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Cembrane diterpenoids from the Taiwanese soft coral *Sinularia flexibilis*

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ABSTRACT

Chemical investigation of the soft coral *Sinularia flexibilis* (Quoy and Gaimard), collected from the southern coast of Taiwan, led to the isolation of 10 new cembrenoid diterpenoids, the flexilarins A–J (**1**–**10**), along with 17 known compounds (**11**–**27**). The structures of these compounds were elucidated by spectroscopic techniques (NMR, MS, UV, IR). The structure of compound **1** was confirmed by X-ray crystallographic analysis. Compound **4** exhibited potent cytotoxicity against Hep2 tumor cells.

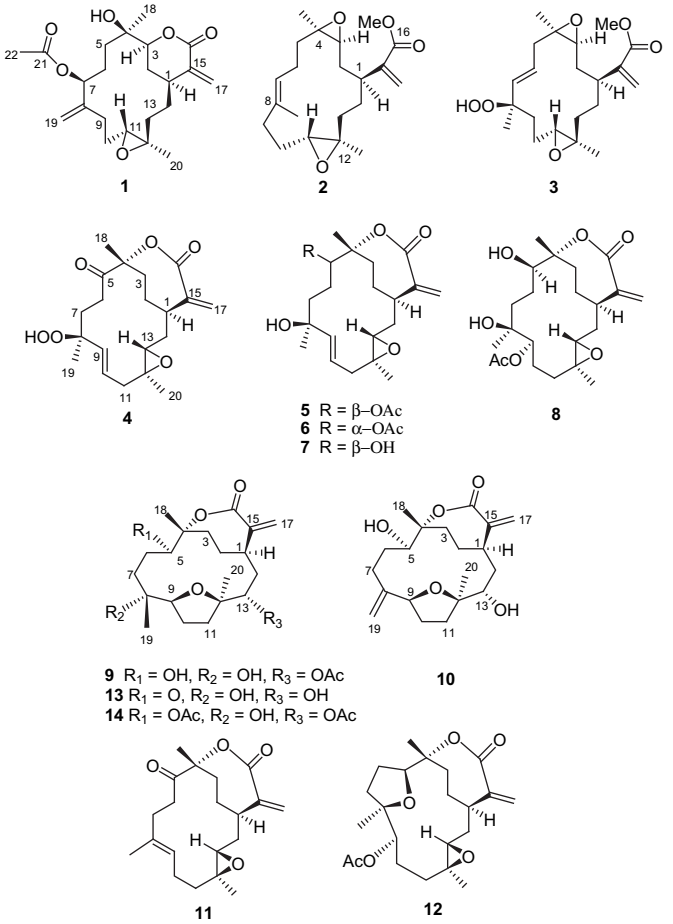
1. Introduction

Marine invertebrates of the family Alcyoniidae have been proven to be a rich source of natural products for biomedical research. Members of the genus *Sinularia* (family Alcyoniidae) have yielded numerous cembrane diterpenoids possessing the classic 14-membered ring.[1–8] Cembrane diterpenes have been reported to have antitumor,[9,10] antimicrobial,[11] and neuroprotective[12] activities. Among the early discovered diterpenes isolated from the genus *Sinularia*[13,14] were the sinulariolides from the Indonesian *Sinularia flexibilis*[15] and pukalide from the Hawaiian *Sinularia abrupta*.[16] The soft coral, *S. flexibilis* (Quoy and Gaimard) occurs in many diverse regions of the Indo-pacific, and several collections have been chemically examined by workers who have reported the isolation of a series of cembranoid diterpenes.[17–23] Most are derivatives of sinulariolide or flexibilide, and sinularin, which contain a 14-membered cembrene ring fused to 6 or 7-membered lactone functionality. This paper reports the isolation and structural elucidation of new cembrane diterpenoids flexilarins A–J (**1**–**10**) from this source collected off the coast of Taiwan. The cytotoxicity of these compounds was also tested and evaluated.

2. Results and discussion

2.1. Structure of compound 1

Compound **1** was obtained as colorless prisms. The HRESIMS revealed a pseudo-molecular ion peak at *m*/*z* 415.2097 $[M+Na]^+$, consistent with the molecular formula $C_{22}H_{32}O_6$ having seven



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degrees of unsaturation. The IR spectrum suggested the absorption bands diagnostic of hydroxyl (3404 cm^{-1}) and α,β-unsaturated ester (1714 cm^{-1}) functionalities. The ^{13}C NMR spectrum showed resonances for 22 carbons, which can be differentiated by DEPT experiments into 3 methyl, 9 methylene, 4 methine, and 6 quaternary carbons. The ^{1}H NMR spectrum of **1** showed two *gem*-methylene doublets at δ_H 6.48 and 5.70 (δ_C 128.9), which showed HMBC correlations with a quaternary carbon (δ_C 139.9), a carbonyl carbon (δ_C 167.1), and a methine carbon (δ_C 34.5) signal. The latter carbon correlates to a ^{1}H NMR methine multiplet at δ_H 2.61, which showed COSY and NOESY correlations with a methylene multiplet at δ_H 1.40 (δ_C 27.0) and a methine doublet at δ_H 3.98 (δ_C 82.0). The assemblage of the above fragments indicates the presence of an *exo*-methylene conjugated lactone ring. The protons of the methyl singlet at δ_H 1.38 (δ_C 24.6) were linked by HMBC correlations with an oxygenated quaternary carbon (δ_C 72.7) and the adjacent methine carbon (δ_C 82.0) on lactone ring, together suggesting extension of one carbon from the lactone ring with methyl and hydroxyl functional groups. The signals of another set of methylene protons at δ_H 5.19 (δ_C 111.5) were linked by HMBC correlations to a quaternary carbon (δ_C 142.1), the adjacent methylene carbon (δ_C 29.8), and oxygenated methine carbon (δ_C 73.5). The latter methine proton multiplet at δ_H 5.24 was in turn correlated with a carbonyl carbon at δ_C 170.0 that was assigned to an acetoxyl group (δ_H 2.12 and δ_C 21.1). Thus, the above correlations revealed a fragment composed of exocyclic double bond with adjacent acetoxyl methine carbon. The ^{1}H NMR methyl singlet at δ_H 1.33 was correlated with a quaternary oxygenated carbon (δ_C 59.3) and an oxygenated methine carbon (δ_C 63.8), the latter of which correlates with a methine proton at δ_H 2.92 (dd), implicating the presence of an epoxy group flanking by a methyl group. By deducing the unsaturations of the *exo*-methylene conjugated lactone ring, acetoxyl, exocyclic methylene, and the epoxy ring, the remaining one unsaturation strongly indicated that compound **1** is a member of the cembrene diterpene class. The assignments of each fragment on the cembrene ring were ascertained by COSY and HMBC experiments (Fig. 1). The structural features of flexarin A (**1**) were very similar to the known compound sinuflexolide,[24] in which the acetoxyl group was eliminated, the exocyclic methylene group rearranged into an endocyclic double bond between C-7 and C-8, and the epoxy ring opened up to give adjacent dihydroxyl groups. In the NOESY spectrum of **1** (Fig. 2), the correlations between Me-18/H-3α; H-3α/H-1α; H-1α/Me-20; Me-18/H-5α/H-6α; and H-6α/H-7α were observed, therefore, favoring the orientations of H-1α, H-3α, H-7α, H-11β and Me-20α. Finally, the structure of **1** was conclusively assigned by single-crystal X-ray diffraction analysis and an ORTEP diagram showing the relative configuration of **1** was illustrated in Figure 3. The configurations at the lactone centers, C-1 and C-3, and the adjacent quaternary carbon C-4 are similar to that of the known sinuflexolide.[24] This series of compounds was given a general name of flexilarin, and flexilarin A was assigned to as compound **1**.

