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Eight new diterpenoids from soft coral *Cespitularia hypotentaculata*

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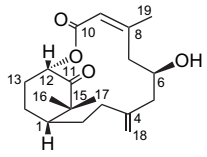
Abstract—Chemical investigation of the soft coral *Cespitularia hypotentaculata* resulted in the isolation of eight new diterpenes, cespihypotins E–L (**1**–**8**). The new metabolites comprise of six verticillene-type diterpenes, one cespitularane derivative, and one derivative with 14-membered lactone ring. The structures were determined through detailed spectroscopic analyses, especially high resolution ESIMS and 2D NMR techniques. The relative stereochemistry was deduced from NOESY spectrum and application of Mosher's ester technique. Immunomodulatory and antiviral activities of **1**–**8** were tested and evaluated. The biogenetic pathways for **1**–**8** were also proposed.

1. Introduction

The soft coral *Cespitularia* (Xeniidae) live in colonies with polyps occurring on the branches with white, cream, blue, brown, or iridescent-green surface.[1] This genus elaborate varied diterpenoids of cembrane, neodolabellane, cespitularane, and verticillane skeleton,[2–7] and some of these compounds demonstrated cytotoxic activities.[7–9] The verticillene skeleton is basically bicyclic[9.3.1]diterpenes with some resemblance to taxane diterpenes isolated from various species of terrestrial *Taxus* trees, especially the β-*gem*-dimethyl attached to the cyclohexane group.[10] Some nor-verticillene has been also isolated, together with cespitularane with 14-membered lactone ring between C-10 and C-12.[7,11] Chemical investigation of the *Cespitularia hypotentaculata* Roxas resulted in the isolation of eight new diterpenes, cespihypotins E–L (**1**–**8**). The new metabolites comprise of six verticillene-type diterpenes **2**–**5**, **7**–**8**, one cespitularane derivative **6**, and one derivative with 14-membered lactone ring **1**. The biological activities of compounds **1**–**8** were tested against HSV-1 virus and evaluated with peripheral blood mononuclear cell (PBMC) proliferation induced by phytohemaglutinin (PHA).



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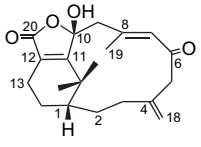

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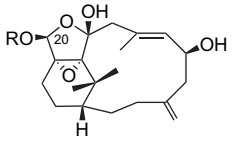

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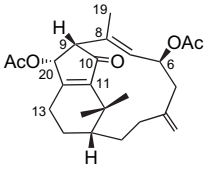
4 R = OCOCH=CH$_2$ (21-23)
5 R = Ac



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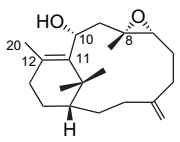

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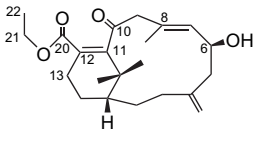

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2. Results and discussion

2.1. Structure of compound 1

The molecular formula of cespihypotin E (**1**), $[\alpha]_D^{26}$ +52.1 (EtOAc), was established as $C_{19}H_{28}O_4$ from HRESIMS.

Keywords: *Cespitularia hypotentaculata*; Verticillene diterpenes; Cespihypotins; Immunomodulatory activity.
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The UV and IR spectra revealed α,β-unsaturated ester, hydroxyl, and carbonyl groups. The ^{13}C NMR data unveiled an exomethylene, trisubstituted double bond, a carbonyl (δ_C 210.6), a conjugated ester carbonyl (δ_C 166.5), *gem*-methyls (δ_C 28.1, 22.9), and a vinyl methyl (δ_C 20.3) suggesting a bicyclic[6.3.1]nor-diterpene. The ^1H NMR spectrum revealed an oxymethine proton at δ_H 3.67 (H-6) by virtue of HMBC correlation to C-4, and COSY correlations to two CH_2 (H-5 and H-7). HMBC correlations between H-6/C-8; Me-19/C-7, C-9; between H-9/C-7, and between H-9/C-10 located unsaturation at C-8 and the ester carbonyl at C-10. The *gem*-protons (H-16 and H-17) displayed correlations to C-1 and the carbonyl (C-11), while correlation of H-12/C-10, C-13, C-11 allowed assigning the latter carbonyl to C-11 in the cyclohexanone ring that was attached at C-12 to the conjugated carbonyl forming 14-membered lactone ring. The NOESY spectrum of **1** revealed correlations between Me-19/H-6, H-9; H-9/H_α-7; H_β-3/Me-16, Me-17; and Me-16/H-1, H-12, Me-17 indicating the α-configuration of H-6 and the β-configuration of H-12. The NOESY correlation between M-19/H-9 was in accordance with *Z*-geometry of the 8,9-double bond. These data were closely similar to those reported for cespitulactone A but with different position and arrangement of the double bond.[8]

