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Pachyclavulariolides G–L and secopachyclavulariaenone A, seven novel diterpenoids from the soft coral *Pachyclavularia violacea*

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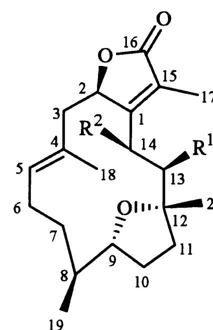
Abstract—Seven novel diterpenoids pachyclavulariolides G–L (**1–6**), secopachyclavulariaenone A (**7**) along with two known compounds, pachyclavulariolide (**8**) and pachyclavulariolide E (**9**) have been isolated from the soft coral *Pachyclavularia violacea*. The structures of **1–6** were established by spectroscopic and chemical methods along with extensive single crystal X-ray analyses. The structure of **8** was further confirmed and the relative configuration of **9** was fully established also by X-ray structure analyses. Cytotoxicity of these compounds toward various cancer cell lines also is described. © 2001 Elsevier Science Ltd. All rights reserved.

1. Introduction

Marine octocorals are important sources of structurally novel and bioactive diterpenoids.[1] We have previously isolated several cytotoxic briarane diterpenoids, named excavatolides and briaexcavatolides from a gorgonian *Briareum excavatum*.[2,3] In our continuing survey of Formosan marine invertebrates with promising cytotoxicity against the growth of a variety of cancer cell lines, we encountered the octocoral *Pachyclavularia violacea*, which was collected along the coast of Kenting, located in the southernmost tip of Taiwan, in September 1995, and was found to be very similar in colonial morphology with those of *Briarem* spp.[4,5] The investigation on the chemical constituents of *P. violacea* was then carried out in our laboratory due to the significant in vitro cytotoxicity of its organic extract toward P-388 tumor cells (ED_{50}=0.3 μg/mL). Our previous study has led to the isolation of three novel briarellin-type diterpenoids.[6] Our continuing investigation on the secondary metabolites of *P. violacea* has further resulted in the isolation of seven new diterpenoids, pachyclavulariolides G–L (**1–6**) and secopachyclavulariaenone A (**7**), along with two known compounds, pachyclavulariolide (**8**)[7] and pachyclavulariolide E (**9**).[8] The relative stereochemistry of **9** has not been fully established in the previous study. Our present work also unambiguously confirmed the relative configuration of pachyclavulariolide E (**9**) by a single-crystal X-ray analysis.



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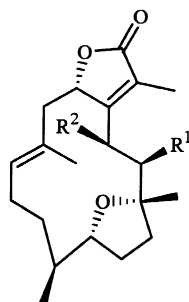

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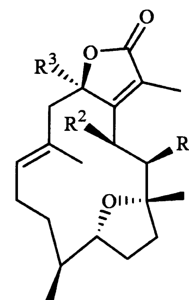

4 R[1] = OAc R[2] = OCOPr R[3] = OH
6 R[1] = R[2] = R[3] = OH
9 R[1] = R[2] = OAC R[3] = OH
10 R[1] = R[2] = R[3] = OAc

Keywords: *Pachyclavularia violacea*; diterpenoids; pachyclavulariolides; secopachyclavulariaenone; soft coral; cytotoxicity.

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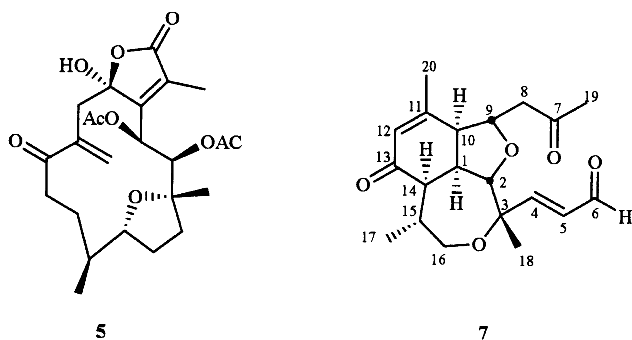
Table 1. ^{1}H and ^{13}C NMR chemical shifts, ^{1}H–^{1}H COSY, HMBC, and NOESY correlations for **1**

C/H	^{1}H[a]	^{13}C[b]	^{1}H–^{1}H COSY	HMBC	NOESY
1		159.9 s[d]		H-2, H_2-3, H-14, H_3-17	
2	4.73 d (10.0)[c]	78.3 d	H_2-3	H_2-3, H-14	H-3′
3	2.78 dd (13.6, 10.0)	43.1 t	H-2, H-3′	H_3-18	H-5
3′	2.64 d (13.6)		H-3′		H-2
4		130.6 s		H_2-3, H_3-18	
5	5.80 dd (10.7, 5.6)	131.5 d	H_2-6	H_2-3, H_3-18	H-3
6	2.20 m	25.0 t	H-5, H-6′, H_2-7		
6′	2.10 m		H-5, H-6, H_2-7		
7	1.84 m	32.1 t		H_3-19	
7′	1.15 m				
8	1.08 m	39.9 d	H-9, H_3-19	H_3-19	
9	3.71 ddd (9.6, 9.6, 6.9)	85.1 d	H-8, H_2-10	H_3-19	H_3-19
10	2.10 m	31.0 t	H-9, H_2-11		
10′	1.60 m		H-9, H_2-11		
11	1.88 m	39.0 t	H_2-10	H-13, H_3-20	
11′	1.63 m		H_2-10		
12		83.2 s		H-13, H_3-20	
13	4.75 s	73.1 d	H-14	H_3-20	H-14, H_3-20
14	6.14 s	70.0 d	H-13	H-13	H-13
15		125.6 s		H-2, H_3-17	
16		173.4 s		H_3-17	
17	1.92 s	9.8 q			
18	1.76 s	16.1 q		H_2-3	
19	0.86 d (6.3)	15.9 q	H-8		H-9
20	1.44 s	23.4 q		H-13	
Acetate					
CH_3	2.04 s	20.6 q			
CO		170.0 s		H-13	
Acetate					
CH_3	2.18 s	20.6 q			
CO		169.2 s		H-14	

[a] Spectra recorded at 300 MHz in $CDCl_3$.
[b] Spectra recorded at 75 MHz in $CDCl_3$.
[c] *J* values (in Hz) in parentheses.
[d] Multiplicity deduced by DEPT and indicated by usual symbols. The values are downfield in ppm from TMS.



