The presence of the hexanoate group was confirmed by the NMR data [δ_H 0.89 (3H, t, *J* = 7.0 Hz), ca. 1.33 (4H, overlapped), 1.62 (2H, overlapped), 2.28 (2H, m), δ_C 15.8 (q), 22.4, 24.9, 31.3, and 34.7 (each t), 173.1 (CO)].

The relative configuration of briareolate ester M (**2**) was assumed to be the same as that of **1** due to the similarity of proton−proton coupling constants and ^1H and ^{13}C chemical shifts. Thus, briareolate ester M was assigned as **2**.

Briareolate ester N (**3**) was isolated as a colorless oil. The molecular formula of briareolate ester N (**3**), $C_{33}H_{50}O_9$, that was determined from the HRESIMS of the $[M + Na]^+$ ion at *m*/*z* 613.3350 required 9 degrees of unsaturation. Initial analysis of the NMR suggested that briareolate ester N (**3**) had the same carbon skeleton as briareolate ester B (**5**), except that the hydroxy group at C-12 was replaced by a hexanoate group. The ^1H and ^{13}C NMR data of **3** were similar to those of **5**, except that H-12 (δ_H 5.13, m) was shifted downfield by 1.27 ppm as compared with that of **5**.[2] In addition, in the ^{13}C NMR spectrum, resonances of C-12 (δ_C 72.4) were shifted downfield by 1.8 ppm and those of C-11 (δ_C 32.3) and C-13 (δ_C 31.4) were shifted upfield by 1.2 and 2.2 ppm, respectively, in comparison with those of **5**. An HMBC correlation observed from H-12 (δ_H 5.13) to the ester carbonyl carbon at δ_C 172.9 allowed placement of the hexanoate group at the C-12 position. The similarity of proton−proton coupling constants and ^1H and ^{13}C chemical shifts together with a ROESY spectrum of **3** showed

Table 1. ^{1}H NMR Data (500 MHz) for Briareolate Esters **1**– **3**

position	**1**[a] (J in Hz)	**2**[b] (J in Hz)	**3**[b] (J in Hz)
2	5.50, br d (8.0)	5.51, br d (7.5)	4.98, d (9.5)
3α	2.48, dd (16.5, 11.0)	2.48, dd (16.0, 11.0)	2.13, m
3β	1.26, m	1.26, m	
4α	2.29, m	2.35, m	2.22, m
4β	2.17, m	2.22, m	1.38, m
6	6.21, br s	6.13, br s	5.62, d (9.0)
7	7.68, br s	7.64, br s	5.96, d (9.5)
10	3.80, d (11.5)	3.70, d (11.0)	3.68, d (12.0)
11	2.28, m	2.15, m	2.26, m
12	3.77, br q (3.0)	4.82, br q (3.0)	5.13, m
13α	2.07, dt (16.0, 3.0)	2.18, dt (13.0, 3.0)	2.10, m
13β	1.89, dt (16.0, 3.0)	1.87, dt (13.0, 3.0)	2.10, m
14	4.81, br s	5.08, br s	4.92, t (3.0)
15	0.87, s	0.87, s	1.42, m
16	1.73, s	1.75, s	1.38, m
17	3.52, q (6.0)	3.51, q (6.5)	3.76, q (7.0)
18	1.24, d (7.0)	1.28, d (7.0)	1.36, d (7.0)
20	0.89, d (7.0)	0.78, d (7.0)	0.89, d (7.0)
OCH_3	3.60, s	3.69, s	3.87, s
C-2 ester	2.20, m	2.22, m	2.20, m
	1.60, sextet (8.0)	1.64, m	1.61, m
	0.93, t (7.0)	0.93, t (7.0)	0.96, t (7.0)
C-12 ester		2.28, m	2.32, m
		1.62, m	1.69, m
		1.33, m	1.34, m
		1.33, m	1.34, m
		0.89, t (7.0)	0.93, t (7.0)
C-14 ester	2.00, s	2.04, s	1.98, s

[a] Measured in CD_3OD, [b] Measured in $CDCl_3$.

the same relative configuration as **5** at all eight chiral centers. Thus, briareolate ester N was assigned as **3**.