2.2. Structure of compound 2

The HRESIMS of **2**, [α] −25.1 (acetone), revealed that cespihypotin F had a molecular formula $C_{20}H_{26}O_4Na$. The IR spectrum displayed absorption band diagnostic of lactone (1752 cm^{-1}) and conjugated carbonyl (1682 cm^{-1}) groups. The ^{13}C NMR data showed two carbonyls (δ_C 199.0, 170.1), an exomethylene double bond (δ_C 143.7, 115.9), a trisubstituted double bond (δ_C 129.8, 150.3), and a double bond adjacent to a carbonyl, which implied a tricyclic compound. The ^1H NMR spectrum (Table 1) displayed an olefinic proton singlet (δ_H 6.40), two exomethylene singlets (δ_H 4.94 and 4.85), and three methyl singlets. HMBC revealed correlations of H-19/C-7, C-8, C-9, and correlations of H-7/C-8, C-6. Moreover, the two geminal protons (H-5) correlated to C-18 and the carbonyl (C-6). Each of the methyl singlets correlated with one another and with C-15, C-1, and C-11. COSY connectivities between CH_2-3/H_2-2/H-1/H_2-14/H_2-13, and HMBC correlations of H-1/C-11, C-13, C-15 suggested that the two *gem*-methyls are attached to a quaternary carbon in a cyclohexene ring. The presence of γ-hydroxy-α,β-unsaturated-γ-lactone was evident from conjugated carbonyl at δ_C 170.1 (C-20), 129.1 (C-12), 167.2 (C-11), and 108.2 (C-10). The aforementioned data were in accordance with those of cespitularin D, a 1*S*-verticillene-type diterpene previously isolated from *C. hypotentaculata*, in which a hydroxyl group at C-6 was replaced by a carbonyl in **2**.[7] The relative stereochemistry of **2** was determined on basis of biogenetic consideration and analysis of NOESY spectrum. The correlations between H_β-13/H-16, H-1; H-17/H-1, H_β-14; and H-7/H_β-5, H-17 indicated that H-1, H-7, Me-16, and Me-17 were on β-face of the molecule. Additionally, NOESY correlation between Me-19/H_α-9 suggested that Me-19 was on the α-side of the molecule, while absence of Me-19/H-7 favored the *E*-geometry of the 7,8-double bond.

2.3. Structure of compound 3

Compound **3**, [α] −15.6 (*c* 0.6, acetone), had a molecular formula $C_{20}H_{30}O_5$ as deduced from HRESIMS. The IR spectrum of **3** unveiled the absence of carbonyl group. The NMR data of **3** were similar to those of **5** with the exception of absence of signals attributable to an acetyl group. The oxymethine proton at δ_H 4.49, having COSY correlation with H-5, H-7 and HMBC correlation to C-7, C-5, was assigned to H-6. A second oxymethine resonating at δ_H 4.70 was assigned to H-20 as a result of its HMBC correlations to C-12 (δ_C 78.2), C-11 (δ_C 74.3) as well as the acetal

Table 1. ^1H NMR data ($CDCl_3$, 300 MHz) for compounds **1–8** (δ in parts per million, *J* in hertz)[a]