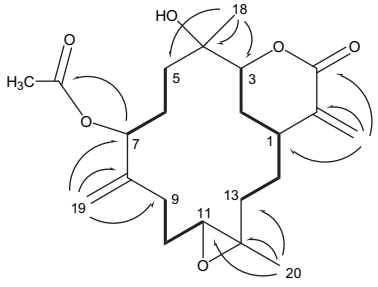


Figure 1. Selected HMBC (hooks) and COSY (bold line) correlations of **1**.

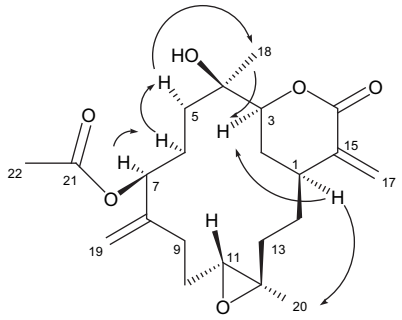


Figure 2. Key NOESY correlations of **1**.

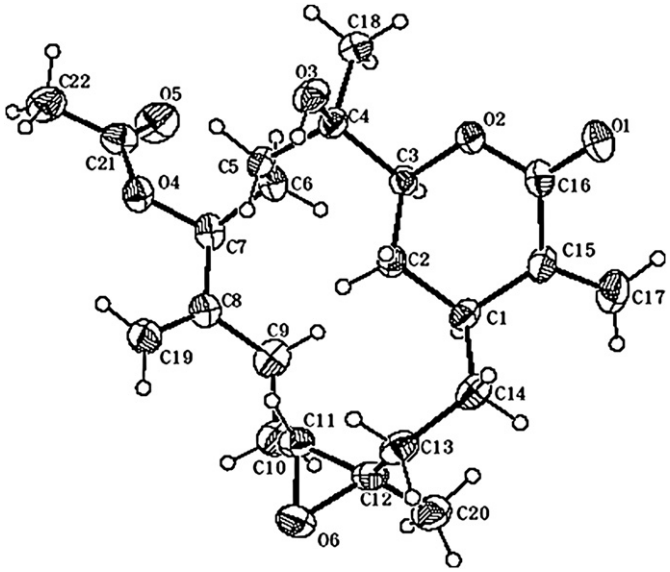


Figure 3. ORTEP diagram showing the crystallographic and relative configuration of **1**.

2.2. Structure of compound 2

The molecular formula of compound **2** was established as $C_{21}H_{32}O_4$ by HRFABMS (*m*/*z* 349.2379 $[M+H]^+$), containing six degrees of unsaturation. Analysis of ^{1}H NMR data indicated a similar *exo*-methylene group, with protons at δ_H 5.48 (br s, H-17a) and 6.26 (br s, H-17b) (δ_C 124.4) that have HMBC correlations with a quaternary carbon (δ_C 142.6, C-15), and a carbonyl carbon (δ_C 167.3, C-16). The latter carbon was in turn linked with a methoxyl signal at δ_H 3.75 (δ_C 52.0), which implies a methyl ester conjugated with an α-*exo*-methylene group. The signals of the *exo*-methylene protons also showed HMBC correlations with a methine carbon at δ_C 35.2 (C-1) (δ_H 3.63, m), indicating attachment of the α,β-unsaturated ester group at the C-1 position. The carbon backbone contains an additional double bond, as illustrated by resonances at δ_C 134.2 (qC) and 126.5 (δ_H 5.17, t), both of them showing HMBC correlations with the protons of a methyl signal at δ_H 1.62 (δ_C 15.3, C-19). Two pairs of epoxide carbon signals were observed at δ_C 60.1(CH)/60.2 (qC) and 61.9 (CH)/60.9 (qC). The former pair was linked to a methyl singlet at δ_H 1.26 (δ_C 17.8, C-18) and the latter to a methyl singlet at δ_H 1.20 (δ_C 17.0, C-20), allowing the positions of the epoxide rings to be assigned at C-3/C-4 and C-11/C-12, respectively. The COSY NMR spectrum of **2** showed the correlations between H-7/H-6/H-5 and H-9/H-10/H-11, thus revealing the linkage of two ethane bridges with both epoxide rings (Fig. 4). The geometry of the double bond at C-8 was determined to be *E* from the chemical shift of the olefinic methylcarbon at δ_C 15.3 (C-19).[25] The relative configuration of each substituent around C-1, C-4, and C-12 centers is assumed to be the same as those found in compound **1**, which are also the same as

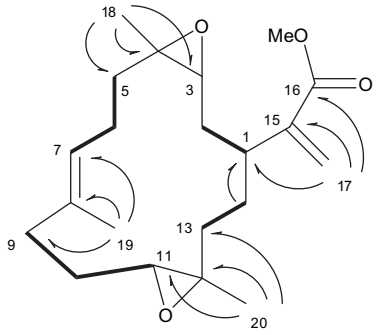


Figure 4. Selected HMBC (hooks) and COSY (bold line) correlations of **2**.

those in sinuflexibilin,[24] a derivative of **2** with the two epoxide rings opened to give 1,2 diols at each site. In the NOESY spectrum of **2** (Fig. 5), proton correlations between H-1α/H-3α/Me-4α, H-11β/H-13β, and H-13α/Me-20α were observed that confirmed the assigned relative configuration around C-1, C-3, C-4, C-11, and C-12. Compound **2** was assigned the name flexilarin B in this series of compounds.

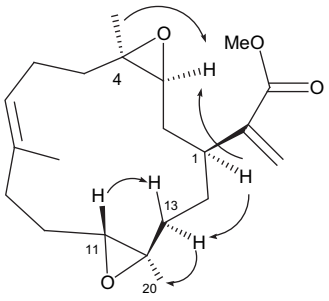
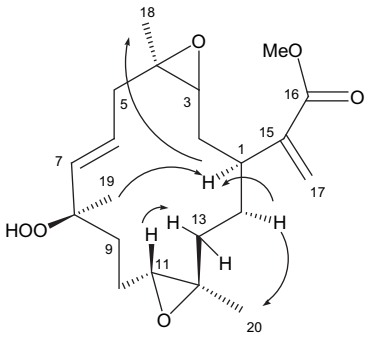


Figure 5. Key NOESY correlations of **2**.

