Oxygenated Cembranoids from the Cultured and Wild-Type Soft Corals *Sinularia flexibilis*

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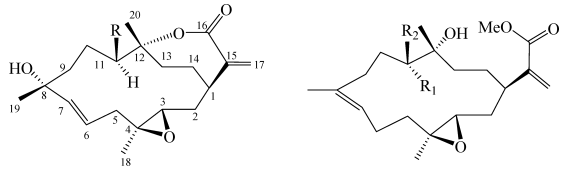
Received February 3, 2009; accepted August 20, 2009; published online August 24, 2009

Two new cembranoids, flexibilisolide A (1) and flexibilisin A (2), along with one known combranoid 5 have been isolated from the cultured soft coral *Sinularia flexibilis*. Furthermore, two new cembranoids, flexibilisolide B (3) and flexibilisin B (4), along with two known combranoids (5, 6), have been isolated from the wild-type soft coral *S. flexibilis*. The structures of the new metabolites were determined on the basis of extensive spectroscopic analysis and by comparison of NMR data with those of known compounds. The metabolites 5 and 6 have been shown to exhibit weak cytotoxic activity against MCF-7 cancer cell line.

Key words cembranoid; *Sinularia flexibilis*; cytotoxicity

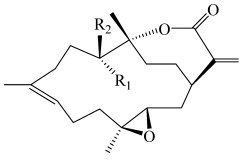
In previous studies a series of novel secondary metabolites, including cembranes,[1—12] eunicellin-based compounds,[13] and other metabolites,[14—17] have been isolated from the soft coral *Sinularia flexibilis* (Quoy and Gaimard). Some of these were found to exhibit cytotoxic activity against the growth of various cancer cell lines.[1,3—6,10—12] During the course of our investigation on new natural substances from the cultured and wild-type soft corals *S. flexibilis*, four new cembranoids (**1**—**4**) along with two known metabolites, 11-*epi*-sinulariolide acetate (**5**)[12,18] and sinulariolide (**6**)[1,19] have been isolated. New metabolites flexibilisolide A (**1**) and flexibilisin A (**2**) were isolated from the cultured soft coral, and flexibilisolide B (**3**) and flexibilisin B (**4**) were obtained from the wild-type soft coral. The cytotoxicity of compounds **1**—**6** against human cervical epitheloid (Hela), laryngeal (Hep 2), medulloblastoma (Daoy) and breast (MCF-7) carcinoma cells was studied. The results showed that 5 and 6 exhibited weak cytotoxicity towards MCF-7 cell line.

Flexibilisolide A (**1**) was obtained as a white powder. The HR-electrospray ionization (ESI)-MS spectrum of **1** exhibited a molecular ion peak at *m*/*z* 415.2099 $[M+Na]^+$, consisting with a molecular formula $C_{22}H_{32}O_6$ and implying seven degrees of unsaturation. The IR spectrum revealed the presence of carbonyl (ν_{max} 1739, 1713 cm^{-1}) and hydroxy groups (ν_{max} 3461 cm^{-1}). The ^{13}C-NMR (Table 1) spectrum of **1**, showed signals of twenty-two carbons, which were further identified by the assistance of distortionless enhancement by polarization transfer (DEPT) spectrum as four methyls, six sp^3 methylenes, three sp^3 methines (including two oxymethines), one sp^2 methylene, two sp^2 methines, three sp^3 quaternary carbons and three sp^2 quaternary carbons (including two ester carbonyls). From ^{1}H- and ^{13}C-NMR spectra, **1** was found to possess one acetoxy group [δ_C