In addition to isolating three new briareolate esters, the previously reported compound briareolate ester G (**4**) was isolated and identified from identical spectroscopic data (MS, IR, UV, optical rotation, ^{1}H and ^{13}C NMR data).[4] In the original publication the geometry of the C-7−C-8 double bond was not determined due to decomposition. In the ROESY experiment of **4**, the olefinic proton H-6 exhibited NOE correlations to both H-7 and the olefinic methyl H_3-16 and allowed assignment of the geometry of the C-7−C-8 double bond as *Z*. NOE correlations from H-7 to H-17 placed H-7 on the outside of the 10-membered ring and confirmed *Z* configuration of the C-5−C-6 double bond. Further NOE correlations confirmed the relative configuration of the compound with that of the previously reported compound **4**.

hESCs are a unique cell type isolated from the inner cell mass of preimplantation blastocysts.[5,6] The advent of defined media and controlled differentiation has enabled screening of hESCs to discover molecules that impact growth, differentiation, or apoptosis in undifferentiated and differentiating populations. The cell growth inhibitory activities of compounds **1**−**6** were evaluated against hESCs (BG02) and a pancreatic cancer cell line (BxPC-3) using an RT-CES system.[7] Briareolate ester L (**1**) showed the greatest growth inhibition against both the BG02 and BxPC-3 cells with EC_{50} values of 2.4 and 9.3 μM, respectively (see Figure S2, SI). Briareolate ester G (**4**) did not show any cytotoxic effects against the BG02 or the BxPC-3 cells at 20 μM (see Figure S3, SI). Compound **2** showed reduced cytotoxic activity against the BG02 cells with an EC_{50} value of 8.0 μM and only some cytostatic effects at 13.0 and 17.0 μM against the BxPC-3 cells. No inhibitory activity was found for compounds **3**, **5**, and **6** at 40 μM.

The screening results raised the question why briareolate G (**4**), which differs only in the geometry of the double bond between C-7−C-8, was not biologically active. The most significant spectral differences observed between **1** and **4** were the chemical shifts of the protons and carbons of the dienone. In particular, the downfield shift of the olefinic proton H-7 at δ_H6.62 (δ_C138.1) in **4** to δ_H7.64 (δ_C145.2), the upfield shift of the carbons of the C-5C-6 double bond [δ_C 144.8 (C-5), δ_C 123.5 (C-6) for **4**; and δ_C 140.2 (C-5), δ_C 116.4 (C-6) for **1**], and the upfield shift of the olefinic methyl group H_3-16 from δ_H 2.17 (δ_C 27.2) in **4** to δ_H 1.73 (δ_C 26.5).

The chemical shift differences indicated that the C-5−C-6 double bond in **1** is less conjugated with the C-7−C-8 double bond compared to **4**, and the (*E,Z*)-dienone is twisted out of plane due to restriction of the 10-membered macrocycle. The observation of a shorter UV absorption maxima at 284 nm for **1** compared to 288 nm for **4** is consistent with less conjugation. This suggested that **1** could be "spring-loaded" or cocked for nucleophilic attack at the conjugated and electrophilic C-7 position to relieve ring strain energy in the macrocycle. To the best of our knowledge, this is the first example of a 10-membered macrocyclic ring containing an (*E,Z*)-dieneone and could be a possible explanation for the difference in biological activity of **1** and **4**.

To explore the "spring-loaded" Michael acceptor hypothesis, solutions of **1** and **4** were reacted with a series of model nucleophiles (eq 1). Briareolate ester L (**1**) was found to react with the sulfur-based nucleophile thiophenol at 23 °C in methanol-d_4 to afford the enol 1,4 addition product (**7a**) quickly ($<$5 min), quantitatively (^{1}H NMR analysis; see Figure S4, SI), and stereoselectively (1D NOESY; see Figure S5, SI). In addition, **1** was found to react with the amino acid derivative cysteine methyl ester in the presence of DMAP overnight to afford the keto 1,4 addition product (**7b**, Nu = L-Cys-OMe; see Figure S6, SI).