2.3. Structure of compound 3

The molecular formula of compound **3** was found to be $C_{21}H_{32}O_6$ by observation of a pseudo-molecular ion at *m*/*z* 403 $[M+Na]^+$ in the ESI mass spectrum and a fragment ion at *m*/*z* 387.2150 $[M+Na-O]^+$ in HRESI mass spectrum. Analysis of the IR spectrum of **3** suggested the presence of hydroxyl and α,β-unsaturated ester functionalities (λ_{max} 3419, 1713 cm^{-1}). Comparison of ^1H, ^{13}C, and HMBC NMR data of **3** with those of **2** indicated that they share similar structural features, including the α-*exo*-methylene conjugated methyl ester at C-1, and two epoxide rings positioned at C-3/C-4 and C-11/C-12. Similarity in the NOESY data of both compounds indicated similar relative arrangements of substituents around C-1, C-4, and C-11. The new structural features were revealed by two olefinic carbons at δ_C 125.5 (δ_H 5.70, m) and 135.8 (δ_H 5.64, d), and one dioxygenated quaternary carbon at δ_C 84.1 (C-8), which was linked by HMBC data with the methyl singlet at δ_H 1.42 (δ_C 23.6, C-19), thus locating a double bond at C-6/C-7 and a hydroperoxyl group at C-8. The latter functional group was assigned based upon from the fact that two extra oxygen atoms remained after deducing all those same atoms contained in other functionalities accounted so far, and the hydroxyl absorption in the IR spectrum. Also the carbon resonance of C-8 appeared in low field range in comparison with the resonances of the same carbon in compounds **5**–**7** that share similar arrangement of a double bond adjacent to the quaternary carbon. The COSY spectrum exhibited correlations of H-5/H-6/H-7 and H-9/H-10/H-11, which clearly indicated the relative position of the double bond with respect to two epoxide rings. Analysis of NOESY NMR data (Fig. 6) from **3** showed the cross-ring correlation of H_3-19 with H-1α, requiring that Me-19 be in an α-orientation, and thus C-8 was



3

Figure 6. Key NOESY correlations of **3**.

assigned as *R* configuration. Compound **3** was assigned flexilarin C in this series of compounds.

2.4. Structure of compound 4

The molecular formula of flexilarin D (**4**) was established to be $C_{20}H_{28}O_6$ by an observation of the pseudo-molecular ion at *m*/*z* 387 $[M+Na]^+$ in the low-resolution ESI mass spectrum and a fragment ion peak at *m*/*z* 371.1836 $[M+Na-O]^+$ in the HRESI mass spectrum. This formula was supported by ^{13}C NMR data, which contains seven degrees of unsaturation. The IR spectrum of **4** showed a broad absorption between 3000 and 3400 cm^{-1} (OH stretching), and a strong absorption at 1698 cm^{-1}, suggesting the presence of α,β-unsaturated ester. In the ^1H NMR spectrum two singlet protons were observed at δ_H 5.50 and 6.35 (δ_C 125.0), which showed HMBC correlations with an olefinic quaternary carbon (δ_C 143.8), the adjacent keto carbon (δ_C 167.7), and a methine carbon (δ_C 36.3, C-1; δ_H 2.20, m) (Fig. 7). The protons of the methyl singlet at δ_H 1.52 (δ_C 28.7, C-18) were in turn linked to an oxygenated tertiary carbon signal at δ_C 91.1 (C-4) and the adjacent methylene carbon at δ_C 32.8 (C-3). COSY correlations between H-1/H-2/H-3 revealed the linkage of an ethane bridge between C-1 and C-4, thus suggesting the presence of a seven-membered δ-lactone ring in conjugation with an exocyclic methylene. The angular methyl protons at δ_H 1.52 (C-18) showed additional HMBC correlation with a keto carbon at δ_C 210.1 (qC, C-5), suggesting an adjacent keto group beside the lactone ring. The methyl protons at δ_H 1.26 (δ_C 16.0, C-20) were correlated with a pair of oxygenated carbons at δ_C 60.7 (qC) and 61.0 (CH, δ_H 2.80, dd), thus positioning an epoxide ring between C-12/C-13. Analysis of HMBC NMR data experiments also linked the methyl singlet at δ_H 1.41 (δ_C 25.2, C-19) with an dioxygenated tertiary carbon at δ_C 84.0 (qC) and a pair of double bond carbons at δ_C 133.7 (δ_H 5.43, d) and 125.0 (δ_H 5.52, m), thus implying an endocyclic double bond between C-9 and C-10, adjacent to the hydroperoxyl bearing

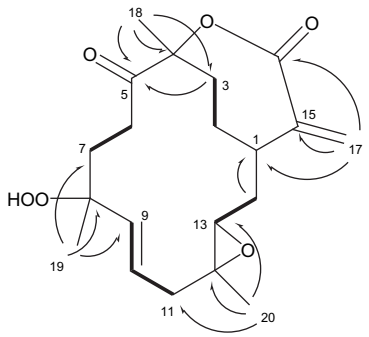


Figure 7. Selected HMBC (hooks) and COSY (bold line) correlations of **4**.

quaternary carbon at C-8. The value of coupling constant between H-9/H-10 (16.2 Hz) suggested an *E* configuration for this double bond. The relative positions of each fragments within the cembranoid ring was further defined by COSY correlations between H-6/H-7, H-9/H-10/H-11, and H-13/H-14, in conjunction with interpretation of HMBC experiments (Fig. 7). The assembled structure **4** is very similar to the known compound 5-dehydrosinulariolide (**11**),[17,18] in which a double bond was introduced between C-8/C-9 without the presence of hydroperoxyl group. The structure of **11** was defined by X-ray crystallographic analysis for the first time in this lab (Fig. 8). In the NOESY NMR spectrum of **4**, the correlations between H_3-18/H-7β and H_3-19α/H-7α justify the assigned relative configuration at C-8, while the correlations between H_3-20/H-14α/H-1α are consistent with the stereochemical arrangements around C-1 and C-12. The name flexilarin D was given to compound **4**.

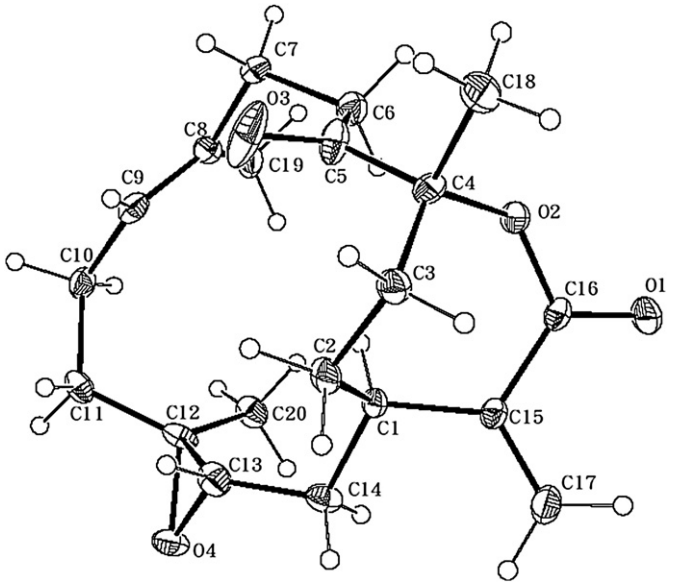


Figure 8. X-ray crystallographic diagram for compound **11**.

2.5. Structure of compound 5

The HRESIMS spectrum of flexilarin E (**5**) exhibited a pseudomolecular ion peak at *m*/*z* 415.2097 ([M+Na]$^+$), consistent with the molecular formula $C_{22}H_{32}O_6$ having seven degrees of unsaturation. The IR absorptions of **5** showed the presence of hydroxyl (3447 cm^{-1}) and ester carbonyl (1728 cm^{-1}) groups. Comparison of the NMR data of **5** with those of **4** revealed that both structures are very similar, with the sole exception that the ketocarbonyl carbon (C-5) in **4** was replaced by an acetoxyl group-bearing methine carbon in **5**. The NMR data for **5** displayed signals relating to the acetoxyl group with a methyl singlet at δ_H 2.13 (δ_C 21.1) and the carbonyl carbon at δ_C 171.4. The methyl singlet protons at δ_H 1.34 (δ_C 24.2, C-18) showed HMBC correlations with the oxygenated tertiary carbon C-4 (δ_C 87.2, qC), the methylene carbon C-3 (δ_C 32.4), and the oxygenated methine carbon C-5 (δ_C 75.1). The reverse correlation of the methine proton at δ_H 5.56 (d) on C-5 with the oxygenated tertiary carbon at C-4 further supports the location of the acetoxyl group at C-5. The NOESY spectrum of **5** showed cross correlations between H-1α/H-5α, H_3-18/H-3β/H-2β/H-13β, and H_3-19α/H-7α/H-5α that confirmed the assigned relative configuration around C-8 (Fig. 9). Therefore, the orientations of H_3-18β, H-5α, and H_3-19α were unambiguously assigned. The configurations at C-1, C-4, C-5, C-12, and C-13 are the same as those found in the known compound sinulariolide,[17,18] which was determined on the basis of X-ray analysis. Compound **5** was given the name flexilarin E.