2. Results and discussions

Pachyclavulariolide G (**1**) was obtained as a white solid and could be recrystallized from ethyl acetate to form colorless prisms. Its HREIMS exhibited a molecular ion at *m*/*z* 434.2307, establishing a molecular formula for this compound of $C_{24}H_{34}O_7$. Thus, eight degrees of unsaturation were determined for the molecule of **1**. The EIMS of **1** showed peaks at *m*/*z* 434 $[M]^+$, 374 $[M-HOAc]^+$, and 314 $[M-2\ HOAc]^+$, suggesting the presence of two acetoxyl groups in the molecule of **1**. Subtracting the acetate carbons from the molecular formula of **1** revealed that metabolite **1** had a diterpenoid skeleton. From the ^{13}C NMR spectral data of **1** (Table 1), a suite of resonances at δ 173.4 (C-16, s), 159.9 (C-1, s), 125.6 (C-15, s), 78.3 (C-2, d), and 9.8 ppm (C-17, q), could be assigned to the α-methyl-γ-butenolide substructure by comparison with the ^{13}C NMR data of the known metabolite pachyclavulariolide,[7] and could be further confirmed by an UV absorption at 218 nm. Additional unsaturated functionalities were indicated by ^{13}C NMR resonances at δ 170.0 (s), 169.2 (s), 131.5 (C-5, d), and 130.6 (C-4, s), suggesting the presence of two esters and a trisubstituted olefin, respectively. The absence of ^{13}C NMR signals for other unsaturated functional groups thus required that **1** had to be tricyclic. The ^{1}H NMR spectrum of **1** showed the presence of six methyl groups: the first a doublet (δ 0.86, couple to a methine proton at δ 1.08), and then a singlet at δ 1.44 representing a methyl group on a quaternary carbon atom bearing an oxygen, a methyl group on a carbon–carbon double bond (δ 1.76, brs, coupled to the olefinic proton at δ 5.80), a methyl group on the butenolide moiety (δ 1.92, s), and two acetyl methyls (δ 2.04, s; 2.18, s). The structure and all of the ^{1}H and ^{13}C chemical shifts of **1** were further determined by the assistance of 2D NMR studies (^{1}H–^{1}H COSY, HMQC, HMBC, and NOESY). From the ^{1}H–^{1}H COSY spectrum of **1**, it is possible to establish the proton sequences from H-2 to H_2-3; H-5 (an olefinic proton) to H_2-6; H-8 to H_2-11; H-13 to H-14; and H-8 to H_3-19. A vinyl methyl group attached at C-4 position was confirmed by the HMBC correlations between H_3-18 to C-3, C-4, and C-5; H_2-3 to C-1, C-2, C-4, C-5 and C-18. Furthermore, the HMBC correlations observed between the ester carbonyl resonance at δ 170.0 (s) and a methine

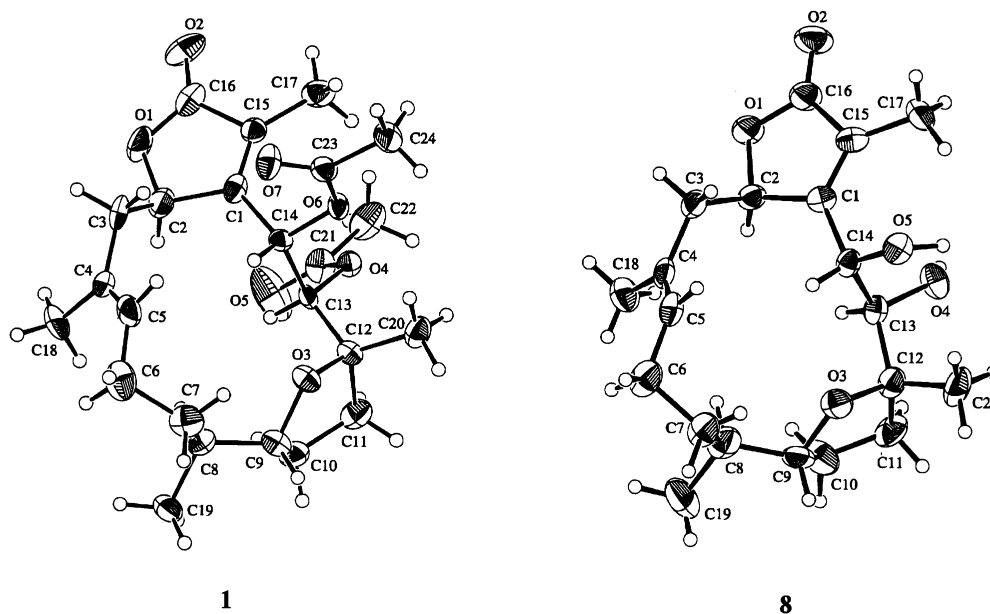


Figure 1. Computer-generated ORTEP plots of **1** and **8** showing the relative configurations.

Table 2. ^{1}H and ^{13}C NMR chemical shifts for diterpenoids **2–4**

C/H	**2**		**3**		**4**	
	^{1}H^{a}	^{13}C^{b}	^{1}H^{a}	^{13}C^{b}	^{1}H^{a}	^{13}C^{b}
1		158.8 s[d]		161.0 s		157.0 s
2	5.41 d (13.2)[c]	79.2 d	5.23 dd (7.2, 3.9)	80.9 d		106.8 s
2-OH					4.99 s	
3	2.84 d (16.5)	40.5 t	2.82 dd (14.1, 3.9)	43.3 t	2.90 s	45.9 t
3′	2.24 dd (16.5, 13.2)		2.46 dd (14.1, 7.2)		2.90 s	
4		130.6 s		130.8 s		131.8 s
5	5.59 t (7.2)	126.1 d	5.35 t (6.9)	129.9 d	5.91 dd (10.6, 5.2)	131.8 d
6	2.17 s	25.1 t	2.11 m	25.0 t	2.17 m	25.0 t
6′	2.10 m		1.92 m		2.02 m	
7	1.84 m	32.9 t	1.66 m	34.4 t	1.85 m	32.1 t
7′	1.15 m		1.22 m		1.13 m	
8	1.18 m	35.9 d	1.00 m	39.0 d	1.29 m	39.4 d
9	3.70 dd (9.5, 7.2)	85.2 d	3.71 ddd (9.9, 9.9, 6.4)	84.7 d	3.71 ddd (9.6, 9.6, 6.2)	85.3 d
10	1.90 m	26.8 t	2.00 m	29.0 t	2.17 m	31.4 t
10′			1.66 m		1.59 m	
11	1.69 m	37.8 t	1.66 m	36.6 t	1.81 m	39.7 t
11′			1.22 m		1.67 m	
12		83.2 s		85.7 s		82.9 s
13	4.95 d (1.8)	76.8 d	3.87 brs	72.6 d	4.91 s	75.2 d
14	5.85 d (1.8)	70.6 d	4.95 brs	66.3 d	6.28 s	70.4 d
15		128.4 s		123.0 s		127.8 s
16		173.6 s		175.0 s		170.4 s
17	1.97 s	10.7 q	1.99 s	9.4 q	1.88 s	9.7 q
18	1.76 s	17.1 q	1.59 s	17.1 q	1.84 s	19.5 q
19	0.83 d (6.3)	16.9 q	0.82 d (6.6)	16.0 q	0.85 d (6.4)	16.0 q
20	1.34 s	23.7 q	1.30 s	22.0 q	1.48 s	23.5 q
Acetate						
CH_3	2.11 s	20.5 q			2.10 s	21.0 q
CO		169.8 s				173.1 s
Acetate						
CH_3	2.07 s	20.9 q				
CO		169.4 s				
n-Butyrate						
CH_3					0.98 t (7.2)	13.8 q
CH_2					1.70 m	18.1 t
CH_2					2.41 t (7.2), 2.42 t (7.2)	35.7 t
CO						171.7 s