1: R= OAc
3: R= OH

2: R_1 = H, R_2 = OAc
2a: R_1 = H, R_2 = OH
4: R_1 = OH, R_2 = H



5: R_1 = H, R_2 = OAc
6: R_1 = OH, R_2 = H

Table 1. ^{13}C-NMR Data for Compounds **1**—**4**

	1[a]	**2**[a]	**3**[b]	**4**[a]
1	35.0 (CH)[c]	36.3 (CH)	36.4 (CH)	38.4 (CH)
2	32.3 (CH_2)	34.9 (CH_2)	33.2 (CH_2)	34.3 (CH_2)
3	61.3 (CH)	59.4 (CH)	60.5 (CH)	61.1 (CH)
4	61.0 (C)	60.8 (C)	60.2 (C)	60.0 (C)
5	42.6 (CH_2)	35.9 (CH_2)	41.6 (CH_2)	37.5 (CH_2)
6	126.1 (CH)	22.3 (CH_2)	124.1 (CH)	23.1 (CH_2)
7	138.8 (CH)	128.4 (CH)	139.1 (CH)	122.6 (CH)
8	72.5 (C)	132.8 (C)	74.3 (C)	136.1 (C)
9	37.2 (CH_2)	34.6 (CH_2)	37.0 (CH_2)	33.7 (CH_2)
10	25.3 (CH_2)	27.8 (CH_2)	27.6 (CH_2)	28.6 (CH_2)
11	75.0 (CH)	75.6 (CH)	70.8 (CH)	73.8 (CH)
12	86.7 (C)	74.7 (C)	88.6 (C)	74.5 (C)
13	32.0 (CH_2)	36.0 (CH_2)	32.5 (CH_2)	37.1 (CH_2)
14	30.4 (CH_2)	25.7 (CH_2)	31.5 (CH_2)	26.4 (CH_2)
15	143.4 (C)	142.3 (C)	144.5 (C)	143.0 (C)
16	169.2 (C)	167.6 (C)	169.2 (C)	167.4 (C)
17	124.9 (CH_2)	124.3 (CH_2)	124.1 (CH_2)	124.6 (CH_2)
18	16.5 (CH_3)	18.4 (CH_3)	16.4 (CH_3)	17.0 (CH_3)
19	29.3 (CH_3)	17.0 (CH_3)	31.1 (CH_3)	18.1 (CH_3)
20	26.0 (CH_3)	23.2 (CH_3)	25.1 (CH_3)	24.8 (CH_3)
OMe		52.0 (CH_3)		51.9 (CH_3)
OAc	21.1 (CH_3)	21.2 (CH_3)		
	171.1 (C)	172.3 (C)		

a) Spectra recorded at 500 MHz in $CDCl_3$. *b*) 400 MHz in $CDCl_3$. *c*) Deduced from DEPT.

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171.1 (C), 21.1 (CH_3), and δ_H 2.14 (s)] and one α-methylene-ε-lactone ring [δ_C 169.2 (C), 143.4 (C), 124.9 (CH_2), 86.7 (CH), 35.0 (CH), 32.0 (CH_2), 30.4 (CH_2) and δ_H 6.31 (s), 5.49 (s)].[12,19] Furthermore, three methyls (δ_C 29.3, 26.0, 16.5), one 1,2-disubstituted double bond (δ 138.8, 126.1), two oxygen-bearing methines (δ_C 75.0, 61.3), and two oxygenated quaternary carbons (δ 72.5, 61.0) were observed. The gross structure of **1** was determined by a detailed analysis of 1D and 2D NMR spectra. From the ^1H–^1H correlation spectroscopy (COSY) spectrum of **1**, it was possible to identify three different structure units, which were assembled with the assistance of a heteronuclear multiple bond connectivity (HMBC) experiment (Fig. 1). Key HMBC correlations between H-3 to C-4 and C-5; H-6 to C-8; H-7 to C-8; H-11 to C-12 and C-13; H_2-13 to C-12; H_2-17 to C-1, C-15, and C-16; H_3-18 to C-3, C-4, and C-5; H_3-19 to C-7, C-8 and C-9; and H_3-20 to C-11, C-12, and C-13 permitted the connection of the molecular skeleton. Furthermore, one acetoxy group positioned at C-11 was confirmed from the HMBC correlations from δ 5.46 (H-11) to the ester carbonyl carbon resonating at δ 171.1 (C). The *J* values for both H-6 and H-7 (15.6 Hz) further confirmed the *E*-configuration of the 6,7-double bond. The relative configurations of the six chiral centers at C-1, C-3, C-4, C-8, C-11, and C-12 in **1** were elucidated by detailed analysis of nuclear Overhauser effect (NOE) correlations, as shown in Fig. 2. Finally, the *J* values of Ha-2/H-3 (10.4 Hz), Hb-5/H-6 (10.4 Hz) and Hb-10/H-11 (12.8 Hz) revealed the *anti* geometries between the above vicinal protons, as shown in Fig. 2. It was found that H_3-18 (δ 1.44, s) showed NOE interactions with both H-1 (δ 2.82, m) and H-6 (δ 5.96, ddd, J=15.6, 10.4, 4.2 Hz), while H-7 (δ 5.73, d, J=15.6 Hz) was NOE correlated with H_3-19 (δ 1.31, s). Therefore, H-1 and H_3-18 are situated on the α-face, and in contrast H_3-19 should be positioned on the β-face. One of the methylene protons at C-10 (δ_H 1.72, m) exhibited NOE correlations with H_3-19 and was assigned as H-10β, while the other (δ_H 1.53, m) was denoted as H-10α. The NOE correlations observed between H_3-20 and H-10β reflected the β-orientations of the methyl substituent at C-12. Furthermore, the NOE interactions found between the H-1 and H-11 and between H-11 and H-10α assigned the β-orientation of the acetoxy group. From the above observations and further analysis of other NOE interactions (Fig. 2), the structure of **1** was fully established.