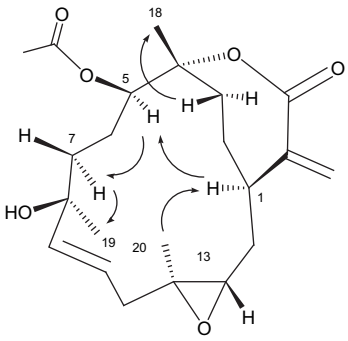


Figure 9. Key NOESY correlations of **5**.

2.6. Structure of compound 6

Flexilarin F (**6**) was an epimeric isomer of **5**, both sharing the same molecular formula of $C_{22}H_{32}O_6$ as determined by HRESI mass spectrometry. The NMR data of **5** and **6** showed close similarity and were comparable to those of 5-dehydrosinulariolide (**11**).[18] The substitution of an acetoxyl group (δ_H 2.14; δ_C 21.0, 171.1) at C-5 was concluded based upon HMBC correlations of the methine proton H-5 (δ_H 5.45, d, *J*=4.0 Hz) with the acetyl carbonyl carbon at δ_C 171.1 (qC), and to C-4 (δ_C 86.7) and C-18 (δ_C 26.0). The NOESY spectrum of **6** displayed a correlation between H_3-18/H-5, indicating a β-orientation for H-5 and an α-orientation for the acetoxyl group at C-5. All the other NMR data were similar to those of **5**, suggesting that **6** is a C-5 epimer of **5**. The name flexilarin F was assigned to compound **6**.

2.7. Structure of compound 7

The molecular formula of flexilarin G (**7**) was found to be $C_{20}H_{30}O_5$ by observation of an HRESI mass spectral pseudo-molecular ion peak at *m*/*z* 373.1990 [M+Na]$^+$, containing six degrees of unsaturation. The IR spectrum of **7** (λ_{max} 3419, 1707 cm^{-1}) suggested the presence of hydroxyl and α,β-unsaturated ester functionalities. Inspection of the NMR spectral data for **7** indicated a strong resemblance with those of compound **5**. However, the disappearance of signals relating to the acetate group indicated that **7** is a deacetylated analogue of compound **5**. A NOESY NMR correlation between Me-18 and H-5 in **7** was missing in contrast to compound **6**, requiring β-orientation for the hydroxyl group at C-5. All other NMR spectral features relating to the δ-lactone, the angular methyl (C-18), the hydroxyl-methylcarbon (C-8), the adjacent double bond, and the epoxide are all similar to those observed in compound **5**. The COSY NMR spectrum showed correlations between H-9/H-10/H-11, which were compatible with assigning the double bond position between C-9 and C-10. The protons of the methyl singlet at δ_H 1.33 (δ_C 30.9, C-19) showed HMBC correlations with the methylene carbon at δ_C 37.1 (C-7), and the double bond carbons at δ_C 74.2 (qC, C-8) and 139.1 (CH, C-9). The relative positions of all functional groups were clearly supported by ^{1}H–^{1}H COSY, HMQC, and HMBC results, indicating similar arrangements to those found in compound **5**. The relative configuration of **7** as assigned by using NOESY correlations is in complete agreement with compound **5**. Thus, flexilarin G (**7**) was assigned as a deacetylated analogue of compound **5**.

2.8. Structure of compound 8

The molecular formula of compound **8** was established as $C_{22}H_{34}O_7$ by HRESIMS (*m*/*z* 433.2204 [M+Na]$^+$). Its IR spectrum (λ_{max} 3421 and 1719 cm^{-1}) suggested the presence of hydroxyl groups and α,β-unsaturated ester. The NMR spectroscopic data of **8**

were closely related to those of compound **12**.[19] The NMR data showed signals of a lactone carbon at δ_C 168.6 (qC), which was conjugated with an *exo*-methylene carbons at δ_C 143.4 (qC) and 125.2 (CH_2; δ_H 5.54, s, and 6.30, s). The protons of the methyl singlet at δ_H 1.36 displayed HMBC correlations with carbon signals at δ_C 90.3 (qC, C-4), 32.5 (CH_2, C-3), and 72.4 (CH, C-5; δ_H 4.78, dd). This revealed a δ-lactone linkage at C-4 and a hydroxyl group at C-5. The pair of oxygenated carbons at δ_C 59.7 (qC) and 62.1(CH; δ_H 3.32, dd) was HMBC correlated with the methyl singlet protons at δ_H 1.31 (δ_C 15.7, C-20), which indicated the presence of an epoxide ring between C-12/C-13. The methyl singlet protons at δ_H 1.21 (δ_C 24.5, C-19) showed HMBC correlations with carbon signals at δ_C 32.9 (CH_2, C-7), 73.2 (qC, C-8), and 73.2 (CH, C-9; δ_H 5.01, d). The latter methine protons were linked by HMBC data with the signal of a carbonyl carbon at δ_C 170.2 (qC) assigned to an acetoxyl group (δ_C 21.2; δ_H 2.08, s). These data allowed an acetoxyl group to be positioned at C-9, an assignment further corroborated by the COSY correlation between H-9 and H-10. The COSY correlations of H-5/H-6/H-7 and H-9/H-10/H-11 showed that C-8 and C-9 are both separated by two carbon units from the hydroxyl methine at C-5 and the epoxide at C-12, respectively. A NOESY NMR correlation between H-17a (δ_H 5.54, s) and H_2-14 pointed out that H-1 was in an α-orientation, as observed previously in all other known analogues. Similarly, NOESY correlations of H_3-20/H-14α (δ_H 2.18, m) and H-14α/H-1 allowed the assignment of H_3-20 in α-orientation. In addition, NOESY correlations between H-5 and H-6α (δ_H 1.76, m) and between H-6α and H_3-19 confirmed the α-orientations for Me-19, and H-5. The configurations at C-1, C-4, C-5, C-12, and C-13 were thus assigned as identical to those found in the known compound sinulariolide,[17] a structure assigned on the basis of X-ray analysis. Compound **8** was assigned as flexilarin H in this series.