Position	1	2	3	4	5	6	7	8
1	1.75 m	1.62 m	1.45 m	1.51 m	1.51 m	1.68 m	1.48 m	1.55 m
2	1.73 m (2H)	1.20 m (2H)	1.43 m (2H)	2.32 m (2H)	2.28 m (2H)	2.28 m, 1.81m	1.81 m, 1.26 m	2.16 m, 1.74 m
3	1.98 m (2H)	1.49 m, 1.25m	2.19 m, 1.09 m	2.25 m (2H)	2.20 m, 2.08 m	2.30 m, 1.96 m	2.01 m, 1.76 m	2.38 m, 2.11 m
5	2.51 m, 2.11 m	3.15 d (13), 2.97 d (13)	2.65 m, 2.21 m	2.61 m, 2.20 m	2.66 m, 2.25 m	2.57 dd (4.4, 4.2), 1.86 m	2.02 m, 1.76 m	2.51 d (11.4), 2.36 m
6	3.67 m		4.49 m	4.51 m	4.50 t (8.5)	5.54 m	2.17 m (2H)	4.36 t (8.1)
7	2.41 m, 2.29 m	6.40 s	5.46 d (8.7)	5.49 d (7.5)	5.46 d (8.4)	5.03 d (9.9)	2.82 d (9.0)	5.54 d (7.8)
9	5.72 s	3.24 d (11.9), 2.96 d (11.9)	3.06 d (14.4), 2.53 d (14.4)	3.08 d (14.6), 2.56 d (14.6)	3.08 d (14.6), 2.53 d (14.6)	3.38 d (5.6)	2.41 d (12.0), 2.06 m	3.50 d (18.0), 3.13 d (18.0)
10							4.58 d (12)	
12	4.98 dd (8.6, 5.8)							
13	2.50 m, 2.35 m	1.51 m (2H)	2.10 m, 1.64 m	1.85 m, 1.59 m	1.70 m, 1.55 m	2.23 m, 2.07 m	2.28 d (10.2), 2.10 m	2.39 m (2H)
14	1.49 m (2H)	2.25 m, 1.20 m	1.08 m (2H)	1.12 m (2H)	1.88 m (2H)	2.06 m, 1.64 m	2.00 m (2H)	1.64 m, 1.50 m
16	1.32 s	1.58 s	0.95 s	1.33 s	1.32 s	1.40 s	1.09 s	1.21 s
17	1.12 s	1.31 s	1.32 s	0.98 s	0.97 s	1.16 s	1.18 s	1.24 s
18	4.89 s (2H)	4.94 s, 4.85 s	4.92 s (2H)	4.95 s (2H)	4.93 s (2H)	4.89 s, 4.84 s	4.73 s, 4.67 s	4.87 s, 4.84 s
19	2.07 s	2.01 s	1.81 s	1.83 s	1.81 s	1.75 s	1.36 s	1.62 s
20			4.70 s	5.86 s	5.62 s	6.01 d(5.6)	1.85 s	
21								4.17 q (7.2)
22				6.18 m				1.28 t (7.2)
23				5.93 d (10), 6.51 d (15.7)				
6-Ac						2.01 s		
20-Ac					2.11 s	2.12 s		

[a] Assignments were made by COSY, HMQC, and HMBC techniques.

carbon at δ_C 94.7 (C-10). The NOESY correlation between H-6/H_α-5, H-19; H-19/H_α-9; H_β-9/H-7, H-16, H-17; and H-1/H-16, H-17, H-20 revealed the α-orientation of H-6 and β-orientation of H-20.

2.4. Structure of compound 4

The molecular formula $C_{23}H_{32}O_6$ was assigned to **4** based on its HRESIMS (*m/z* 427.2096, $[M+Na]^+$). The NMR data (Tables 1 and 2) were in accordance with a verticillene-type diterpene ester. The NMR spectra disclosed signals at δ_C 164.8 (conjugated carbonyl), δ_C 127.8, δ_C 132.5, as well as characteristic cis- and trans-couplings at δ_H 6.51 (d, *J*=15.7 Hz), 5.93 (d, *J*=10.0 Hz), and 6.18 (m) with HMBC correlations between these protons and the carbonyl, thereby proving the presence of acrylate ester. Other ^1H and ^{13}C NMR signals as well as COSY of **4** were very similar to those of **3**. The oxymethine at δ_H 5.86 (s, H-20) correlated in HMQC spectrum with *C*H at δ_C 96.6, and in HMBC with the ester carbonyl (C-21), C-10, and *C*H_2-13. The eight degrees of unsaturation required additional ring that was deduced to be an epoxy ring involving two oxyquaternary carbons at δ_C 78.8 and δ_C 75.1. This was proved by HMBC correlations of H-16, H-17 to C-11 and of H-20 to C-11 and C-12. The relative configuration of **4** was established on the basis of correlations with **3** and NOESY experiments. NOESY correlations between H-1/H_β-13, H-16, H_β-14, H-17, H-20 and H-7/H_β-5, H-17 indicated that H-1, H-7, Me-16, Me-17, and H-20 were on β-face (Fig. 1). NOESY correlation between Me-19/H_α-9 and H-6/H_α-5, H-19 suggested that H-6 was α-oriented.

2.5. Structure of compound 5

Compound **5**, [α] −15.6 (acetone), had a molecular formula $C_{22}H_{32}O_6$ as derived from HRESIMS at *m/z* 415.2098 ($[M+Na]^+$). The UV bands and IR adsorptions were similar to those of **3** and **4**, suggesting a close analog. The ^1H and ^{13}C NMR data of **5** were also similar to those of **4** with the exception of the absence of signals assignable to the acrylate ester and presence of signals of acetate ester at δ_C 170.1(s), δ_C 20.9 (q), and δ_H 2.11(s). The oxymethine at δ_H 5.62 (s, H-20) had HMBC correlations to the acetate carbonyl (δ_C 170.1) as well as C-13 validating the attachment of acetoxyl group to C-20. HMBC and NOESY correlations were identical to those of **4**.

