Communication

Cespitulones A and B, Cytotoxic Diterpenoids of a New Structure Class from the Soft Coral *Cespitularia taeniata*

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**Abstract:** Two novel diterpenoids, cespitulones A (1) and B (2), were isolated from extracts of the soft coral *Cespitularia taeniata*. Both compounds possess an unprecedented bicyclo [10.3.1] ring system with C-C bond connections between C-10 and C-20, and between C-20 and C-11. Their structures were elucidated on the basis of extensive spectroscopic analyses. Compound 1 exhibited significant cytotoxicity against human medulloblastoma and colon adenocarcinoma cancer cells.

**Keywords:** *Cespitularia taeniata*; diterpenoid; cespitulone; cytotoxicity

1. **Introduction**

Marine soft corals are an excellent source of secondary metabolites with novel structures and interesting biological functions [1–5]. Members of the genus *Cespitularia*, along with morphologically similar *Xenia* species, inhabit the coral reefs along the coasts of Taiwan. These interesting cnidarians have brilliant colors and their outer layers are covered with thick mucilage. Previously, several
members of the genus *Cespitularia* were reported to contain a series of complex terpenoids, including cespitularins, nitrogen-containing diterpenoids, cespihytins, cespitulins and cespitulactones [6–12]. These diterpenoids are thought to be derived from geranylgeranyl pyrophosphate and 1S-verticillene, involving interesting biogenetic pathways similar to those that generate the taxane diterpenes [13–15].

To explore novel bioactive metabolites from these invertebrates, we continued our study on *Cespitularia taeniata*, and have now isolated two novel diterpenoids, designated as cespitulones A (1) and B (2) (Figure 1). Both compounds possess an unprecedented bicyclic [10.3.1] skeleton.

### Figure 1. Cespitulones A (1) and B (2) isolated from soft coral *Cespitularia taeniata*.

2. Results

Cespitulone A (1) was obtained as an amorphous solid that analyzed by HRESIMS for the molecular formula C$_{20}$H$_{30}$O$_{5}$, having six degrees of unsaturation. The presences of hydroxyl and carbonyl functions were indicated by IR absorptions at 3419 and 1703 cm$^{-1}$. The $^{1}$H- and $^{13}$C NMR spectra (Table 1), along with DEPT NMR data, confirmed the presence of two carbonyls (δ$_{C}$ 212.4 and 207.4), and illustrated a trisubstituted olefin (δ$_{C}$ 131.7 CH, 133.6 C; δ$_{H}$ 5.58 d, J = 9.1 Hz), a 1,1-disubstituted olefin (δ$_{C}$ 144.9) with an exomethylene group (δ$_{C}$ 115.5 CH$_{2}$; δ$_{H}$ 4.87s, 4.92s), and one aliphatic quaternary carbon (δ$_{C}$ 45.6, C-15). In addition, two oxygenated methine carbons (δ$_{C}$ 69.8 CH, 77.4 CH), an oxygenated tertiary carbon 34.3 and δ$_{C}$ 24.6), and three methyl groups (δ$_{C}$ 26.9, 27.2, (δ$_{C}$ 89.1 C), six methylene carbons (δ$_{C}$ 31.5, 38.7, 46.9, 47.7, 18.6) with their corresponding proton chemical shifts (δ$_{H}$ 1.54, 1.32, 1.88) were observed. Since 1 contained two carbonyls and two double bonds, the carbon framework of cespitulone A must be bicyclic. Analysis of the COSY NMR data for 1 established the connectivities of H-9/H-9, Me-19/H-7/H-6, H-6/H-5/H-18/H-3/H-2/H-1 and H-13/H-14/H-1. These coupled with the HMBC NMR correlations of H-20/C-10, C-11, C-12, and H-9/C-10, and H-13/C-12, allowed the positions of the carbonyls at C-10 and C-12 and the hydroxyl at C-20 to be assigned. Thus, C-20 could be positioned between the C-10 carbonyl and the tertiary oxygenated C-11 carbon. This suggested that 1 contains an unusual bicyclic system in which the C-20 methyl group (as in cespitularines) was somehow modified and incorporated into the ring system. Analysis of other HMBC correlations, including Me-16/C-11, C-15; Me-17/C-11, C-15; H-9/C-7, C-8 as well as H-5/C-4, C-6, C-18, allowed the proposed bicyclo [10.3.1] ring system to be assigned (Figure 2). The relative configuration of 1 was determined by analysis of NOESY NMR data.
based upon the assumption that 1 has the same absolute C-1 (H-1β) configuration as that of the Cespitularia-derived cespitulactams, cespitularines, cespihypotins and toxoids [16,17].