Although the 1,4 addition products were stable enough to obtain full NMR data sets, the adducts formed were found to be labile toward purification on HPLC (C18 and PRP-1) and could not be purified chromatographically without inducing reversal (**7** → **1**). All attempted addition of oxygen- and nitrogen-based nucleophiles (e.g., phenol, aniline, *N*-Boc L-tyrosine methyl ester, *N*-Boc L-serine methyl ester, L-histidine methyl ester HCl, and

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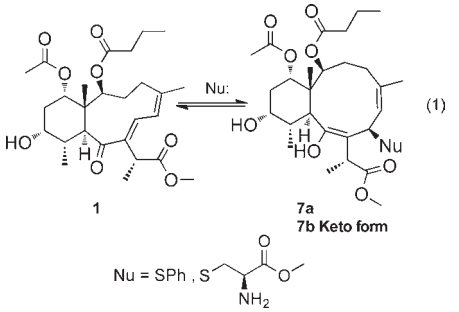
N-benzoyl-L-arginine ethyl ester) with and without DMAP failed to produce any detectable 1,4 addition products in reactions with **1**. Briareolate ester G (**4**) did not react with any of the nucleophiles tested and indicated that the twisted or "spring-loaded" (*E,Z*)-dienone is required for nucleophilic addition reactions to occur.



To better understand the reactivity of the compounds, the optimized molecular structures and energies of **1** and **4** were calculated using density functional theory in Gaussian 09[8] employing the B3LYP functional and 6-31G(d) basis set (Figure 2 for **1**; and see Figure S7 for **4**). The (*Z,Z*)-dieneone isomer **4** was found to be more stable than the (*E,Z*)-dieneone isomer **1** with calculated total energies of −1654.75577047 and −1654.69805219 Ha, respectively. This corresponds to an energy difference of 36.22 kcal/mol.

The energies of the two isomers were also calculated in methanol and water solvents at the same level of theory using the CPCM solvent model. In both cases the (*Z,Z*)-dieneone **4** was found to be the more stable isomer with energy differences of 29.75 and 29.58 kcal/mol in methanol and water, respectively. Although solutions of **1** in benzene or methanol were found to be thermally stable (> 60 °C), direct irradiation (200 W Hg lamp) for five days led to complete photoisomerization (see Figure S8, SI) to give **4**, which had identical NMR and optical rotation data, and is in agreement with the calculated stability of the (*Z,Z*)-dieneone.

In conclusion, we have isolated three new C-19 methyl ester briarane diterpenoids. Briareolate ester L (**1**) and M (**2**) are the first natural products possessing a 10-membered macrocyclic ring with an (*E,Z*)-dieneone and exhibit growth inhibition activity against both BG02 and BxPC-3 cell lines. We found that the α,*β*,*γ*,*δ*-unsaturated ketone

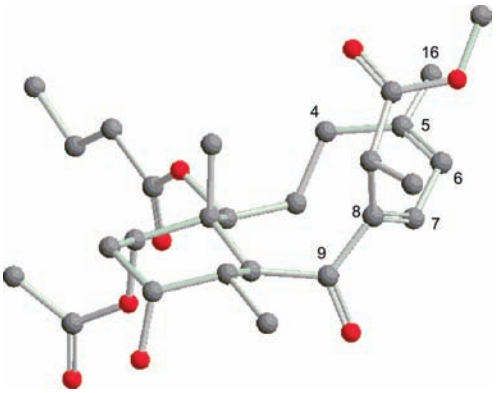


Figure 2. Optimized molecular structure of briareolate L (**1**).

in conjunction with the double bond configuration is required for biological activity. In addition, we showed that **1** contains a "spring-loaded" Michael acceptor that can form a reversible covalent bond to model sulfur-based nucleophiles and provides a possible mechanism of bioactivity of **1** and **2**.

Recently there has been a renewed interest in Michael acceptors in particular compounds that can undergo reversible reactions with thiols such bardoxolone methyl (RTA402).[9,10] Bardoxolone methyl is a synthetic homotriterpenoid that induces Nrf2 (NF-E2-related factor 2), a transcription factor of the antioxidant response, and is currently in late-stage clinical development for chronic kidney disease.[11] Therefore, the identification of novel structural motifs capable of reversible thiol addition reactions such as in **1** and **2** could have utility in the drug discovery process.

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Supporting Information Available. Experimental procedures, cytotoxic responses of **1** and **4**, key NOE correlations of **1**, Michael addition reactions, key NOE correlations of **7a-SPh**, optimized molecular structure of **4**, photoisomerization reaction, and NMR spectra for **1**, **2**, **3**, **7a-SPh**, and **7b-L-Cys-OMe** are available including ^{1}H, ^{13}C, COSY, HSQC, HMBC, and ROESY. This material is available free of charge via the Internet at http://pubs.acs.org.

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