[a] Spectra recorded at 300 MHz in $CDCl_3$.
[b] Spectra recorded at 75 MHz in $CDCl_3$.
[c] *J* values (in Hz) in parentheses.
[d] Multiplicity deduced by DEPT and indicated by usual symbols. The values are downfield in ppm from TMS.

resonance at δ 4.75 (H-13) and a methyl resonance at δ 2.04 (acetoxyl CH_3), and between the second ester carbonyl resonance at δ 169.2 and a methine resonance at δ 6.14 (H-14) and a methyl resonance at δ 2.18 (acetoxyl, CH_3), methine resonance at δ 6.14 (H-14) and a methyl resonance at δ 2.18 (acetoxyl, CH_3), revealed the presence of two acetates attached to C-13 and C-14, respectively. Based on the consideration of molecular formula, one more oxygen atom had to be placed between C-9 and C-12 to form a tetrahydrofuran ring. These data, together with the HMBC correlations from H-2 to C-1 and C-15; H_3-17 to C-1, C-15 and C-16; H_3-19 to C-7, C-8, and C-9; H-13 to C-11, C-12, C-14, and C-20; H-14 to C-1 and C-2, unambiguously established the molecular framework of **1**. The geometry of the C-4/C-5 double bond in **1** was determined by a NOESY experiment. H_3-18 exhibited NOE responses with H_2-6, but not with H-5, demonstrating the *trans* configuration of $\Delta^{4,5}$ olefin. However, due to the conformational mobility of the fourteen membered ring, the relative stereochemistries at the chiral centers of **1** could not be fully determined by the NOESY experiment. Fortunately, acetylation of a known diterpenoid pachyclavulariolide (**8**), which was obtained as a white solid[9] and could be crystallized from slow evaporation of the ethyl acetate solution and the structure was unambiguously confirmed by single crystal X-ray structure analysis (Fig. 1) for the first time, gave the diacetate **1**, and established the structure of **1**. Also, it was found that slow evaporation of the ethyl acetate solution of **1** could afford colourless crystals. Thus, the relative stereochemistry of **1** could be further confirmed by a single crystal X-ray structure analysis. The X-ray structure demonstrates the relative configurations of the chiral centers at C-2 (R^*), C-8 (S^*), C-9 (R^*), C-12 (S^*), C-13 (R^*), and C-14 (S^*) in pachyclavulariolide G (**1**).

Pachyclavulariolide H (**2**) was found to be slightly more polar than **1** and was isolated as a white solid. The molecular formula of $C_{24}H_{34}O_7$ was established by HRFABMS. Thus, **2** is an isomer of **1**. The FABMS of **2** also showed peaks at *m/z* 435 $[M+H]^+$, 375 $[M-HOAc+H]^+$, and 315 $[M-2HOAc+H]^+$, implying metabolite **2** also contained two acetoxyl groups. ^1H and ^{13}C NMR spectral data (Table 2) of **2** were assigned by the assistance of 2D NMR experiments ($^1H-^1H$ COSY, HMQC, and HMBC) and were found to be closed to those of **1**, suggesting that the structure of **2** should be very similar to that of **1**. The most obvious difference is the chemical shift of H-2, which showed signal at δ 4.73 in **1**, was downfield shifted to δ 5.41, revealing that **2** could be the C-2 epimer of **1**. However, even though H-2 showed NOE differences with H_3-18, H_2-3, H-5, H-13, and H-14, the high conformational mobility of the fourteen membered carbocyclic ring made it difficult for us to determine the relative configurations at six chiral centers of pachyclavulariolide H (**2**), although we knew at this stage that **2** is highly possible to be the C-2 epimer of **1**. In order to establish the relative stereochemistry of **2** by a X-ray diffraction analysis, we attempted the recrystallization of **2**. Fortunately, the single crystals of **2** could be obtained from the slow evaporation of the ethyl acetate solution of **2**. A single crystal X-ray diffraction analysis was then carried out and the ORTEP drawing of the structure of **2** is shown in Fig. 2, which unambiguously confirmed the relative configurations of the all chiral centers at C-2 (S^*), C-8 (S^*), C-9 (R^*), C-12 (S^*), C-13 (R^*), and C-14 (S^*) in metabolite **2**.

Pachyclavulariolide I (**3**) was obtained as a white solid. Its HRFABMS gave a $[M+H]^+$ peak at *m/z* 351.2172 and established the molecular formula $C_{20}H_{30}O_5$. ^1H and ^{13}C spectral data (Table 2) of **3** were assigned by the assistance of 2D NMR experiments ($^1H-^1H$ COSY, HMQC, and HMBC) and were found to be very similar to those of pachyclavulariolide (**8**),[7] suggesting that **3** could be the diastereomer of **8**. Acetylation of **3** gave a less polar product