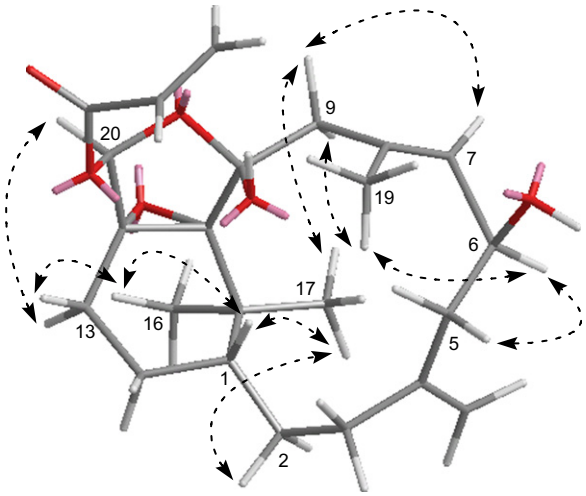


Figure 1. Key NOESY correlations of **4**.

2.6. Structure of compound 6

Compound **6**, [α] +57.6 (CH_2Cl_2) possessed a molecular formula $C_{24}H_{32}O_5$ as established from HRESIMS. The

Table 2. ^{13}C NMR data ($CDCl_3$, 75 MHz) for compounds **1**–**8**[a]

C	1	2	3	4	5	6	7	8
1	43.3 d	44.2 d	44.3 d	44.2 d	44.1 d	41.7 d	42.6 d	42.3 d
2	29.2 t	17.2 t	25.8 t	26.1 t	25.4 t	22.2 t	27.0 t	24.4 t
3	32.0 t	33.8 t	37.7 t	37.8 t	37.8 t	30.7 t	31.9 t	33.8 t
4	146.1 s	143.7 s	145.6 s	145.9 s	145.8 s	144.7 s	151.2 s	146.7 s
5	43.6 t	54.4 t	45.8 t	45.9 t	45.9 t	42.5 t	29.7 t	44.1 t
6	70.1 d	199.0 s	69.2 d	69.3 d	69.3 d	69.9 d	30.3 t	69.7 d
7	46.4 t	129.8 d	133.6 d	133.8 d	133.6 d	127.8 d	65.2 d	136.9 d
8	151.2 s	150.3 s	131.8 s	132.5 s	132.5 s	134.9 s	59.5 s	130.9 s
9	119.7 d	49.2 t	41.2 t	40.7 t	40.8 t	60.8 d	47.9 t	54.9 t
10	166.5 s	108.2 s	94.7 s	94.7 s	94.7 s	202.6 s	67.1 d	207.4 s
11	210.6 s	167.2 s	74.3 s	75.1 s	72.5 s	147.9 s	140.2 s	160.7 s
12	76.8 d	129.1 s	78.2 s	78.8 s	79.7 s	165.7 s	133.9 s	124.9 s
13	26.1 t	36.0 t	30.9 t	30.7 t	26.3 t	22.7 t	33.8 t	21.6 t
14	19.0 t	24.9 t	33.9 t	33.9 t	33.9 t	28.5 t	31.8 t	32.9 t
15	49.4 s	38.5 s	37.5 s	37.5 s	37.7 s	33.9 s	37.1 s	38.9 s
16	28.1 q	34.2 q	24.9 q	26.1 q	26.4 q	23.9 q	24.9 q	25.4 q
17	22.9 q	24.6 q	26.1 q	25.1 q	25.2 q	30.7 q	33.4 q	33.5 q
18	114.2 t	115.9 t	115.7 t	116.0 t	115.8 t	112.8 t	109.6 t	114.7 t
19	20.3 q	19.2 q	17.2 q	17.5 q	17.3 q	18.9 q	17.2 q	18.5 q
20		170.1 s	98.8 d	96.6 d	100.9 s	74.2 d	21.7 q	166.9 s
21				164.8 s				61.2 t
22				127.8 d				14.1 q
23				132.5 t				
6-Ac						170.5 s 21.4 q		
20-Ac					170.1 s 20.9 q	170.5 s 21.1 q		

[a] Assignments were aided by DEPT, HMQC, and HMBC experiments.