Flexibilisin A (**2**) was found to possess the molecular formula $C_{23}H_{36}O_6$, as deduced from the HR-ESI-MS and NMR spectroscopic data. A detailed comparison of the NMR spectroscopic data of **2** (Tables 1, 2) with those of 11-*epi*-sinulariolide acetate (**5**) showed that both compounds have similar

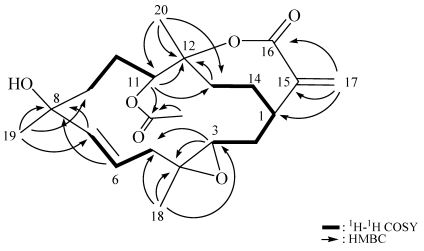

Fig. 1. Key ^1H–^1H COSY and HMBC Correlations for **1**

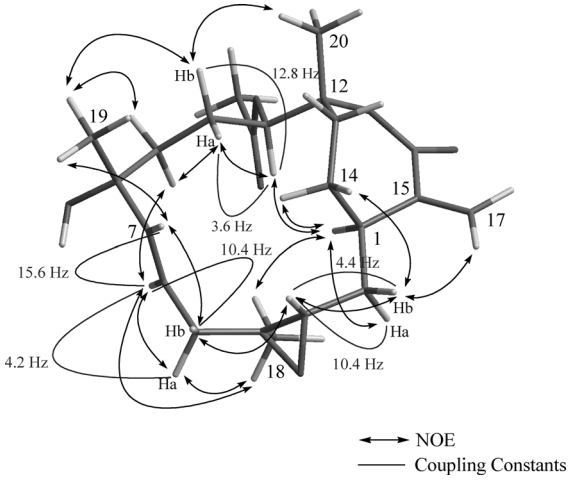

Fig. 2. Selective NOESY Correlations and Coupling Constants (*J*) of **1**

Table 2. ^1H-NMR Data for Compounds **1**—**4**

	1[a]	**2**[a]	**3**[b]	**4**[a]
1	2.82 m	3.02 tt (11.5, 4.0)	2.51 m	2.70 m
2	2.08 m; 1.47 m	1.90 m; 1.50 m	2.10 m; 1.40 m	2.79 m; 1.58 m
3	2.90 dd (10.4, 4.4)[c]	2.91 dd (10.5, 3.5)	3.05 dd (10.0, 4.5)	2.84 dd (7.5, 5.5)
5	2.76 dd (13.2, 4.2); 1.80 m	1.98 m; 1.78 m	2.63 dd (13.0, 7.5); 1.87 m	2.00 m; 1.40 m
6	5.96 ddd (15.6, 10.4, 4.2)	2.09 m; 1.97 m	5.73 m	2.14 m
7	5.73 d (15.6)	5.12 dd (9.0, 3.5)	5.70 d (16.0)	5.10 t (6.5)
8				
9	1.70 m; 1.60 m	2.08 m; 1.94 m	1.92 m; 1.87 m	2.20 m; 2.13 m
10	1.72 m; 1.53 m	1.62 m; 1.55 m	1.74 m; 1.67 m	1.72 m; 1.56 m
11	5.46 dd (12.8, 3.6)	5.08 dd (10.0, 4.0)	4.03 dd (11.0, 3.0)	3.51 d (10.5)
12				
13	1.99 m; 1.90 m	1.54 m	2.10 m; 1.89 m	1.59 m; 1.49 m
14	2.26 m; 1.35 m	1.73 m; 1.66 m	2.32 m; 1.27 m	1.62 m; 1.50 m
17	6.31 s; 5.49 s	6.30 s; 5.50 s	6.29 s; 5.44 s	6.27 s; 5.58 s
18	1.44 s	1.36 s	1.24 s	1.29 s
19	1.31 s	1.65 s	1.38 s	1.67 s
20	1.36 s	1.10 s	1.38 s	1.24 s
OMe		3.75 s		3.75 s
OAc	2.14 s	2.14 s		