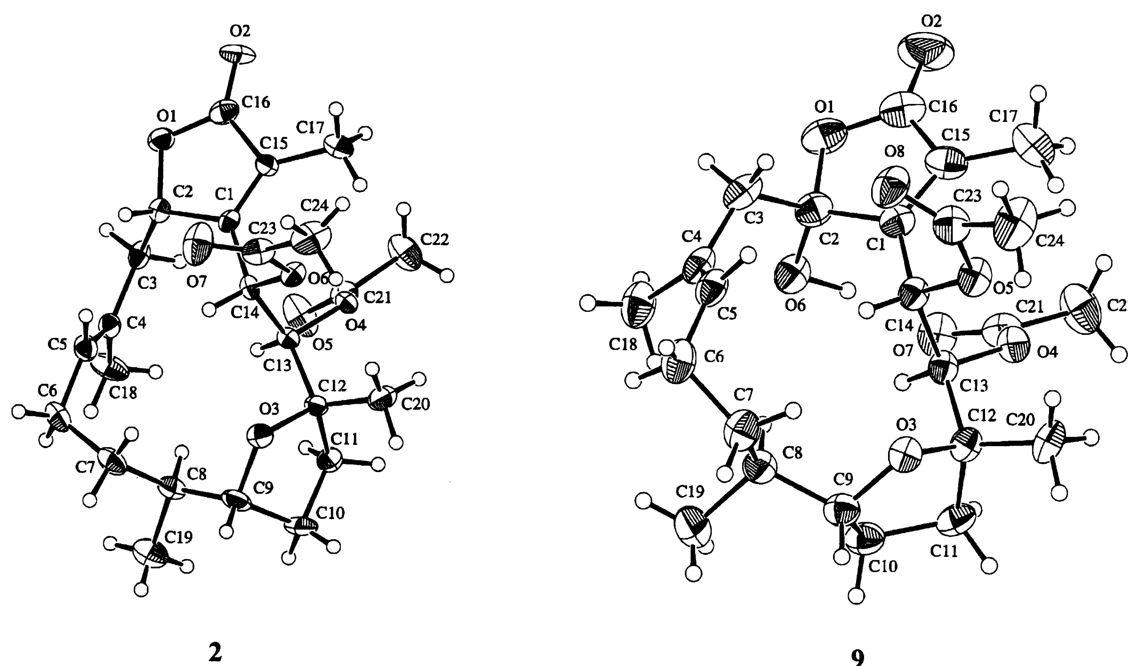


Figure 2. Computer-generated ORTEP plots of **2** and **9** showing the relative configurations.

Table 3. ^{1}H and ^{13}C NMR chemical shifts for **5**, **6** and **9**

C/H	5		6		9	
	^{1}H[a]	^{13}C[b]	^{1}H[c]	^{13}C[d]	^{1}H[a]	^{13}C[b]
1		154.9 s[f]		164.5 s		156.6 s
2		105.7 s		108.6 s		106.6 s
2-OH	5.25 s		5.01 s		5.01 s	
3	3.76 d (14.4)[e]	40.4 t	2.85 brd (14.0)	48.8 t	2.90 d (15.6)	45.9 t
3′	2.64 d (14.4)		2.19 brd (14.0)		2.86 d (15.6)	
4		139.6 s		132.2 s		131.6 s
5		207.2 s	5.56 brs	130.7 d	5.89 dd (10.8, 5.0)	131.6 d
6	3.31 dd (9.3, 3.9)	36.6 t	2.08 m	25.1 t	2.15 m	25.0 t
6′	2.50 d (9.3)				2.00 m	
7	1.93 m	31.1 t	2.01 m	34.0 t	1.85 m	32.1 t
7′	1.61 m		1.01 m		1.16 m	
8	1.20 m	39.5 d	1.51 m	39.9 d	1.26 m	39.3 d
9	3.66 ddd (9.6, 9.6, 6.3)	85.1 d	3.51 ddd (9.2, 9.2, 5.9)	86.3 d	3.73 ddd (9.6, 9.6, 6.0)	85.2 d
10	2.50 m	31.6 t	2.06 m	32.5 t	2.13 m	31.4 t
10′	1.40 m		1.45 m		1.63 m	
11	1.87 m	38.0 t	2.08 m	40.3 t	1.80 m	39.6 t
11′	1.53 m				1.67 m	
12		83.4 s		86.1 s		82.8 s
13	5.15 s	72.8 d	3.43 s	76.8 d	4.91 s	75.0 d
14	5.81 s	69.2 d	5.01 s	69.8 d	6.30 s	70.6 d
15		128.2 s		129.3 s		127.8 s
16		170.3 s		174.6 s		170.1 s
17	1.90 s	9.5 q	1.96 s	10.4 q	1.89 s	9.6 q
18	6.66 s 6.46 s	135.9 t	1.70 s	19.4 q	1.84 s	19.4 q
19	0.83 d (6.6)	14.8 q	0.73 d (6.3)	15.9 q	0.85 d (6.3)	15.9 q
20	1.30 s	23.1 q	1.36 s	23.3 q	1.49 s	23.4 q
Acetate						
CH_3	2.08 s	20.6 q			2.11 s	20.6 q
CO		169.7 s				168.8 s
Acetate						
CH_3	2.16 s	20.9 q			2.18 s	21.0 q
CO		169.2 s				172.9 s

[a] Spectra recorded at 300 MHz in $CDCl_3$.
[b] Spectra recorded at 75 MHz in $CDCl_3$.
[c] Spectra recorded at 400 MHz in MeOH-d_4.
[d] Spectra recorded at 100 MHz in MeOH-d_4.
[e] J values (in Hz) in parentheses.
[f] Multiplicity deduced by DEPT and indicated by usual symbols. The values are downfield in ppm from TMS.

which was found to be identical with pachyclavulariolide H (**2**) by comparison of the physical and spectral data, and unambiguously confirmed the molecular structure, including the relative configuration of diterpenoid **3**. Thus, **3** is the C-2 diastereomer of pachyclavulariolide **8**.[7]

Pachyclavulariolide J (**4**) was isolated as an amorphous solid that gave a $[M]^+$ ion at m/z 478.2569 in HREIMS, appropriate for a molecular formula of $C_{26}H_{38}O_8$ requiring eight degrees of unsaturation. The gross structure of **4** established by a series of 2D NMR experiments (^{1}H–^{1}H COSY, HMQC, and HMBC) was found to be very similar to those of **1**–**3**. The ^{13}C NMR spectrum of **4** displayed a set of signals at δ 170.4 (C-16, s), 157.0 (C-1, s), 127.8 (C-15, s), 106.8 (C-2, s), and 9.7 (C-17, q) which were determined to be the signals of an α-methyl-γ-hydroxybutenolide functionality by comparison with the carbon shifts of pachyclavulariolide E (**9**). In the ^{13}C NMR spectrum of **4**, two additional carbonyl resonances were appeared at δ 173.1 (s) and 171.7 (s), and further confirmed the presence of two other ester groups in **4**. In the ^{1}H NMR spectrum, one acetyl methyl was observed (δ 2.10, 3H, s). The other acyl group was found to be an n-butyryloxyl group based on the ^{1}H NMR studies, including an ^{1}H–^{1}H COSY experiment which revealed seven contiguous protons (δ 0.98, 3H, t, J=7.2 Hz; 1.70, 2H, m; 2.41, ^{1}H, t, J=7.2 and 2.42 Hz, ^{1}H, t, J=7.2 Hz). In the HMBC spectrum of **4**, the carbon signal at δ 171.7 (s) which showed a correlation with H-14 (δ 6.28, s) was found to be correlated with the signals of the methylene protons of the n-butyryloxyl at δ 2.41 and 2.42, and was assigned as the carbon atom of the n-butyryloxyl carbonyl. Thus, the n-butyryloxyl group should be positioned at C-14. The location of the acetoxyl group at C-13 also could be confirmed by using the similar approach. Thus, metabolite **4** was found to be a structurally similar analogue of pachyclavulariolide E (**9**). However, similar to pachyclavulariolide E (**9**), the relative configuration at C-2 in **4** could not be successfully assigned by the NOESY experiment due to conformational mobility of the fourteen membered carbocyclic ring.