spectroscopic data of **6** clearly indicated the presence of two acetate moieties (δ_H 2.01, 2.12). One was attached to C-6, as indicated by correlations of H-6 to C-4 (δ_C 144.7), ester carbonyl, and C-8 (δ_C 134.9). The ^{13}C NMR spectrum revealed the presence of α,β-unsaturated ketone (δ_C 202.6, 147.9, and 165.7), which was verified by COSY and HMBC. COSY correlation of H-20/H-9 and HMBC correlations of H-20/C-8 and H-20/acetate carbonyl (δ_C 170.5) implied acetoxyl group at C-20. Furthermore, the HMBC correlations between H-9/C-7, C-8, C-10, C-11, C-12, C-20; H-20/C-8, C-11, C-12; H-13/C-12; and H-16/C-1, C-11, allowed to assign α,β-unsaturated-γ-acetyloxy-cyclopentenone. These data were analogous to those of cespitularin F previously isolated from *Cespitularia taeniata* with an extra-acetyl group at C-6.[8] NOESY correlations between H-1/H-9, H-17; H-9/H-20; and H-7/H-17 suggested that H-1, H-9, H-20, H-16, and H-17 were on the β-face of the molecule and H-6 was α-oriented (Fig. 2). Absence of correlation between H-7 and H-19 was in agreement with *E*-arrangement of the 7,8-double bond.

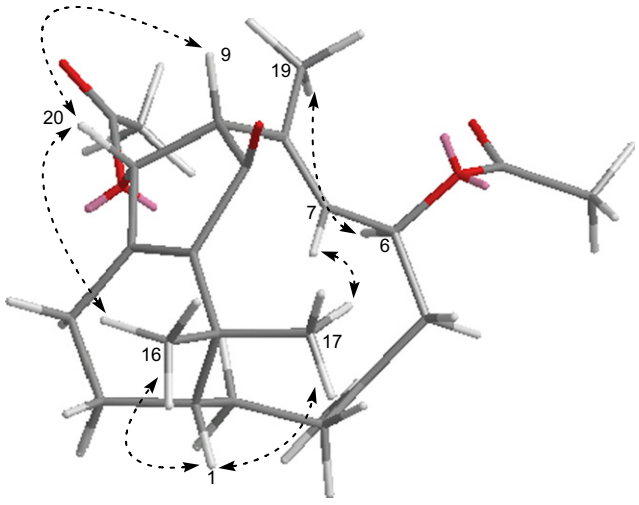


Figure 2. Key NOESY correlations of **6**.

2.7. Structure of compound 7

Cespihypotin K (**7**), [α] −5.8 (acetone), was analyzed for molecular formula $C_{20}H_{32}O_2$ from its HRESIMS. The COSY spectrum exhibited connectivities between H-5/H-6/H-7 and between H-9/H-10. In addition to exomethylene double bond (δ_C 109.6, 151.2), a tetra-substituted double bond was detected at δ_C 133.9 (C-12) and 140.2 (C-11) by HMBC (C-11/Me-16, Me-17, Me-20 and Me-20/C-11, C-12, C-13). An oxymethine carbon (δ_C 67.1, C-10) was directly bonded to H-10 and the latter correlated to C-11, C-12 and C-15 (δ_C 37.1). The signals of δ_C 65.2, δ_C 59.5, and δ_H 2.82 were diagnostic of epoxy ring that was placed at 7,8-position based on HMBC correlations between H-5/C-7; H-7/C-6; H-19/C-7, C-8, C-9; and H-10/C-8. The NOESY correlations between H-19/H-7, H-10 and H-17/H-1, H-10, H-16 were in agreement with β-orientation of H-7, H-10, and Me-19 as well as the α-form of the epoxy ring.

2.8. Structure of compound 8

Compound **8**, [α] −121.8 (CH_2Cl_2), proved to have molecular formula $C_{22}H_{32}O_4$ by HRESIMS. The ^1H and ^{13}C NMR spectroscopic data (Tables 1 and 2) indicated the same sequence from C-1 to C-9 as those of **5**. The ^{13}C NMR disclosed two carbonyls at δ_C 207.4 (C-10) and 166.9 (C-20). The H-9 (δ_H 3.50 and 3.13) had HMBC correlations to C-7 (δ_H 5.54) and C-10. Each of Me-16, Me-17, and H_2-13 exhibited correlation to C-11. An ethyl moiety was detected at δ_C 61.2 and δ_C 14.1. It was concluded that an ethoxy group was attached to C-20 that was confirmed through HMBC correlation of H-21 (δ_H 4.17) and C-20. The NOESY correlations between H_α-5/H-6, H-18; H-19/H-6, H_α-9; H_β-9/H-7, H-16, H-17; and H-16/H-1, H-17 suggested that H-6, H-18, and Me-19 were α-face of the molecule, while H-1, H-7, Me-16, and Me-17 had the β-orientation. The absolute configuration at C-6 was further confirmed by modified Mosher's method.[13] The difference values for right-sided protons H-7, H-9, and H-19 were (+0.16), (+0.06), and (+0.01), respectively, while values for left-sided protons were H-5 (−0.11) and H-18 (−0.05). The results demonstrated the (*S*)-configuration at C-6.