2.9. Structure of compound 9

The HRFABMS spectrum of compound **9** exhibited a pseudo-molecular ion peak at *m/z* 411.2383 ($[M+H]^+$), consistent with the molecular formula $C_{22}H_{34}O_7$ containing six degrees of unsaturation. IR absorption showed the presence of hydroxyl (3421 cm^{-1}) and ester carbonyl (1718 cm^{-1}) groups in compound **9**. Comparison of the NMR data of **9** with those of sinulariolone (**13**)[23] revealed that both structures are very similar. The difference is that the carbonyl carbon at C-5 in **13** was replaced by a hydroxyl-bearing methine carbon in **9**, and one of the hydroxyl groups was acetylated. Because the chemical shift of H-13 (δ_H 4.97) was shifted downfield in comparison with the same proton shift value in **13** (δ_H 3.80), the acetoxyl group was positioned at C-13. The relative positions of each functional group were examined by ^1H–^1H COSY, HMQC, HMBC, and NOE NMR experiments (Fig. 10) and were similar to those found in **13**. Furthermore, in conjunction with the NOESY correlations, molecular modeling of compound **9** was carried out by MM2 force field calculations. The results clearly show the proximity of correlated proton pairs in space confirming the assignments (Fig. 11). An *R** configuration at C-5 was also in consistent with the above results. The assigned structure was further supported by acetylation of **9** with acetic anhydride in pyridine to yield a 5,13-diacetoxyl product **14** that is identical in every aspect with querciformolide C.[23] Compound **9** was thus assigned the name flexilarin I in this series.

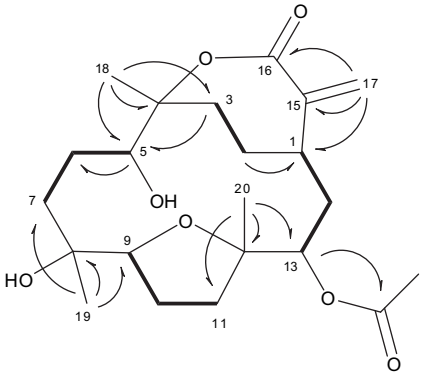


Figure 10. Selected HMBC (hooks) and COSY (bold line) correlations of **9**.

2.10. Structure of compound 10

The molecular formula $C_{20}H_{30}O_5$ (six degrees of unsaturation) of compound **10** was derived from the pseudo-molecular ion peak in HRESIMS at *m/z* 373.1989 $[M+Na]^+$. IR bands indicated the presence of hydroxyl (3423 cm^{-1}) and carbonyl (1734 cm^{-1}) groups in **10**, which were similar to those observed from compound **9**. Inspection of ^1H and ^{13}C NMR spectral data also indicated a close resemblance, suggesting that **10** is an analogue of **9**. However, a new structural feature was revealed by the appearance of a pair of exocyclic methylene proton singlets at δ_H 5.07 and 5.25 (δ_C 115.4, CH_2, C-19) that were attached to a quaternary carbon at C-8 (δ_C 151.2) (Tables 1 and 2). Thus, it was suggested that compound **10** might be a dehydration derivative of compound **9**. Furthermore, the planar structure of **10**, including the relative positions of the above-mentioned double bond and the hydroxyl groups, was determined by ^1H–^1H COSY and HMBC correlations. The relative configuration of **10** could be established by using NOESY correlations in comparison with identical data derived from compound **9**. Compound **10** was assigned flexilarin J among this series of compounds.

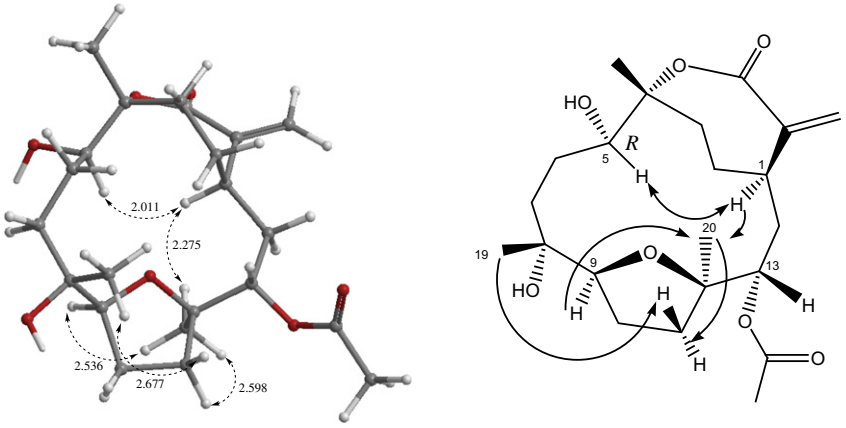


Figure 11. Molecular modeling and key NOESY correlations of **9**.

Table 1
^{1}H NMR spectroscopic data ($CDCl_3$, 300 MHz) for flexilarins A–J (**1**–**10**)

	1	2	3	4	5[a]	6[b]	7[a]	8[a]	9[a]	10[a]
1	2.61, m	3.63, m	2.68, m	2.20, m	2.75, m	2.82, m	2.46, m	2.73, m	3.02, m	3.16, m
2	1.40, m	1.60, m; 1.69, m	1.52, m; 1.90, m	2.06, m	2.19, m	1.36, m; 2.26, m	1.84, m	2.98, m	1.85, m	1.03, m
3	3.98, d (10.8)	2.80, dd (3.0, 4.5)	2.96, m	2.35, dd (4.5, 10.8)	1.94, m	1.94, m	1.85, m; 2.15, m	1.93, m; 2.02, m	1.87, m; 1.89, m	1.94, m; 1.96, m
5	1.70, m	1.57, m; 1.95, m	1.83, m; 2.73, d (4.5)		5.56, d (11.4)	5.45, d (3.5)	4.00, dd (3.6, 7.6)	4.78, dd (2.0, 10.4)	4.79, dt (7.2, 13.2)	4.71, d (7.5)
6	1.97, m	2.19, m	5.70, m	2.04, m	1.65, m	1.54, m; 1.70, m	1.44, m; 1.70, m	1.41, m; 1.76, m	1.47, m	1.47, m
7	5.24, br s	5.17, t (4.5)	5.64, d (16.5)	2.01, m	1.62, m; 1.69, m	1.68, m	1.91, m	1.66, m; 1.67, m	1.67, m; 2.08, m	2.21, m; 2.44, m
9	1.94, m; 2.32, m	2.13, m; 2.63, m	1.83, m; 1.99, m	5.43, d (16.2)	5.75, br s	5.72, d (14.5)	5.67, d (16.0)	5.01, d (9.60)	4.06,dt (7.2, 13.6)	4.41, dt (6.0, 8.5)
10	1.65, m	1.32, m; 2.40, m	1.52, m	5.52, m	5.75, br s	5.96, ddd (5.0, 10.5, 15.5)	5.71, m	2.26, m	1.88, m	1.99, m
11	2.92, dd (3.0, 5.1)	2.65, dd (2.4, 5.1)	2.84, dd (3.75, 6.0)	1.68, m; 2.52, m	2.68, m; 1.78, m	1.79, dt (2.0, 12.5) 2.7, dd (3.5, 8.0)	1.82, m; 2.60, dd (6.0, 6.8)	1.19, m; 2.05, m	1.72, m	2.29, m
13	2.03, m	1.15, m; 1.71, m	1.63, m	2.80, dd (3.0, 8.1)	2.96, dd (4.8, 5.4)	2.92, dd (4.0, 6.0)	3.01, dd (4.4, 6.0)	3.32, dd (4.0, 6.4)	4.97, dd (4.8, 11.6)	3.77, m
14	2.00, m	1.20, m	1.48, m	1.39, m; 1.91, m	1.42, m; 1.91, m	1.46, m; 2.08, m	1.26, m; 2.28, m	1.31, m; 2.18, m	1.78, m	1.80, m
17	5.70, d (2.1); 6.48, d (1.5)	5.48, s; 6.26, s	5.56, s; 6.28, s	5.50, s; 6.35, s	5.49, s; 6.30, s	5.48, s; 6.31, s	5.41, s; 6.26, s	5.54, s; 6.30, s	5.47, s; 6.28, s	5.49, s; 6.27, s
18	1.38, s	1.26, s	1.36, s	1.52, s	1.34, s	1.36, s	1.33, s	1.36, s	1.39, s	1.17, s
19	5.19, br s	1.62, s	1.42, s	1.41, s	1.28, s	1.30, s	1.33, s	1.21, s	1.15, s	5.07, s; 5.25, s
20	1.33, s	1.20, s	1.20, s	1.26, s	1.48, s	1.46, s	1.22, s	1.31, s	1.15, s	1.32, s
OAc	2.12, s				2.13, s	2.14, s		2.08, s	2.06, s	
OMe		3.75, s	3.77, s							

[a] Measured at 400 MHz.
[b] Measured at 500 MHz.