a) Spectra recorded at 500 MHz in $CDCl_3$. *b*) 400 MHz in $CDCl_3$. *c*) *J* values (Hz) in parentheses.

structures. However, an additional methoxy group (δ 52.0, CH_3; δ 3.75, s) was observed in **2**. In addition, the methoxy group positioned at C-16 was confirmed by the HMBC correlation between the oxymethyl protons (δ_H 3.75) and the carbonyl carbon (δ_C 167.6, C, C-16). Moreover, H_3-20 (δ_H 1.10) and C-12 (δ_C 74.7) of **2** displayed signals at upper field in comparison with the corresponding signals of **5** (δ_H 1.37, H_3-20; δ_C 87.2, C-12),[12] implying the presence of a hydroxy group at C-12. In order to confirm the structure, including the stereochemistry of **2**, a base-catalyzed hydrolysis of **5** was performed and the reaction was found to afford **2** and its acetyl derivative **2a**.[18] Thus, the structure of **2** was fully established.

HR-ESI-MS of flexibilisolide B (**3**) exhibited a $[M+Na]^+$ peak at *m/z* 373.1992 (Calcd for $C_{20}H_{28}O_5Na$, 373.1991) and established the molecular formula $C_{20}H_{30}O_5$, implying six degrees of unsaturation. The IR spectrum also revealed the presence of carbonyl (ν_{max} 1714 cm^{-1}), and hydroxy (ν_{max} 3421 cm^{-1}) moieties. The NMR spectra of **3** (Tables 1, 2) were quite similar to those of **1**. However, resonances appropriate for the acetoxyl in **1** were absent from ^1H- and ^{13}C-NMR spectra of **3**. In addition, the proton of the acetoxyl-containing methine which showed resonance at δ 5.46 (dd, J=12.8, 3.6 Hz) in **1** was replaced by a proton which was upfield-shifted to δ 4.03 (dd, J=11.0, 3.0 Hz) in **3**, revealing **3** as a deacetyl derivative of **1**. These observations could be further confirmed by the correlations observed in the 2D NMR (including ^1H–^1H COSY, heteronuclear multiple quantum correlation (HMQC) and HMBC) experiments of **3**. HR-ESI-MS of flexibilisin B (**4**) established the molecular formula $C_{21}H_{34}O_5$, implying five degrees of unsaturation. The observed strong IR absorption at 1716 cm^{-1} suggested the presence of an α,β-unsaturated ester group. The ^1H- and ^{13}C-NMR spectral data (Tables 1, 2) of **4** were found to be very similar to those of **6**, except for the disappearance of the lactone. This was further supported by the mild base-catalyzed hydrolysis of **6** in MeOH to give a methyl ester, which was found to be identical to **4**.

The cytotoxicity of **1**—**6** against four human cancer cell lines, including Hela (cervical epitheloid carcinoma), Hep 2 (laryngeal carcinoma), Daoy (medulloblastoma) and MCF-7 (breast carcinoma) cells, was assayed. The results showed that only compounds **5** and **6** exhibited weak cytotoxicity against the proliferation of MCF-7 cells (ED_{50}'s 11.5 and 16.9 μg/ml) and other metabolites were inactive (ED_{50}'s $<$20 μg/ml) towards these cancer cell lines.