Plausible biogenetic pathways of new compounds were proposed as shown in Scheme 1 based on recently published diterpenoids.[8,11,12] Cespitularin C, derived from GGDP via 1*S*-verticillene might be the precursor of all the isolated diterpenes. Compounds **2**–**5** may be transformed from cespitularin D. Compound **6** might be transformed from intermediate **a**, an important analogue of **8**. The occurrence of the latter is of significance from a biogenetic point of view.

The isolated diterpenes **1**–**8** were tested in vitro against HSV-1 virus. As indicated in Table 3, they exhibited weak activity as compared with acyclovia. A preliminary study on resting cells and cells activated with PHA was tested with compounds **1**–**8** at 100 μM. The inhibition or enhancement of cell proliferation was determined by tritiated thymidine uptake. As indicated in Table 4, compound **7** showed significant enhancement of cell proliferation, while compound **8** exhibited inhibition on peripheral blood mononuclear cells (PBMC) proliferation induced by phytohemaglutinin (PHA).

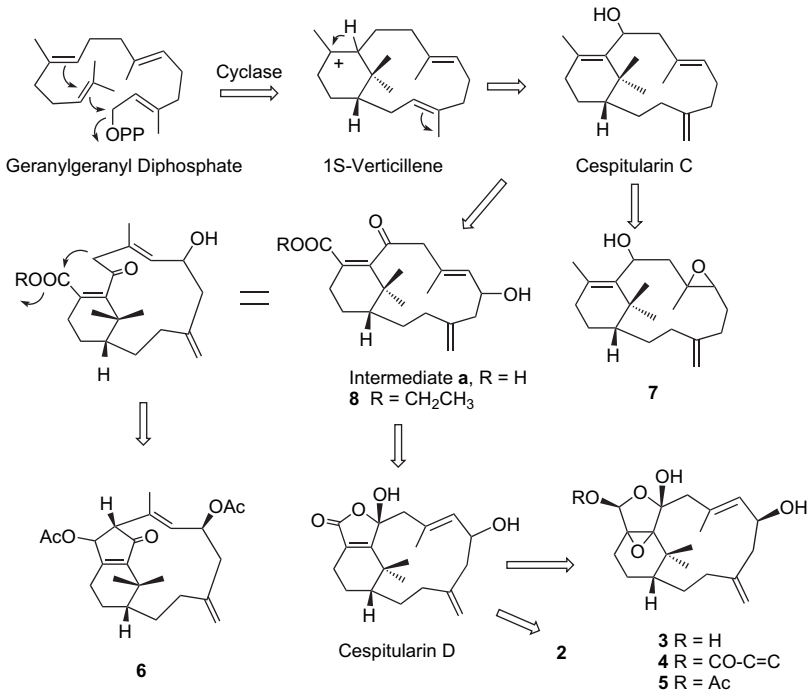
3. Experimental

3.1. General

Optical rotations were recorded on a JASCO DIP-1000 polarimeter. IR spectra were measured on Hitachi U-3210 spectrophotometers. The ^1H, ^{13}C NMR, COSY, HMQC, HMBC, and NOESY spectra were recorded on a Bruker FT-300 spectrometer using TMS as internal standard. The chemical shifts are given in δ (ppm) and coupling constants in hertz. Low resolution EIMS and high resolution ESI-MS were operated on JEOL JMS-HX 110 mass spectrometer. Silica gel 60 (Merck) was used for column chromatography (CC), and pre-coated silica gel plates (Merck, Kieselgel 60 F_{254}, 1 mm) were used for preparative TLC. Sephadex LH-20 (Amersham Pharmacia Biotech AB, Uppsala, Sweden) was used for separation.

3.2. Animal material

The soft coral *C. hypotentaculata* Roxas (Xeniidae) was collected at Green island, off the eastern coast of Taiwan,



Scheme 1. Plausible biogenetic pathways of **2**–**8**.

Table 3. Inhibition of HSV-1 replication by compounds **1**–**8**[a]

Compound (100 μM)	Inhibitory activity (%)
1	18.5
2	5.2
3	19.4
4	6.6
5	13.7
6	21.8
7	10.0
8	22.3

[a] HSV-1: Herpes simplex virus type 1.