The cytotoxic activity of the isolated cembranoids was tested in vitro against HeLa (human cervical epitheloid carcinoma), Daoy (human medulloblastoma), Hep2 (human hepatocarcinoma), and MCF-7 (human breast carcinoma) tumor cell lines. As illustrated in Table 3, flexilarin D (**4**) exhibited potent cytotoxicity against Hep2 tumor cells with ED_{50} at 0.07 μg/mL, comparable to the standard mitomycin C (0.09 μg/mL). Compound **4** also showed moderate cytotoxic activity against HeLa, Daoy, and MCF-7 cell lines. Compounds **7** and **11** had moderate cytotoxicity against four tumor cell lines, while compounds **15** and **18** were selectively active against HeLa and Hep2 tumor cells, respectively, while other flexilarins were weak or inactive.

3. Experimental

3.1. General

Optical rotations were recorded on a Jasco P-1020 polarimeter. UV and IR spectra were taken with a Jasco V-650 and a Jasco FT/IR-4100 spectrophotometers, respectively. ^{1}H and ^{13}C NMR spectra, as well as 2D NMR spectra (COSY, HMQC, HMBC, and NOESY) were recorded in $CDCl_3$ using Bruker DRX NMR spectrometer operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C using the solvent peak as internal standard, and Varian 400MR FT-NMR (or Varian Unity INOVA 500 FT-NMR) instrument at 400 MHz (or 500 MHz) for ^{1}H and 100 MHz (125 MHz) for ^{13}C in $CDCl_3$ using TMS as internal standard. Low-resolution ESIMS spectra were recorded on a VG Quattro 5022 mass spectrometer. High-resolution ESIMS spectra were measured on a JEOL HX 110 mass spectrometer. LiChrospher Si 60 (5 μm, Merck) and LiChrospher 100 RP-18e (5 μm, Merck) were used for NP-HPLC and RP-HPLC (Hitachi), respectively.

3.2. Animal material

The soft coral *S. flexibilis* was collected near Kenting in the southern coast of Taiwan in March 2007. The specimen was collected at a depth of 10 m and kept frozen until use. This animal was identified by one of the authors (Y.-S.L.). A voucher specimen (KT-0332) was deposited in National Sun Yat-sen University, Kaohsiung, Taiwan.

3.3. Extraction and isolation

The animal material (wet, 6.1 kg) was chopped and extracted three times with CH_2Cl_2/MeOH (1:1, 6 L) at room temperature and then was concentrated under vacuum to obtain a crude extract. The extract was partitioned between H_2O/EtOAc to yield an EtOAc-soluble portion (42 g), which was separated on Sephadex LH-20 (MeOH) into two fractions, L1 and L2. Fraction L2 (16 g) was further fractionated on a silica gel column using a gradient of *n*-hexane/EtOAc to furnish 12 fractions (L2-1 to L2-12). Fraction L2-2 (2 g) was further separated by silica gel column (*n*-Hex/EA, 10:1 to 1:1) to yield **2** (32 mg). Fraction L2-4 (2 g) was purified by silica gel column (*n*-Hex/EA, 10:1 to 1:1) to give five fractions (a–e). Fraction L2-4a was separated by using RP-HPLC (MeOH/H_2O/CH_3CN, 6:3:1) to yield **11**[17] (34 mg) and **12**[19](3 mg). Fraction L2-4d was separated by the same method to yield **3** (6 mg) and **4** (5 mg). Fraction L2-5 (320 mg) was chromatographed on a silica gel column using a gradient of *n*-hexane/EtOAc to furnish two fractions (a–b), and then separated by RP-HPLC (MeOH/H_2O/CH_3CN, 55:40:5) to yield **17** (4 mg),[26] **21** (18 mg),[18] and **22** (7 mg).[27] Fraction L2 (320 mg) was fractionated on a silica gel column using a gradient of *n*-hexane/EtOAc, and then separated by RP-HPLC (MeOH/H_2O/CH_3CN, 55:40:5) to yield **19** (3 mg).[7] Fraction L2-8 (1.4 g) was purified on Sephadex LH-20 using MeOH to give three fractions (a–c). Fraction L2-8a (240 mg) was separated on an RP-HPLC column using (MeOH/H_2O/CH_3CN, 55:40:5) to give flexilarin **1** (6 mg), **8** (7 mg),

Table 2
^{13}C NMR spectroscopic data ($CDCl_3$, 75 MHz) for flexilarins A–J (**1**–**10**)

	1	**2**[a]	**3**	**4**	**5**[a]	**6**[b]	**7**[a]	**8**[a]	**9**[a]	**10**[b]
1	34.5 CH	35.2 CH	36.9 CH	36.3 CH	34.9 CH	35.0 CH_2	36.4 CH	35.6 CH	33.0 CH	32.6 CH
2	27.0 CH_2	27.3 CH_2	27.4 CH_2	27.3 CH_2	29.3 CH_2	30.5 CH_2	27.5 CH_2	29.4 CH_2	29.1 CH_2	29.7 CH_2
3	82.0 qC	60.1 CH	60.5 CH	32.8 CH_2	32.4 CH_2	32.0 CH_2	32.5 CH_2	32.5 CH_2	33.1 CH_2	32.8 CH_2
4	72.7 qC	60.2 qC	61.0 qC	91.1 qC	87.2 qC	86.7 qC	88.7 qC	90.3 qC	91.5 qC	90.9 qC
5	31.0 CH_2	36.8 CH_2	42.3 CH_2	210.1 qC	75.1 CH	75.1 CH	70.7 CH	72.4 CH	73.2 CH	72.9 CH
6	26.1 CH_2	22.1 CH_2	125.5 CH	25.6 CH_2	23.6 CH_2	25.3 CH_2	25.3 CH_2	25.3 CH_2	27.1 CH_2	29.2 CH_2
7	73.5 CH	126.5 CH	135.8 CH	28.7 CH_2	35.4 CH_2	37.2 CH_2	37.1 CH_2	32.9 CH_2	36.7 CH_2	31.0 CH_2
8	142.1 qC	134.2 qC	84.1 qC	84.0 qC	73.4 qC	72.4 qC	74.2 qC	73.2 qC	74.6 qC	151.2 qC
9	29.8 CH_2	36.0 CH_2	34.2 CH_2	133.7 CH	140.4 CH	138.8 CH	139.1CH	73.2 CH	85.4 CH	82.9 CH
10	23.5 CH_2	24.5 CH_2	25.9 CH_2	125.0 CH	124.2 CH	126.1 CH	124.0 CH	25.3 CH_2	25.4 CH_2	29.3 CH_2
11	63.8 CH	61.9 CH	60.8 CH	42.5 CH_2	42.5 CH_2	42.6 CH_2	41.6 CH_2	34.5 CH_2	40.1 CH_2	37.7 CH_2
12	59.3 qC	60.9 qC	60.7 qC	60.7 qC	60.5 qC	60.9 qC	60.3 qC	59.7 qC	86.0 qC	86.0 qC
13	34.4 CH_2	33.0 CH_2	32.4 CH_2	61.0 CH	60.5 CH	61.3 CH	60.6 CH	62.1 CH	76.6 CH	72.7 CH
14	32.2 CH_2	29.7 CH_2	34.4 CH_2	31.2 CH_2	32.0 CH_2	32.3 CH_2	31.4 CH_2	33.5 CH_2	33.6 CH_2	37.9 CH_2
15	139.9 qC	142.6 qC	143.4 qC	143.8 qC	143.1 qC	143.4 qC	144.4 qC	143.4 qC	143.9 qC	144.6 qC
16	167.1 qC	167.3 qC	167.6 qC	167.7 qC	168.5 qC	168.4 qC	169.2 qC	168.6 qC	169.3 qC	169.6 qC
17	128.9 CH_2	124.4 CH_2	124.4 CH_2	125.0 CH_2	124.8 CH_2	124.9 CH_2	124.2 CH_2	125.2 CH_2	124.3 CH_2	123.9 CH_2
18	24.6 CH_3	17.8 CH_3	16.8 CH_3	28.7 CH_3	24.2 CH_3	26.0 CH_3	25.0 CH_3	22.6 CH_3	23.3 CH_3	22.8 CH_3
19	111.5 CH_2	15.3 CH_3	23.6 CH_3	25.2 CH_3	33.3 CH_3	29.3 CH_3	30.9 CH_3	24.5 CH_3	19.1 CH_3	115.4 CH_2
20	15.6 CH_3	17.0 CH_3	19.0 CH_3	16.0CH_3	16.7 CH_3	16.5 CH_3	16.3 CH_3	15.7 CH_3	17.1 CH_3	19.2 CH_3
OAc	170.0 qC;				171.4 qC;	171.1 qC;		170.2 qC;	170.0 qC;	
	21.1 CH_3				21.1 CH_3	21.0 CH_3		21.2 CH_3	21.2 CH_3	
OMe		52.0 CH_3	52.0 CH_3							