Pachyclavulariolide K (**5**) was obtained as a white solid that gave a molecular ion peak in the HREIMS at 464.2046, appropriate for the molecular formula of $C_{24}H_{32}O_9$. Thus, metabolite **5** was found to possess nine degrees of unsaturation. The gross structure of **5** and all of the ^{1}H and ^{13}C chemical shifts (Table 3) associated with the molecule were determined by a series of 2D NMR experiments (^{1}H–^{1}H

Table 4. ^1H and ^{13}C NMR chemical shifts, ^1H–^1H COSY, HMBC, and NOESY correlations for **7**

C/H	^1H[a]	^{13}C[b]	^1H–^1H COSY	HMBC	NOESY
1	2.77 m	38.0 d[d]	H-2, H-10, H-14		H-10, H-14, H_3-17
2	3.84 d (9.9)[c]	85.6 d	H-1	H_3-18	H-9, H_β-16, H_3-18
3		77.2 s		H-4, H_3-18	
4	6.88 d (15.6)	159.1 d	H-5	H_3-18	
5	6.51 dd (15.6, 7.8)	131.2 d	H-4, H-6		
6	9.69 d (7.8)	193.5 s	H-5		
7		205.6 s		H_3-19	
8	2.73 dd (16.6, 4.6)	50.0 t	H-9	H_3-19	
8′	2.55 dd (16.6, 7.6)		H-9		
9	4.63 t (6.5)	78.5 d	H_2-8, H-10	H-8	H-2, H_3-20
10	2.82 brs	50.8 d	H-1, H-9	H_3-20	H-1
11		156.6 s			
12	5.96 s	127.3 d		H_3-20	
13		197.2 s			
14	2.49 dd (2.8, 1.6)	48.6 d	H-1, H-15	H_3-17	H-1, H_3-17
15	2.80 m	30.3 d	H_3-17	H_3-17	
16β	3.63 d (13.5)	64.9 t		H_3-17	H-2
16α	3.58 dd (13.5, 2.4)				H_3-17
17	1.12 d (7.2)	16.9 q	H-15		H-1, H-14, H_α-16
18	1.35 s	24.5 q			H-2
19	2.18 s	30.5 q			
20	2.04 s	21.7 q			H-9

[a] Spectra recorded at 300 MHz in $CDCl_3$.
[b] Spectra recorded at 75 MHz in $CDCl_3$.
[c] *J* values (in Hz) in parentheses.
[d] Multiplicity deduced by DEPT and indicated by usual symbols. The values are downfield in ppm from TMS.

COSY, HMQC, and HMBC). The presences of two singlets in the ^1H NMR spectrum at δ 6.46 and 6.66 (1H each) and the disappearance of the olefinic methyl at C-4 suggested that the 4,5-trisubstituted double bond was converted to an α,β-unsaturated ketone functionality. This could be further confirmed by ^{13}C NMR spectrum which showed resonances at 207.2 (C-5, s), 139.6 (C-4, s), and 135.9 (C-18, t). The ^{13}C NMR spectrum of **5** obtained a set of signals at 170.3 (C-16, s), 154.9 (C-1, s), 128.2 (C-15, s), 105.7 (C-2, s), and 9.5 (C-17, q) that were also determined to be a α-methyl-γ-hydroxybutenolide in comparison with the ^{13}C NMR spectral data of pachyclavulariolide E (**9**) and **4**. Additional unsaturated functionalities were revealed by ^{13}C NMR resonances at δ 169.2 (s) and 169.7 (s), suggesting the presence of two esters in the molecule of **5**. These observations, together with the ^1H–^{13}C long-range correlations observed in an HMBC experiment, unambiguously established the framework of the molecule. It was found that compound **5** has similar structure in comparison with those of **1**–**3**, except that **5** has different functionalities at C-2, C-4, C-5, and C-18. However, similar to pachyclavulariolide E (**9**)[8] which also contained a γ-hydroxybutenolide structural unit in the molecule, the relative configuration at C-2 in metabolite **5** is still unknown at this stage.

This investigation also has led to the isolation of a white solid having the molecular formula $C_{22}H_{34}O_8$. This compound, **9**, was found to have very similar molecular structure as that of **4** by comparison of both ^1H and ^{13}C NMR spectral data (Table 3). The only difference is that the *n*-butyryloxyl group in **4** is replaced by an acetoxyl group in **9**. Thus, compound **9** has the same molecular framework as that of pachycalvulariolide E (**9**). Slow evaporation of the ethyl acetate solution of **9** gave colorless crystals which were suitable for X-ray crystallographic studies. An ORTEP drawing of the structure of **9** determined by a single crystal X-ray diffraction analysis (Fig. 2) demonstrated the relative configurations of the chiral centers at C-2 (R^*), C-8 (S^*), C-9 (R^*), C-12 (S^*), C-13 (R^*), and C-14 (S^*) in compound **9**, which was assumed to have the same molecular structure as that of pachyclavulariolide E, as the ^1H and ^{13}C NMR spectral data measured in C_6D_6 were found to be in fully agreement with the reported data of pachyclavulariolide E measured under the same condition.[8] Because pachyclavulariolides J and K (**4** and **5**), were found to have a γ-hydroxybutenolide moiety in the molecules as that of **9**, and were isolated with **9** from the same organism, it is reasonable to assume that these two metabolites also have R^* configuration at C-2. Thus, the relative stereochemistry of the structurally close related metabolites **4** and **5**, and pachyclavulariolide E (**9**), could be established as described by formula **4**, **5**, and **9**, respectively.

Pachyclavulariolide L (**6**) was found to be more polar and was isolated as a white solid. The molecular formula of $C_{20}H_{30}O_6$ was established by HRFABMS. Thus, six degrees of unsaturation were determined for the molecule of **6**. The FABMS of **6** showed peaks at *m/z* 367 $[M+H]^+$, 349 $[M+H-H_2O]^+$, 331 $[M+H-2H_2O]^+$, and 313 $[M+H-3H_2O]^+$, suggesting the presence of three hydroxyl groups in the molecule of **6**. It was found that the ^{13}C spectrum of **6** in $CDCl_3$ revealed mostly broad and/or very weak signals when measured at rt. However, the sharpened ^{13}C NMR signals of **6** (Table 3) could be obtained in MeOH-d_4 at rt. The ^{13}C NMR spectrum of **6** displayed a set of peaks at δ 174.6 (C-16, s), 164.5 (C-1, s), 129.3 (C-15, s), 108.6 (C-2, s), and 10.4 (C-17, q), which were found to be the characteristic signals of the α-methyl-γ-hydroxybutenolide moiety by comparing the related carbon shifts of pachyclavulariolides J (**4**) and K (**5**). The spectral data (^1H and ^{13}C NMR) of **6** (Table 3) were found to be similar to those of metablites **4**