Table 4. Effects of compounds **1**–**8** on PBMC proliferation induced by PHA

Compound (100 μg/ml)	Activity (%)	
	Resting[a]	PHA (5 μg/ml)
1	−61.9±4.5	89.4±2.4
2	7.0±1.0	−8.6±0.3
3	37.8±8.9	26.7±0.2
4	−37.8±2.6	−2.6±4.0
5	4.3±4.6	−27.8±6.5
6	31.6±8.9	2.9±0.1
7	45.7±8.5	162.6±63.7
8	11.5±1.1	−84.2±1.5
IL-2 (10 U/ml)	95.3±10.1	208±25.7
Cyclosporine A (2.5 μg/ml)	−15.9±4.4	−92.2±6.8

[a] Negative represents inhibitory activity; positive represents enhancement of proliferation.

in December 2004, by scuba diving at a depth of 15 m. The fresh coral was immediately frozen after collection and kept at −20 °C until processed. A voucher specimen (NTUO-5) was deposited in School of Pharmacy, College of Medicine, National Taiwan University, Taiwan.

3.3. Extraction and isolation

The soft coral (wet, 8 kg) was extracted with CH_2Cl_2/MeOH (1:1, 3×10 L) at rt and the extract was concentrated under vacuum. The crude extract (20 g) was partitioned between EtOAc and H_2O (1:1). The EtOAc-soluble portion was subjected to flash column (silica gel, *n*-hexane/EtOAc 100:0→0:100). The fraction eluted with *n*-hexane/EtOAc (4:1) was separated on Sephadex LH-20 using CH_2Cl_2/MeOH (1:1) to furnish five fractions (S_1–S_5). This was followed by fractionation of S_3 by silica gel column (70–230 mesh) eluting gradiently with *n*-hexane/EtOAc (15:1→0:1) (F_1–F_{16}). Fraction F_8 eluted with *n*-hexane/EtOAc (8:1) was chromatographed on silica gel column (230–400 mesh) using a gradient of *n*-hexane/CH_2Cl_2/MeOH. Fraction eluted with the previous solvent (ratio, 20:20:1) was further subjected to separation on NP-HPLC using *n*-hexane/acetone (5:1) to yield **2** (6 mg), and **7** (4 mg), while fraction eluted with ratio (18:18:1) was separated on NP-HPLC using *n*-hexane/acetone (9:2) to yield **4** (6 mg) and **1** (14 mg). Fraction F_9 eluted with *n*-hexane/EtOAc (7:1) was chromatographed on silica gel column using a gradient of *n*-hexane/acetone (4:1) followed by NP-HPLC using *n*-hexane/CH_2Cl_2/MeOH (12:12:1) to give **5** (9 mg). Fraction F_{10} eluted with *n*-hexane/EtOAc (6:1) was chromatographed on RP-HPLC using MeOH/H_2O/MeCN (70:25:5) to produce **3** (7 mg), **6** (5 mg), and **8** (16 mg).

3.3.1. Cespihypotin E (1). $[\alpha]_D^{26}$ +52.1 (*c* 0.25, EtOAc); UV λ_{max} (log ε) 219 (3.8) nm; IR (CH_2Cl_2) ν_{max} 3448 (OH), 1737 (C=O), 1662 (double bond), 1256, 898 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) Table 1; ^{13}C NMR (75 MHz, $CDCl_3$) Table 2; HRESIMS *m/z* 343.1882 $[M+Na]^+$ (calcd for $C_{19}H_{28}O_4Na$, 343.1885).

3.3.2. Cespihypotin F (2). $[\alpha]_D^{26}$ −25.1 (*c* 0.25, acetone); UV λ_{max} (log ε) 226 (4.0) nm; IR (CH_2Cl_2) ν_{max} 3417 (OH), 2926 (C–H), 1752 (lactone), 1682 (conj. C═O), 1614 (double bond), 1267, 910, 828, 735 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) Table 1; ^{13}C NMR (75 MHz, $CDCl_3$) Table 2; HRESIMS *m/z* 353.1731 $[M+Na]^+$ (calcd for $C_{20}H_{26}O_4Na$, 353.1729).

3.3.3. Cespihypotin G (3). $[\alpha]_D^{26}$ −15.6 (*c* 0.6, acetone); UV λ_{max} (log ε) 208 (3.2) nm; IR (CH_2Cl_2) ν_{max} 3422 (OH), 2931, 1645 (double bond), 998 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75 MHz, $CDCl_3$) Tables 1 and 2; HRESIMS *m/z* 373.1991 $[M+Na]^+$ (calcd for $C_{20}H_{30}O_5Na$, 373.1998).

3.3.4. Cespihypotin H (4). $[\alpha]_D^{26}$ −2.2 (*c* 0.25, acetone); UV λ_{max} (log ε) 215 (3.7) nm; IR (CH_2Cl_2) ν_{max} 3447 (OH), 2925, 1711 (conj. ester), 1636 (double bond), 1266, 983, 886 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75 MHz, $CDCl_3$) Tables 1 and 2; HRESIMS *m/z* 427.2096 $[M+Na]^+$ (calcd for $C_{23}H_{32}O_6Na$, 427.2093).