Table 1. ¹H and ¹³C NMR data for 1<sup>a</sup>.

| No | δ<sub>H</sub> (mult, J, Hz)<sup>b</sup> | δ<sub>C</sub><sup>c</sup> | HMBC<sup>1H-¹³C</sup> | COSY<sup>¹H-¹H</sup> |
|----|----------------------------------|----------------|---------------------|---------------------|
| 1  | 1.54 (m)                         | 45.3           | 11, 15, 16          | 2, 14               |
| 2  | 1.10 (m), 1.46 (m)               | 31.5           | 1                   | 1, 3                |
| 3  | 2.00 (m), 2.23 (m)               | 38.7           | 2                   | 18                  |
| 4  |                                 | 144.9          |                     |                     |
| 5  | 2.14 (m), 2.68 (m)               | 46.9           | 4, 6, 18            | 6, 18               |
| 6  | 4.52 (td, 9.0, 5.5)              | 69.8           | 4, 5, 7             | 7                   |
| 7  | 5.58 (d, 9.0)                    | 131.7          | 6                   | 19                  |
| 8  |                                 | 133.6          |                     |                     |
| 9α | 2.63 (d, 14.0)                   | 47.7           | 6, 7, 8, 10         | 9b                  |
| 9β | 4.00 (d, 14.0)                   |                | 7, 8, 10, 19        | 9a                  |
| 10 |                                 | 212.4          |                     |                     |
| 11 |                                 | 89.1           |                     |                     |
| 12 |                                 | 207.4          |                     |                     |
| 13 | 2.77 (m), 2.53 (m)               | 34.3           | 12                  | 14                  |
| 14 | 2.00 (m), 1.60 (m)               | 24.6           | 1                   | 13                  |
| 15 |                                 | 45.6           |                     |                     |
| 16 | 1.54 (s)                         | 26.9           | 1, 11, 15, 17       |                     |
| 17 | 1.32 (s)                         | 27.2           | 1, 11, 15, 16       |                     |
| 18 | 4.87 (s), 4.92 (s)               | 115.5          | 3, 5                | 3, 5                |
| 19 | 1.88 (s)                         | 18.6           | 7, 8, 9             | 7                   |
| 20 | 4.14 (d, 3.0)                    | 77.4           | 10, 11, 12, 15      | OH                  |
| 20-OH | 4.30 (d, 3.0)                   |                | 11, 20              |                     |

<sup>a</sup>Data were recorded in CDCl<sub>3</sub> on 500 MHz; chemical shifts (δ) and coupling constants are given in ppm and Hz, respectively; <sup>b</sup>Assignments made by COSY and HMQC; <sup>c</sup>Assignments made by HMQC and HMBC; Multiplicities determined by DEPT.