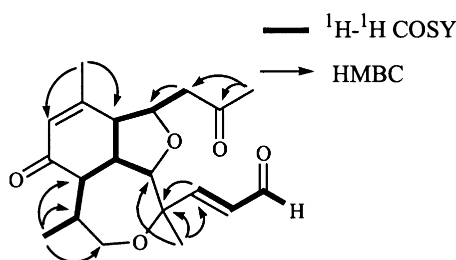


Figure 3. ^{1}H–^{1}H COSY and HMBC correlations of secopachyclavulariaenone A (**7**).

and **5**, however, the acetoxyl and/or *n*-butyryloxyl groups in **4** and **5** were disappeared. Also, the ^{1}H and ^{13}C NMR spectra revealed that the signals corresponding to the two hydroxy-bearing C-13 and C-14 methine groups in **6** (δ_H 3.43, s; δ_C 76.8, d; and δ_H 5.01, s; δ_C 69.8, d) were shifted to upfield in comparison with those of the pachyclavulariolides **1–5**, indicating that diterpenoid **6** is a 2,13,14-trihydroxyl cembranoid. Acetylation of diterpenoid **6** gave a triacetyl cembranoid which was found to be identical with compound **10**, which also was the acetyl derivative of pachyclavulariolide E (**9**), by comparison of physical and spectral data. Thus, the structure of pachyclavulariolide L (**6**), including the relative stereochemistry, was determined unambiguously as described by formula **6**.

Our present study also led to the isolation of a new compound **7**. The molecular formula of $C_{20}H_{26}O_5$ was deduced from HREIMS. Thus, eight degrees of unsaturation was determined for **7**. Inspection of the NMR spectral data (Table 4) for **7** by the assistance of DEPT spectrum revealed the presence of four methyls, two methylenes, six sp^3-hydridized methines, one quaternary sp^3-carbon, one trisubstituted double bond, one 1,2-disubstituted double bond, and three carbonyls (including two ketones and one aldehyde) carbons. Compound **7**, therefore, possessed three rings in its structure. The ^{1}H NMR spectrum also showed the presence of four methyl groups including a methyl attached to methine carbon (δ 1.12, 3H, d, J=7.2 Hz), a methyl attached to an oxygen-bearing carbon (δ 1.35, 3H, s), one olefinic methyl group (δ 2.04, 3H, s), and a methyl attached to a carbonyl carbon (δ 2.18, 3H, s). The signal at δ 5.96 (1H, s) was assigned as one proton attached to α-carbon of the enone moiety. Three protons showed signals at δ 6.51 (1H, dd, J=15.6, 7.8 Hz), 6.88 (1H, d, J=15.6 Hz), and 9.69 (1H, d, J=7.8 Hz) were assigned as the α,β olefinic, and the aldehydo proton of the α,β-unsaturated aldehyde containing a *trans*-disubstituted carbon, carbon double bond. By comparison of the ^{1}H and ^{13}C NMR spectral data of **7** with those of pachyclavulariaenones A–C,[6] it was found

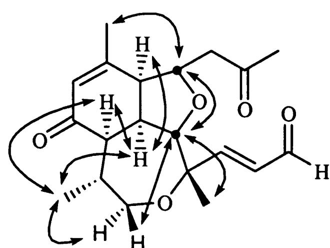
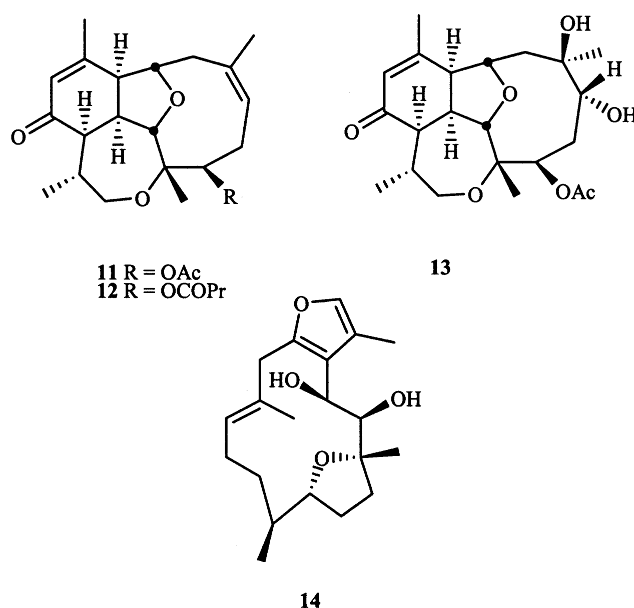


Figure 4. Selective NOE correlations of secopachyclavulariaenone A (**7**).

that resonances at δ 3.84 (1H, d, J=9.9 Hz) and 4.63 (1H, t, J=6.5 Hz) were attributed to the protons of two oxymethines in the THF structural unit. The second ether ring was further identified by the appearance of an oxymethylene group (δ 3.58, 1H, dd, J=13.5, 2.4 Hz; 3.63, 1H, d, J=13.5 Hz). The ^{13}C NMR spectrum showed signals at δ 205.6 (s), 193.5 (s), 159.1 (d), and 131.2 (d), and further supported the presence of a normal ketone and an α,β-unsaturated aldehyde. A conjugated enone moiety could also be established by resonances at δ 197.2 (s), 156.6 (s), and 127.3(d). The chemical shifts of three methine protons, located at three ring-junction carbons of the six-membered ring and two ether rings, δ 2.82 (1H, brs, H-10), 2.77 (1H, m, H-1), 2.49 (1H, dd, J=2.8, 1.6 Hz), and that of another methine proton H-15 (δ 2.80, 1H, m), were assigned based on the results of ^{1}H–^{1}H COSY and HMQC experiments. Based on the above observations and by analyses of ^{1}H–^{1}H COSY and HMBC spectral data as shown in Fig. 3, the molecular framework of **7** was established. Furthermore, the relative stereochemistry of **7** was determined by a NOESY experiment, and the results (Table 4) were illustrated in Fig. 4. Strong NOE correlations were observed for H-1 with H-10, H-14, and H_3-17, suggesting that all these protons are on the same side of the molecule and assigned as the α face. The oxymethine proton H-2 (δ 3.84) failed to reveal NOE response with H-1 (and H-10), in turn, it exhibited NOE's with $H_β$-16 (δ 3.63) and H_3-18 (δ 1.35). Thus, H-2 and H_3-18 should be positioned on the β face. H-9 showed correlations with H_3-20 and H-2, and was assigned as on the β face. Thus, the structure, including the relative stereochemistry of **7** was established unambiguously. Since **7** could be assumed to be arised from the oxidative cleavage of 6,7-bond and the following elimination of the corresponding carboxylic acid from the know diterpenoids, pachyclavulariaenone A (**11**), B (**12**), or C (**13**),[6] metabolite **7** was named as secopachyclavulariaenone A.