3.3.5. Cespihypotin I (5). $[\alpha]_D^{26}$ −15.6 (*c* 0.8, acetone); UV λ_{max} (log ε) 206 (3.2) nm; IR (CH_2Cl_2) ν_{max} 3421 (OH), 2929, 1747 (ester C═O), 1638 (double bond), 1219, 948, 892, 855 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75 MHz, $CDCl_3$) Tables 1 and 2; HRESIMS *m/z* 415.2098 $[M+Na]^+$ (calcd for $C_{22}H_{32}O_6Na$, 415.2096).

3.3.6. Cespihypotin J (6). $[\alpha]_D^{26}$ +57.6 (*c* 0.25, CH_2Cl_2); UV λ_{max} (log ε) 232 (4.1) nm; IR (CH_2Cl_2) ν_{max} 2928, 1736 (ester), 1695 (conj. C═O), 1641 (double bond), 1232 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75 MHz, $CDCl_3$) Tables 1 and 2; HRESIMS *m/z* 423.2147 $[M+Na]^+$ (calcd for $C_{24}H_{32}O_5Na$, 423.2150).

3.3.7. Cespihypotin K (7). $[\alpha]_D^{26}$ −5.8 (*c* 0.25, acetone); UV λ_{max} (log ε) 207 (4.5) nm; IR (CH_2Cl_2) ν_{max} 3420 (OH), 2928, 1645 (double bond), 1265 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75 MHz, $CDCl_3$) Tables 1 and 2; HRESIMS *m/z* 327.2300 $[M+Na]^+$ (calcd for $C_{20}H_{32}O_2Na$, 327.2303).

3.3.8. Cespihypotin L (8). $[\alpha]_D^{26}$ −121.8 (*c* 0.25, CH_2Cl_2); UV λ_{max} (log ε) 226 (4.2) nm; IR (CH_2Cl_2) ν_{max} 3435 (OH), 2932 (C–H), 1715 (ester), 1684 (conj. C═O), 1633 (double bond), 1246 (C–O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75 MHz, $CDCl_3$) Tables 1 and 2; HRESIMS *m/z* 383.2198 $[M+Na]^+$ (calcd for $C_{22}H_{32}O_4Na$, 383.2196). Preparation of (*R*)- and (*S*)-MTPA esters of (**8**). *S*-(+)- or *R*-(−)-MTPA chloride (1.5 mg) was added to a solution of **8** (3 mg in 0.5 ml pyridine) and the solution was allowed to stand at room temperature for 7 h. After purification using preparative TLC, each ester (1.8 mg, 90% yield) was submitted to ^1H NMR analysis and $\Delta\delta = \delta_S - \delta_R$ was calculated.

3.4. Cell culture and viruses

Vero cells were cultured in minimal essential medium (MEM; GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin and incubated at 37 °C in a 5% CO_2 incubator. To prepare HSV-1 (KOS strain, VR-1493, ATCC) stocks, Vero cells were infected by HSV-1 at a multiplicity of infection of three plaque forming units (PFU)/cell and harvested at 24 h post-infection and centrifuged at 1500×*g* (Centrifuge 5810 R, Eppendrof) at 4 °C for 20 min. The supernatant was collected and stored at −70 °C for use.

3.5. Plaque reduction assay

The assay followed procedures described previously.[14] Acyclovir was used as a positive control. Vero cells (3.5×10^5/dish) were incubated with 100 PFU of HSV-1 and various compounds (10 μM) or acyclovir (2.5 μM) were added to the cells. The viruses were adsorbed for 1 h at 37 °C and 1% methylcellulose was added to each well. After 5 days, the virus plaques formed in HeLa cells were counted by crystal violet staining. The activities of various compounds and acyclovir for inhibition of plaque formation were calculated.

3.6. Lymphoproliferation test

The lymphoproliferation test was modified from previously described.[15,16] The density of PBMC was adjusted to 2×10^6 cells/ml before use. Cell suspension of 100 μl was applied into each well of a 96-well flat-bottomed plate (Nunc 167008, Nunclon, Raskilde, Denmark) with or without PHA (Sigma). Various compounds were added to the cells at 100 μM. The plates were incubated in 5% CO_2–air humidified atmosphere at 37 °C for 3 days. Subsequently, tritiated thymidine (1 μCi/well, NEN) was added into each well. After a 16 h incubation, the cells were harvested on glass fiber filters by an automatic harvester (Dynatech, Multimash 2000, Billingshurst, U.K.). Radioactivity in the filters was measured by a scintillation counting. IL-2 (interleukin 2) and cyclosporine A were used as positive and negative standard compounds, respectively.

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