Experimental

Melting points were determined using a Fisher-Johns melting point apparatus. Optical rotations were measured on a JASCO P-1020 polarimeter. IR spectra were recorded on a JASCO FT/IR-4100 infrared spectrophotometer. UV spectra were recorded on a JASCO V650 spectrophotometer. The NMR spectra were recorded on a Varian 400MR FT-NMR (or Varian Unity INOVA 500 FT-NMR) instrument at 400 MHz (or 500 MHz) for ^1H and 100 MHz (or 125 MHz) for ^{13}C in $CDCl_3$. LR-MS and HR-MS were obtained by ESI on a Bruker APEX II mass spectrometer. Silica gel (Merck, 230—400 mesh) was used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60 F-254, 0.2 mm) were used for analytical TLC. High-performance liquid chromatography was performed on a Hitachi L-7100 HPLC apparatus with the Merck Hibar Si-60 column (250×21 mm, 7 μm).

Animal Material Specimens of the cultured soft coral *Sinularia flexibilis* (specimen no. CSC-1) were collected by hand in a 80 ton cultivating tank located in the National Museum of Marine Biology and Aquarium (NMMBA), Taiwan, in July 2006. The wild-type soft coral *S. flexibilis* (specimen no. 20040112-8) was collected by hand using SCUBA off the coast of Southern Pingtung of Taiwan, in January 2004 at depths of 10 to 15 m. Two voucher samples were deposited at the Department of Marine Biotechnology and Resources, National Sun Yat-sen University.

Extraction and Isolation The sliced bodies of the cultured soft coral *Sinularia flexibilis* (1.5 kg, wet weight) were minced and extracted exhaustively with EtOH (1 l×6). The EtOH extract was filtered and concentrated under reduced pressure. The residue was partitioned between CH_2Cl_2 and H_2O. The CH_2Cl_2-soluble fraction was concentrated and the residue (9.2 g) was chromatographed on Si gel by CC and eluted with EtOAc in *n*-hexane (0—100%, gradient) to yield 25 fractions. Fraction 16, eluted with EtOAc–*n*-hexane (1 : 1), was further purified by normal phase HPLC using EtOAc–*n*-hexane (1 : 3) to yield **5** (12.1 mg) and **2** (6.9 mg). Fraction 19, eluted with EtOAc–*n*-hexane (2 : 1), was further purified by normal phase HPLC using EtOAc–*n*-hexane (1 : 1) to yield **1** (3.7 mg).

The wild-type soft coral *S. flexibilis* (1.2 kg, wet weight) was exhaustively extracted with EtOH (1 l×6). The EtOH extract was filtered and concentrated under vacuum, and the residue of aqueous suspension was partitioned between EtOAc and H_2O. The solvent-free extract EtOAc (15.9 g) was subjected to CC on Si gel and eluted with EtOAc in *n*-hexane (0—100%, gradient) to yield 26 fractions. Fraction 13, eluted with EtOAc–*n*-hexane (1 : 2), was further purified over Si gel using acetone–*n*-hexane (1 : 4), followed by normal phase HPLC using EtOAc–*n*-hexane (1 : 3 to 1 : 1) to yield **5** (17.1 mg), **6** (12.6 mg) and **4** (5.2 mg). Fraction 16, eluted with EtOAc–*n*-hexane (2 : 1), was purified by normal phase HPLC, using EtOAc–CH_2Cl_2 (1 : 1) to give **3** (4.7 mg).

Flexibilisolide A (**1**): White powder; mp 67—69 °C; $[\alpha]_D^{25}$ −41.9 (c=0.37, $CHCl_3$); IR (neat) ν_{max} 3461, 2929, 1739, 1713, 1629, 1460, 1373, and 1231 cm^{-1}; UV (MeOH) λ_{max} 216 (log ε=3.8); ^{13}C- and ^1H-NMR data, see Tables 1 and 2; ESI-MS *m/z*: 415 $[M+Na]^+$; HR-ESI-MS *m/z*: 415.2099 $[M+Na]^+$ (Calcd for $C_{22}H_{32}O_6Na$, 415.2096).

Flexibilisin A (**2**): Colorless oil; $[\alpha]_D^{25}$ +31.6 (c=0.69, $CHCl_3$); IR (neat) ν_{max} 3482, 2928, 1736, 1719, 1627, 1439, 1373, 1244 cm^{-1}; UV (MeOH) λ_{max} 216 (log ε=3.8); ^{13}C- and ^1H-NMR data, see Tables 1 and 2; ESI-MS *m/z*: 431 $[M+Na]^+$; HR-ESI-MS *m/z*: 431.2407 $[M+Na]^+$ (Calcd for $C_{23}H_{36}O_6Na$, 431.2409).