[a] Measured at 100 MHz.
[b] Measured at 125 MHz.

Table 3
Cytotoxicity of Isolated cembranoids against human tumor cells (IC_{50}, μg/mL)[a,c]

Compound	Hela	Daoy	Hep2	MCF-7
2	(−)[b]	19.7	12.2	(−)
4	0.41	1.24	0.07	1.24
7	8.23	10.5	6.22	10.8
11	3.04	2.46	1.58	3.14
14	17.9	17.7	13.3	(−)
15	6.84	(−)	10.0	(−)
18	10.8	9.34	7.44	(−)
22	15.8	(−)	18.0	(−)
Mitomycin C	0.08	0.06	0.06	0.09

[a] HeLa: human cervical epitheloid carcinoma; Daoy: human medulloblastoma; Hep2: Human hepatocarcinoma; MCF-7: human breast adenocarcinoma.
[b] (−) Represents >20 μg/mL.
[c] Compounds **1**, **5**, **6**, **8**–**10**, **13**, **16**, **17** were inactive (−) in this assay system.

and **18** (3 mg).[24] L2-9 (1.1 g) was purified on a flash column using a gradient of *n*-hexane/EtOAc followed by separation on an RP-HPLC column (MeOH/H_2O/CH_3CN, 65:30:5) to yield **5** (6 mg), **6** (7 mg), and **20** (5 mg).[24] Fraction L2-10 (2.2 g) was purified on a Si gel column and eluted with *n*-hexane/EtOAc (3:1) to obtain **18** (342 mg), **24** (21 mg),[19] and **8** (7 mg). L2-11 (2.4 g) was separated by using a silica gel column using a gradient of *n*-hexane/EtOAc to furnish four fractions a–d. Fraction L2-11a (240 mg) was separated on RP-HPLC using (MeOH/H_2O/CH_3CN, 60:40:5) to give **16** (14 mg).[20] Fraction L2-11b was separated by an RP-HPLC column (MeOH/H_2O/CH_3CN, 55:40:5) to give **15** (16 mg)[20]and **25** (5 mg).[28] Fraction L2-11d was separated by an RP-HPLC column (MeOH/H_2O/CH_3CN, 55:40:5) to afford **26** (13 mg)[23] and **9** (7 mg). Fraction L2-12(320 mg) was fractionated on a silica gel column using a gradient of *n*-hexane/EtOAc, and than separated by RP-HPLC (MeOH/H_2O/CH_3CN, 50:50:5) to furnish **23** (23 mg),[28] **27** (6 mg),[23] and **7** (7 mg).

*3.3.1. Flexilarin A (**1**).* Colorless needless; $[\alpha]_D$ +20 (*c* 0.2, CH_2Cl_2); IR (neat) ν_{max} 3404, 2925, 1714, 1626, 1456, 1373, 1239 cm^{-1}; UV (MeOH) λ_{max} (log ε) 205.6 (3.90) nm; ^{1}H NMR (300 MHz), Table 1; ^{13}C NMR (75 MHz), Table 2; HRESIMS *m*/*z* 415.2097 $[M+Na]^+$ (calcd for $C_{22}H_{32}O_6Na$, 415.2096).

*3.3.2. Flexilarin B (**2**).* Colorless oil; $[\alpha]_D$ +79 (*c* 0.2, CH_2Cl_2); IR (neat) ν_{max} 3395, 2976, 2926, 1735 cm^{-1}; UV (MeOH) λ_{max} (log ε) 204.6 (3.85) nm; ^{1}H NMR (400 MHz), Table 1; ^{13}C NMR (100 MHz), Table 2; HRFABMS *m*/*z* 349.2379 $[M+H]^+$ (calcd for $C_{21}H_{33}O_4$, 349.2379).

*3.3.3. Flexilarin C (**3**).* Colorless oil; $[\alpha]_D$ +23 (*c* 0.2, CH_2Cl_2); IR (neat) ν_{max} 3419, 2924, 1713 cm^{-1}; UV (MeOH) λ_{max} (log ε) 204.5 (3.87) nm; ^{1}H NMR (300 MHz), Table 1; ^{13}C NMR (75 MHz), Table 2; ESIMS *m*/*z* 403 ($C_{21}H_{32}O_6Na$), *m*/*z* 387; HRESIMS *m*/*z* 387.2150 $[M+Na]^+$ (calcd for $C_{21}H_{32}O_5Na$, 387.2147).

*3.3.4. Flexilarin D (**4**).* Colorless oil; $[\alpha]_D$ +32 (*c* 0.2, CH_2Cl_2); IR (neat) ν_{max} 3408, 2925, 1698 cm^{-1}; UV (MeOH) λ_{max} (log ε) 197.8 (3.85) nm; ^{1}H NMR (300 MHz), Table 1; ^{13}C NMR (75 MHz), Table 2; ESIMS *m*/*z* 387, 371; HRESIMS *m*/*z* 371.1836 $[M+Na]^+$ (calcd for $C_{20}H_{28}O_5Na$, 371.1834).