The presence of NOESY correlations among H-1/Me-16/Me-17, H-20/Me-16, H-7/Me-17 agreed with β-configuration of Me-16, Me-17 and H-20, while H-6 was assigned an α-configuration on the base of correlations of H-6/Me-19/H-9α and H-7/H-9β (Figure 2). The configuration of the hydroxyl at C-6 was further determined by Mosher’s reactions to yield compounds 1<sup>a</sup> and 1<sup>b</sup> [18]. The results, illustrated in Figure 3, suggested that the C-6 has the S configuration. A computer-generated perspective structure for 1 is shown in Figure 3 by CS Chem 3D version 9.0 using MM2 force field calculation for energy minimization. The results also suggested that C-6 has S configuration and C-11 hydroxy group is α-oriented.
Cespitulone B (2) was isolated as a colorless amorphous solid. The molecular formula, C_{20}H_{30}O_{5} (Δ = 6), was determined by HRESIMS with a pseudomolecular ion at \( m/z \) 373.1993 [M + Na]^{+}, indicating that it is an isomer of 1. Analysis of IR bands revealed the presence of hydroxyl (3419 cm\(^{-1}\)) and carbonyl (1700 cm\(^{-1}\)) functions. Comparisons of the \(^1\)H- and \(^{13}\)C NMR (Table 2) and DEPT data with those of 1 indicated similar functionalities of both compounds. Analysis of COSY and HMBC NMR correlations also revealed similar arrangement of each functional group around the 13-membered ring, including a 1,1-disubstituted olefin (δ\(_C\) 144.7) with an exomethylene group (δ\(_C\) 115.5 CH\(_2\); δ\(_H\) 4.87 s, 4.95 s), a C-6 oxygenated methine carbon (δ\(_C\) 70.9 CH; δ\(_H\) 4.57 td, \( J = 9.6, 5.7 \) Hz), a trisubstituted olefin (δ\(_C\) 132.1 CH, 135.3 C; δ\(_H\) 5.54 d, \( J = 9.6 \) Hz), a C-10 carbonyl carbon (δ\(_C\) 213.9), a C-20 oxygenated methine carbon (δ\(_C\) 79.3 CH; δ\(_H\) 4.46 d, \( J = 3.0 \) Hz), an oxygenated tertiary carbon (δ\(_C\) 81.6 C), and the C-15 quaternary carbon (δ\(_C\) 49.6) with two attached methyl carbons (δ\(_C\) 24.0 CH\(_3\), 27.5 CH\(_3\)). The positions of two carbonyls at C-10 (δ\(_C\) 213.9) and C-13 (δ\(_C\) 212.9) and two hydroxyl groups at C-20 and C-11 were assigned on the basis of HMBC correlations (H-9/C-10, H-20/C-10, C-11, H-12/C-11,C-13, H-14/C-13, Me-16/C-11, and Me-17/C-11). Thus the only difference revealed in comparison with 1 was the location of the C-13 carbonyl group. Analysis of NOESY correlation data [H-1/Me-16, Me-17, H-20/Me-16, and H-7/Me-17 (Figure 4)], indicated the β-orientation of Me-16, Me-17 and H-20, while H-6 was assigned as α-oriented based upon correlations observed from H-6/Me-19/H-9α and H-7/H-9β. A computer-generated perspective structure for 2 is shown in Figure 4. The results also suggested that C-6 has S configuration and the hydroxyl at C-11 is β-oriented.
Table 2. $^1$H and $^{13}$C NMR data for 2.

| No | $\delta_H$ (mult, $J$, Hz) $^b$ | $\delta_C$ | HMBC $^{1H-13C}$ | COSY $^{1H-1H}$ |
|----|--------------------------------|-----------|-----------------|----------------|
| 1  | 1.32 (m)                        | 47.6      | 11,15, 16       | 2, 14          |
| 2  | 1.88, 1.93 (m)                   | 34.3      | 1               | 1, 3           |
| 3  | 1.62, 2.26 (m)                   | 40.8      | 2, 18           |
| 4  |                                | 144.7     |                 |                |
| 5  | 2.07 (m), 2.81 (m)               | 47.2      | 4, 6, 18        | 6, 18          |
| 6  | 4.57 (td, 9.6, 5.7)              | 70.9      | 4, 5, 7         | 7              |
| 7  | 5.54 (d, 9.6)                    | 132.1     | 6, 19           |
| 8  |                                | 135.3     |                 |                |
| 9α | 2.86 (d, 14.0)                   | 48.9      | 7, 8, 10, 19    |
| 9β | 4.06 (d, 14.0)                   |           |                 |                |
| 10 |                                | 213.9     |                 |                |
| 11 |                                | 81.6      |                 |                |
| 12 | 2.43 (m), 3.33 (m)               | 35.6      | 11, 13          |
| 13 |                                | 212.9     | 12              |
| 14 | 1.67 (m), 1.87 (m)               | 28.2      | 13              | 1              |
| 15 |                                | 49.6      |                 |                |
| 16 | 0.77 (s)                        | 24        | 1, 11, 15, 17   |
| 17 | 1.43 (s)                        | 27.5      | 1, 11, 15, 16   |
| 18 | 4.87 (s), 4.95 (s)               | 115.5     | 3, 5            | 3, 5           |
| 19 | 1.99 (s)                        | 18.8      | 7, 8, 9         | 7              |
| 20 | 4.46 (d, 3.0)                    | 79.3      | 10, 11, 15      |