Besides our investigation on the chemical constituents of *P. violacea*, three previous studies have resulted in the isolation of the cembranoids pachyclavulariadiol (**14**) and its

mono- and diacetyl analogues from specimens collected from Australia,[10] pachycalvulariolide (**8**)[7] from specimens collected Vanuatu,[7] and six new diterpenoids pachyclavulariolides A–F from specimens collected from Papua New Guina.[8] The *P. violacea* specimens studied in our group were collected at a site along the coast of the southernmost tip of Taiwan. This Taiwanese specimens have been found to contain the novel briarellin-type diterpenoids pachyclavulariaenones A–C (**11–13**), as shown by our recent study,[6] and seven new diterpenoids, including six new cembranolides **1–6** and a novel 5,6-secopachyclavulariaeneone **7**, in the present study. The results of the three earlier reports and our investigations of *P. violacea* reveal that the geographic variation of this soft coral significantly affects the diterpenoids content.

The cytotoxicity of the new diterpenoids **1–7** and known compounds **8** and **9** against the growth of P-388, KB, A549 and HT-29 tumor cells were studied. Compound **5** exhibited significant cytotoxicity against P-388 and HT-29 tumor cells with ED_{50}'s of 2.8 and 3.3 μg/mL, and moderate cytotoxicity against KB and A-549 tumor cells with ED_{50}'s of 7.6 and 6.7 μg/mL, respectively. Metabolites **3**, **4**, and **9** also were found to exhibit significant cytotoxicity against P-388 cells with ED_{50}'s of 1.3, 2.5, and 4.0 μg/mL, respectively.[11]

3. Experimental

3.1. General experimental procedures

Melting points were determined using a Fisher–Johns melting points apparatus and were uncorrected. Optical rotations were measured on a Jasco DIP-1000 digital polarimeter. Ultraviolet spectra were recorded on a Hitachi U-3210 UV spectrophotometer, and IR spectra were measured on Hitachi I-2001 and Jasco FT/IR-5300 infrared spectrophotometer. The NMR spectra were recorded on a VXR-300/5 FT-NMR at 300 MHz for ^1H and 75 MHz for ^{13}C or on a Bruker AMX-400 FT-NMR at 400 MHz for ^1H and 100 MHz for ^{13}C, respectively, in $CDCl_3$ or pyridine-d_5 or MeOH-d_4 using TMS as an internal standard, unless otherwise indicated. MS spectra were obtained with a VG QUATTRO GC/MS spectrometer. HRMS spectra were recorded on a JMX-HX 110 mass spectrometer. Silica gel 60 (Merck, 230–400 mesh) was used for CC. Precoated silica gel plates (Merck, Kieselgel 60 F-254, 0.20 mm) were used for analytic TLC.

3.2. Collection, extraction, and separation

The organism *P. violacea* (3.0 kg fresh wt) was collected by hand using SCUBA on reefs at depths of −10 to −15 m, along the coast of Kenting, located in the southernmost tip of Taiwan in September 1995, and was stored in a freezer. A voucher specimen was deposited in the Department of Marine Resources, National Sun Yat-Sen University. The freeze dried organism (711 g) was minced and extracted exhaustively with EtOAc. The organic extract was evaporated to give a dark oily residue (60.0 g). The EtOAc layer was found to exhibit significant cytotoxity against P-388 cell line with ED_{50} of 0.3 μg/mL, and KB cell line with ED_{50} of 4.3 μg/mL. The organic extract was chromatographed on silica gel CC, using hexanes, EtOAc and MeOH mixture of increasing polarity to afford 47 fractions. Fraction 13 with EtOAc–hexanes (1:5), which consisted by TLC analysis of a mixtures of **1**, **2**, **4**, and **9**, was further chromatographed on silica gel using gradient elution with pure CH_2Cl_2 to EtOAC–CH_2Cl_2 (1:1) to give pure diterpenes **1**, **2**, **4**, and **9**. Fraction 16 with EtOAc–hexanes (1:2) further was purified by silica gel chromatography with EtOAc–CH_2Cl_2 (1:3) to give pure **5**. Fraction 18 with EtOAc–hexanes (1:1) was further purified by silica gel chromatography with pure CH_2Cl_2 to EtOAc–CH_2Cl_2 (1:1) to give pure **3**, **7**, and **8**. Fraction 24 with EtOAc–hexanes (5:1) was further purified by silica gel chromatography with EtOAc–CH_2Cl_2 (1:2) to pure EtOAc to MeOH–EtOAc (1:20) to give pure **6**.

3.2.1. Pachyclavulariolide G (1). White solid (173.4 mg); mp 129–130°C; $[\alpha]^{27}_D=-122°$ (*c* 0.86, $CHCl_3$); UV (95% EtOH) λ_{max} 218 nm (ϵ 8345); IR (neat) ν_{max} 1755, 1672, 1450, and 1373 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; EIMS *m/z* 434 [0.4, $(M)^+$], 374 [2, $(M-HOAc)^+$], and 314 [1, $(M-2\ HOAc)^+$]; HREIMS *m/z* 434.2307 (calcd for $C_{24}H_{34}O_7$ 434.2305).

3.2.2. Pachyclavulariolide H (2). White solid (85.4 mg); mp 215–216°C; $[\alpha]^{28}_D=-18°$ (*c* 0.96, $CHCl_3$); UV (95% EtOH) λ_{max} 216 nm (ϵ 15441); IR (neat) ν_{max} 1759, 1743, 1371, and 1224 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; FABMS *m/z* 435 [35, $(M+H)^+$], 375 [3, $(M-HOAc+H)^+$], and 315 [1, $(M-2\ HOAc+H)^+$]; HRFABMS *m/z* 435.2382 (calcd for $C_{24}H_{35}O_7$ 435.2384).

3.2.3. Pachyclavulariolide I (3). White solid (39.8 mg); mp 188–189°C; $[\alpha]^{28}_D=+88°$ (*c* 1.03, $CHCl_3$); UV (95% EtOH) λ_{max} 216 nm (ϵ 7927); IR (neat) ν_{max} 3447, 1745, 1458, 1381, and 1234 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; FABMS *m/z* 351 [2, $(M+H)^+$], 333 [1, $(M-H_2O+H)^+$], and 315 [0.7, $(M-2H_2O+H)^+$]; HRFABMS *m/z* 351.2172 (calcd for $C_{20}H_{31}O_5$ 351.2173).

3.2.4. Pachyclavulariolide J (4). Oil (7.2 mg); $[\alpha]^{25}_D=-47°$ (*c* 0.36, $CHCl_3$); UV (95% EtOH) λ_{max} 207 nm (ϵ 4757); IR (neat) ν_{max} 1735, 1457, 1247, and 1216 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; EIMS *m/z* 478 [1, $(M)^+$], 418 [0.1, $(M-HOAc)^+$], 390 [0.1, $(M-PrCOOH)^+$], and 330 [0.4, $(M-HOAc–PrCOOH)^+$]; HREIMS *m/z* 478.2569 (calcd for $C_{26}H_{38}O_8$ 478.2567).