*3.3.5. Flexilarin E (**5**).* Colorless oil; $[\alpha]_D$ +124 (*c* 0.2, CH_2Cl_2); IR (neat) ν_{max} 3447, 2927, 1728 cm^{-1}; UV (MeOH) λ_{max} (log ε) 198.0 (3.89) nm; ^{1}H NMR (400 MHz), Table 1; ^{13}C NMR (100 MHz), Table 2; HRESIMS *m*/*z* 415.2097 $[M+Na]^+$ (calcd for $C_{22}H_{32}O_6Na$, 415.2096).

*3.3.6. Flexilarin F (**6**).* Colorless oil; $[\alpha]_D$ +96 (*c* 0.2, CH_2Cl_2); IR (neat) ν_{max} 3445, 2925, 1728 cm^{-1}; UV (MeOH) λ_{max} (log ε) 197.8 (3.88) nm; ^{1}H NMR (500 MHz), Table 1; ^{13}C NMR (125 MHz), Table 2; HRESIMS *m*/*z* 415.2097 $[M+Na]^+$ (calcd for $C_{22}H_{32}O_6Na$, 415.2096).

*3.3.7. Flexilarin G (**7**).* Colorless oil; $[\alpha]_D$ +33 (*c* 0.2, CH_2Cl_2); IR (neat) ν_{max} 3419, 2931, 1707, 1382, 1267 cm^{-1}; UV (MeOH) λ_{max} (log ε) 198.0 (3.84) nm; ^{1}H NMR (400 MHz), Table 1; ^{13}C NMR (100 MHz), Table 2; HRESIMS *m*/*z* 373.1990 $[M+Na]^+$ (calcd for $C_{20}H_{30}O_5Na$, 373.1991).

*3.3.8. Flexilarin H (**8**).* Colorless oil; $[\alpha]_D$ +132 (*c* 0.2, CH_2Cl_2); IR (neat) ν_{max} 3421, 2927, 1719 cm^{-1}; UV (MeOH) λ_{max} (log ε) 198 (3.90) nm; ^{1}H NMR (400 MHz), Table 1; ^{13}C NMR (100 MHz), Table 2; HRESIMS *m*/*z* 433.2204 $[M+Na]^+$ (calcd for $C_{22}H_{34}O_7Na$, 433.2202).

*3.3.9. Flexilarin I (**9**).* Colorless oil; $[\alpha]_D$ +174 (*c* 0.2, CH_2Cl_2); IR (neat) ν_{max} 3421, 2925, 1718 cm^{-1}; UV (MeOH) λ_{max} (log ε) 207.2

(3.85) nm; ^{1}H NMR (400 MHz), Table 1; ^{13}C NMR (100 MHz), Table 2; HRFABMS *m*/*z* 411.2383 $[M+H]^+$ (calcd for $C_{22}H_{35}O_7$, 411.2383).

*3.3.10. Acetylation of flexilarin I (**9**).* To a solution of **9** (3 mg) in pyridine (0.3 mL) was added acetic anhydride (0.3 mL) at room temperature for 1 h. The reaction mixture was processed by standard methods to give a monoacetate (**14**), identical with quercitormolide C (^{1}H NMR, MS, and [α]).

*3.3.11. Flexilarin J (**10**).* Colorless oil; $[\alpha]_D$ +8 (*c* 0.2, CH_2Cl_2); IR (neat) ν_{max} 3423, 2935, 1695 cm^{-1}; UV (MeOH) λ_{max} (log ε) 207.6 (3.86) nm; ^{1}H NMR (500 MHz), Table 1; ^{13}C NMR (125 MHz), Table 2; HRESIMS *m*/*z* 373.1989 $[M+Na]^+$ (calcd for $C_{20}H_{30}O_5Na$, 373.1991).

3.4. Single-crystal X-ray structure determination of flexilarin A (1)

A suitable colorless crystal (0.50×0.35×0.21 mm^3) of **1** was obtained by slow evaporation from the mixture acetone/MeOH (1:1) solution. Crystal data: $C_{22}H_{32}O_6$, orthorhombic, M_r=392.48 g/mol; *a*=7.4636(4) Å, *b*=9.2959(3) Å, *c*=30.8355(10) Å, *V*=2139.39(15) Å^3, space group $P2_12_12_1$, *Z*=4, D_{calcd} 1.219 Mg/m^3, λ=0.71073 Å, μ(Mo Kα) 0.087 mm^{-1}, *F*(000)=848, *T*=296(2) K. A total of 12,667 reflections were collected in the range 2.56<*q*<24.98, of which 12,667 unique reflections with $I>2\sigma(I)$ were used for the analysis. The data were solved using the direct method, and the structure was refined by full-matrix least-squares procedure on F^2 values. All non-hydrogen atoms were refined with anisotropic thermal parameters. The hydrogen atom positions were geometrically idealized and allowed to ride on their parent atoms. The final indices were *R* 10.0429, *wR* 20.1145 with goodness-of-fit=1.164. The final X-ray model is shown in Figure 3.

3.5. Single-crystal X-ray structure determination of 11-dehydrosinulariolide (11)

A suitable colorless crystal (0.50×0.41×0.32 mm^3) of **11** was obtained by slow evaporation from the mixture of acetone/MeOH (1:1) solution. Crystal data: $C_{20}H_{28}O_4$, orthorhombic, M_r=332.42 g/mol; *a*=9.4320(2) Å, *b*=10.9676(3) Å, *c*=17.8081(5) Å, *V*=1804.71(8) Å^3, space group $P2_12_12_1$, Z=4, D_{calcd} 1.223 Mg/m^3, λ=0.71073 Å, μ(Mo Kα) 0.084 mm^{-1}, *F*(000)=720, *T*=200(2) K. A total of 10,524 reflections were collected in the range 2.19<*q*<25.05, of which 10,524 unique reflections with $I>2\sigma(I)$ were used for the analysis. The data were solved using the direct method, and the structure was refined by full-matrix least-squares procedure on F^2 values. All non-hydrogen atoms were refined with anisotropic thermal parameters. The hydrogen atom positions were geometrically idealized and allowed to ride on their parent atoms. The final indices were *R* 10.1450, *wR* 20.3765 with goodness-of-fit=1.653. The X-ray crystallographic diagram for **11** is shown in Figure 8.

3.6. Cytotoxicity assay

The cytotoxicity was tested against HeLa (human cervical epitheloid carcinoma), Daoy (human medulloblastoma), Hep2 (human hepatocarcinoma), and MCF-7 (human breast carcinoma) tumor cell lines using an MTT{3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide} colorimetric assay. The cells for assay were cultured in RPMI-1640 medium supplemented with a 5% CO_2 incubator at 37 °C. The cytotoxicity assay depends on the binding of methylene blue to fixed monolayers of cells at pH 8.5, washing the monolayer, and releasing the dye by lowering the pH value. Samples and control standard drugs were prepared at a concentration of 1, 10, 40, and 100 μg/mL. After seeding 2880 cells/well in a 96-well microplate for 3 h, 20 μL of sample or standard agent was placed in each well and incubated at 37 °C for 3 days. After removing the medium from the microplates, the cells were fixed with 10% formaldehyde in 0.9% saline for 30 min, then dyed with 1% (w/v) methylene blue in 0.01 M borate-buffer (100 μL/well) for 30 min. the 96-well plate was dipped into a 0.01 M borate-buffer solution four times in order to remove the dye. Then, 100 μL/well of EtOH/0.1 M HCl (1:1) was added as a dye eluting solvent, and the absorbance was measured on a microtiter plate reader (Dynatech, MR 7000) at a wavelength of 650 nm. The ED_{50} value was defined by a comparison with the untreated cells as the concentration of test sample resulting in 50% reduction of absorbance.

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