$^a$ Data were recorded in CDCl$_3$ on 500 MHz; chemical shifts ($\delta$) and coupling constants are given in ppm and Hz, respectively; $^b$ Assignments made by COSY and HMQC.

Figure 4. Key NOESY correlations and computer-generated perspective model for 2 using MM2 force field calculation.

Scheme 1 illustrates a plausible biogenetic pathway for 1 and 2 based upon previous publications [7,8,16]. Cespitularin C might be a putative precursor of 1 and 2. Compound 1 is probably produced via intermediates a, b and c involving steps of epoxidation, rearrangement (1,2 methyl shift), oxidation, ring expansion, hydroxylation. Compound 2 may be derived from the same pathway, but
through further hydroxylation, dehydration and ketolization of cation c. The Meinwald type rearrangement may be involved to give a ketone directly in the second step [19].

**Scheme 1.** Plausible biogenetic pathway of 1 and 2.

The isolated compounds 1 and 2 were evaluated for cytotoxicity against human medulloblastoma (Daoy) and colon adenocarcinoma (WiDr) cancer cell lines. As a result, cespitulone A showed significant *in vitro* cytotoxicity against human medulloblastoma (Daoy) and colon adenocarcinoma (WiDr) cancer cells with IC$_{50}$ values of 8.7 and 6.7 µM, respectively [20]. Mitomycin was used as a positive control with IC$_{50}$ at 0.3 µM.

3. **Experimental Section**

3.1. **General Experimental Procedures**

Optical rotations were recorded on a JASCO DIP-1000 polarimeter. IR spectra were measured on Hitachi T-2001 spectrophotometer. LRESIMS and HRESIMS were taken on a JEOL JMS-HX 110 mass
spectrometer. The $^1$H, $^{13}$C NMR, $^1$H-$^1$H COSY, HMHC, HMBC and NOESY spectra were recorded on Bruker FT-300 (300 MHz for $^1$H) and a Varian UNITY INOVA 500 (500 MHz for $^1$H and 125 MHz for $^{13}$C) spectrometers. The chemical shifts were given in δ (ppm) and coupling constants in Hz. Silica gel 60 (Merck) was used for column chromatography. Sephadex LH-20 (Amersham Pharmacia Biotech AB, Sweden) was used for separation. LiChrospher® Si 60 (5 μm, 250-10 mm, Merck, Germany) and LiChrospher®100 RP-18e (5 μm, 250-10 mm, Merck, Germany) were used for NP-HPLC and RP-HPLC (Hitachi), respectively.

3.2. Extraction and Isolation

The soft coral (1.1 Kg, freeze-dried), collected at a depth of 20 m in October 2004, was extracted with mixture of CH$_2$Cl$_2$/EtOH (1:1), and the crude extract was partitioned between EtOAc and H$_2$O (1:1). The EtOAc-soluble fraction (100 g) was subjected to a Si gel column (n-hexane/EtOAc, 15:1–0:1; EtOAc/MeOH, 50:1–2:1) to give fractions 1-12. Fraction 6 (3.1 g) was chromatographed on a LH-20 Sephadex column (MeOH) and separated further by HPLC column (Si gel, n-hexane–CH$_2$Cl$_2$–MeOH, 20:20:1) to furnish cespitulone A (1, 3 mg). Fraction 8 (1.7 g) was further separated on a Sephadex LH-20 column using CH$_2$Cl$_2$-MeOH (4:1) to give 5 fractions (8-1~8-5). Fraction 8-4 (779 mg) was further separated with NP-HPLC column (n-hexane–CH$_2$Cl$_2$–MeOH, 20:20:1) and with RP-HPLC column (MeOH–H$_2$O–CH$_3$CN, 65:30:5) to afford 2 (9 mg).