Flexibilisolide B (**3**): White powder; mp 68—70 °C; $[\alpha]_D^{25}$ −104.3 (c=0.17, $CHCl_3$); IR (neat) ν_{max} 3421, 2932, 1714, 1630, 1457, 1381, and 1235 cm^{-1}; UV (MeOH) λ_{max} 216 (log ε=3.6); ^{13}C- and ^1H-NMR data, see Tables 1 and 2; ESI-MS *m/z*: 373 $[M+Na]^+$; HR-ESI-MS *m/z*: 373.1992 $[M+Na]^+$ (Calcd for $C_{20}H_{30}O_5Na$, 373.1991).

Flexibilisin B (**4**): Colorless oil; $[\alpha]_D^{25}$ −28.0 (c=1.52, $CHCl_3$); IR (neat) ν_{max} 3444, 2952, 1716, 1626, 1440, 1381, and 1241 cm^{-1}; UV (MeOH) λ_{max} 216 (log ε=3.8); ^{13}C- and ^1H-NMR data, see Tables 1 and 2; ESI-MS *m/z*: 389 $[M+Na]^+$; HR-ESI-MS *m/z*: 389.2307 $[M+Na]^+$ (Calcd for $C_{21}H_{34}O_5Na$, 389.2304).

Compound 2a ^1H-NMR ($CDCl_3$, 300 NMR) δ: 6.30 (1H, s, H-17a), 5.55 (1H, s, H-17b), 5.18 (1H, dd, J=5.5, 3.5 Hz, H-7), 3.75 (1H, s, OMe), 3.61 (1H, d, J=10.5 Hz, H-11), 2.82 (1H, m, H-3), 2.81 (1H, m, H-1), 2.23 (1H, m, H-9a), 2.12(1H, m, H-6a), 2.10 (1H, m, H-2a), 1.96 (1H, m, H-6b), 1.92 (1H, m, H-5a), 1.80 (1H, m, H-9b), 1.66 (1H, m, H-5b), 1.66 (3H, s, H_3-19), 1.62 (1H, m, H-10a), 1.54 (1H, m, H-10b), 1.49 (2H, m, H-13), 1.46 (1H, m, H-2b), 1.43 (2H, m, H-14), 1.29 (3H, s, H_3-18), 1.08 (3H, s, H_3-20). ^{13}C-NMR ($CDCl_3$, 75 NMR) δ: 166.5 (C-16), 141.7 (C-15), 133.4 (C-8), 126.1 (C-7), 123.7 (C-17), 74.9 (C-12), 71.9 (C-11), 60.9 (C-4), 60.3 (C-3), 52.5 (OMe), 37.1 (C-5), 36.9 (C-1), 36.3 (C-13), 35.4 (C-9), 35.2 (C-2), 29.3 (C-14), 27.5 (C-10), 23.9 (C-20), 23.4 (C-6), 19.1 (C-18), 18.0 (C-19).

Hydrolysis of 5 A solution of **5** (30.0 mg) was dissolved in 5% methanolic NaOH solution (2.7 ml), and the mixture was stirred at 0 °C for 12 h. The mixture was then neutralized with diluted HCl (0.1 N) and evaporated, and the residue was extracted with $CHCl_3$ (2.0 ml×3). The $CHCl_3$-soluble layers were combined, dried over anhydrous $NaSO_4$ and evaporated. The residue was subjected to column chromatograph over silica gel using EtOAc–*n*-hexane (1 : 1) to yield **2** (4.5 mg, 13.7%) and **2a** (20.3 mg, 62.5%).

Hydrolysis of 6 By using the same procedure as for the preparation of **2**, the reaction of **6** (5 mg) with 5% methanolic NaOH solution (1 ml) afforded a crude product which was subjected to column chromatography over silica gel using EtOAc–*n*-hexane (1 : 1) to yield **4** (4.3 mg, 88.0%).

Cytotoxicity Testing Cytotoxicity assays of compounds **1**—**6** were performed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method.[20,21]

Acknowledgments Financial support was provided by Ministry of Edu-

cation (97C031702) and by intramural funding from the National Museum of Marine Biology & Aquarium, Taiwan (NMM98001) awarded to J.-H. Sheu.

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