3.3. Spectroscopic Data

Cespitulone A (1): amorphous solid, [α]$^2^5$D −58.8 (c = 0.2, CH$_2$Cl$_2$); IR (neat) ν$_{max}$ 3419, 2924, 1703 cm$^{-1}$; HRESIMS m/z 373.1989 (C$_{20}$H$_{30}$O$_3$Na$,^+$, calcd 373.1991). $^1$H-NMR and $^{13}$C-NMR (CDCl$_3$, 500/125 MHz) see Tables 1 and 2, respectively.

Cespitulone B (2): colorless amorphous solid; [α]$^2^5$D −63.4 (c 0.2, CH$_2$Cl$_2$); IR (neat) ν$_{max}$ 3419, 2925, 1700, 1445, 1391, 1278 cm$^{-1}$; HRESIMS m/z 373.1993 ([M + Na]$^+$, calcd for C$_{20}$H$_{30}$O$_3$Na$,^+$, 373.1991). $^1$H NMR (CDCl$_3$) and $^{13}$C NMR (CDCl$_3$) data, see Tables 1 and 2, respectively.

Preparation of (S)- and (R)-MPTA esters (1a and 1b) of 1. To a solution of 1 (0.7 mg in 0.5 mL pyridine) was added R-(-)- or S-(+)-MPTA chloride (one drop) and the solution was allowed to stand at room temperature for 12 h. After purification using preparative LC, the ester (0.6 mg, 85% yield) was analyzed by $^1$H NMR spectroscopic measurement, and Δδ = δ$^S$ − δ$^R$ was calculated for 1.

Compound 1a: $^1$H NMR (CDCl$_3$, 300 MHz) 5.698 (1H, td, J = 8.1, 3.0 Hz, H-6), 5.592 (1H, m, H-7), 1.255, 1.384 (6H, s, H-16, -17), 4.922 (1H, s, H-18), 5.041 (1H, s, H-18), 2.000 (3H, s, H-19).

Compound 1b: $^1$H NMR (CDCl$_3$, 300 MHz) 5.655 (1H, td, J = 8.1, 3.0 Hz, H-6), 5.399 (1H, d, J = 8.1 Hz, H-7), 1.109, 1.486 (6H, s, H-16, -17), 4.926 (1H, s, H-18), 5.050 (1H, s, H-18), 1.975 (3H, s, H-19).
3.4. Cytotoxicity Assay

The cytotoxic activities of compounds against human medulloblastoma (Daoy) and colon adenocarcinoma (WiDr) cancer cell lines cells were assayed by the MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay as previously described [21]. 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, 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. Mitomycin was used as a standard compound.

4. Conclusions

Our investigation on constituents of Taiwanese soft coral Cespitularia taeniata has resulted in the isolation of two novel diterpenoids (1 and 2), which possess an unprecedented bicyclo [10.3.1] ring system with C-C bond connections between C-10 and C-20, and between C-20 and C-11. Cespitulone A (1) exhibited significant cytotoxicity against human medulloblastoma (Daoy) and colon adenocarcinoma (WiDr) cancer cells.

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Author Contributions

Ya-Ching Shen led the research team and supervised Ph.D. students, Yu-Chi Lin and Shih-Sheng Wang. Shih-Sheng Wang isolated the metabolites, measured various spectra and operated the reaction. Yu-Chi Lin analyzed the data and determined the structures. Yao-Haur Kuo evaluated the biological activities. Ya-Ching Shen wrote the manuscript and Yu-Chi Lin prepared the tables and figures. Chung-Hsiung Chen and Ya-Ching Shen gave the suggestion of biosynthetic proposal. Chung-Hsiung Chen edited the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.
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