3.2.5. Pachyclavulariolide K (5). White solid (9.3 mg); mp 175–177°C; $[\alpha]^{28}_D=-47°$ (*c* 0.41, $CHCl_3$); UV (95% EtOH) λ_{max} 217 nm (ϵ 8821); IR (neat) ν_{max} 3410, 1747, 1651, and 1371 cm^{-1}; ^1H and ^{13}C NMR data, see Table 3; EIMS *m/z* 464 [2, $(M)^+$], 446 [1, $(M-H_2O)^+$], 404 [3, $(M-HOAc)^+$], 386 [1, $(M-H_2O-HOAc)^+$], and 344 [2, $(M-2\ HOAc)^+$]; HREIMS *m/z* 464.2046 (calcd for $C_{24}H_{32}O_9$ 464.2047).

3.2.6. Pachyclavulariolide L (6). White solid (101.7 mg); mp 194–196°C; $[\alpha]^{29}_D=+53°$ (*c*, 0.22 $CHCl_3$); UV (95% EtOH) λ_{max} 220 nm (ϵ 5749); IR (neat) ν_{max} 3447, 1743, and 1086 cm^{-1}; ^1H and ^{13}C NMR data, see Table 3; FABMS *m/z* 367 [0.4, $(M+H)^+$], 349 [5, $(M-H_2O+H)^+$], and 331

[0.8, $(M-2H_2O+H)^+$]; HRFABMS *m/z* 367.2121 (calcd for $C_{20}H_{31}O_6$ 367.2121).

3.2.7. Secopachyclavulariaenone A (7). Pale oil (9.2 mg); $[\alpha]^{28}_D=-20°$ (*c* 0.02, $CHCl_3$); UV (95% EtOH) λ_{max} 208 nm (ϵ 6963); IR (neat) ν_{max} 1714, 1689, 1671, 1382, and 1205 cm^{-1}; ^1H and ^{13}C NMR data, see Table 4; EIMS *m/z* 346 [0.1, $(M)^+$], 331 [0.2, $(M-CH_3)^+$], 317 [0.2, $(M-CHO)^+$], and 289 [0.4, $(M-CH_2COCH_3)^+$]; HREIMS *m/z* 346.1780 (calcd for $C_{20}H_{26}O_5$ 346.1781).

3.2.8. Pachyclavularolide (8). White solid (78.0 mg); mp 224–226°C; $[\alpha]^{28}_D=-10°$ (*c* 3.59, $CHCl_3$); UV (95% EtOH) λ_{max} 216 nm (ϵ 7927); IR (neat) ν_{max} 3396, 1736, 1140, and 1086 cm^{-1}; FABMS *m/z* 351 [7, $(M+H)^+$], 333 [5, $(M-H_2O+H)^+$], and 315 [0.7, $(M-2H_2O+H)^+$]; HRFABMS *m/z* 351.2171 (calcd for $C_{20}H_{31}O_5$ 351.2172).

3.2.9. Pachyclavulariolide E (9). White solid (262.9 mg); mp 150–152°C; $[\alpha]^{28}_D=-42°$ (*c* 0.41, $CHCl_3$); UV (95% EtOH) λ_{max} 203 nm (ϵ 17102); IR (neat) ν_{max} 3400, 1747, 1651, and 1371 cm^{-1}; ^1H and ^{13}C NMR data, see Table 3; EIMS *m/z* 450 [16, $(M)^+$], 432 [0.3, $(M-H_2O)^+$], 390 [0.8, $(M-HOAc)^+$], 330 [1, $(M-2\ HOAc)^+$], and 312 [1, $(M-H_2O-2\ HOAc)^+$]; HREIMS *m/z* 450.2222 (calcd for $C_{24}H_{34}O_7$ 450.2253).

3.2.10. Acetylation of pachyclavulariolide L (6). A solution of pachyclavulariolide L (**6**) (10.1 mg, 0.028 mmol) in pyridine (2.0 mL) was added with acetic anhydride (1.0 mL) and the mixture was stirred at rt for 18 h. After evaporation of excess reagent, the residue was separated by column chromatography on silica gel to give pure compound **10** (EtOAc–hexanes=1:8, 7.6 mg, 0.018 mmol, 64%). Compound **10** was a white solid; mp 107–110°C; $[\alpha]^{29}_D=-10°$ (*c* 0.20, $CHCl_3$); UV (95% EtOH) λ_{max} 221 nm (ϵ 3830); IR (neat) ν_{max} 1714, 1671, 1382, and 1205 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 6.31 (1H, s, H-14), 6.02 (1H, dd, *J*=10.8, 5.3 Hz, H-5), 4.74 (1H, s, H-13), 3.67, (1H, ddd, *J*=9.9, 9.9, 6.9 Hz, H-9), 3.09 (1H, d, *J*=14.3 Hz, H-3), 2.97 (1H, d, *J*=14.3 Hz, H-3′), 2.18 (1H, m, H-11), 2.17 (3H, s, acetyl, CH_3), 2.15 (1H, m, H-6), 2.12 (1H, m, H-10), 2.03 (1H, m, H-6), 2.04 (3H, s, acetyl, CH_3), 2.01 (3H, s, acetyl, CH_3), 1.87 (1H, m, H-7), 1.80 (3H, s, H-18), 1.59 (1H, m, H-11), 1.54 (1H, m, H-10), 1.42 (3H, s, H-20), 1.16 (1H, m, H-7), 1.16 (1H, m, H-8), 1.00 (3H, s, H-17), 0.86 (3H, d, *J*=6.2 MHz, H-19), ^{13}C NMR (100 MHz, $CDCl_3$) d 170.3 (C-16, s), 170.1 (acetyl, CO, s), 169.0 (acetyl, CO, s), 168.0 (acetyl, CO, s), 153.3 (C-1, s); 134.2 (C-5, d), 131.1 (C-4, s), 129.2 (C-15, s), 106.4 (C-2, s), 84.9 (C-9, d), 83.8 (C-12, s), 73.8 (C-13, d), 70.6 (C-14, d), 46.5 (C-3, t), 39.7 (C-8, d), 39.7 (C-11, t), 32.2 (C-7, t), 31.5 (C-10, t), 25.3 (C-6, t), 23.7 (C-20, q), 22.3 (acetyl, CH_3, q), 20.9 (acetyl, CH_3, q), 20.6 (acetyl, CH_3, q), 19.0 (C-18, q), 15.9 (C-19, q), 10.0 (C-17, q), FABMS *m/z* 433 [6, $(M+H)^+$], 373 [0.5, $(M-HOAc+H)^+$], 313 [0.5, $(M-2\ HOAc+H)^+$], and 253 [0.3, $(M-3\ HOAc+H)^+$].

3.2.11. Acetylation of pachyclavulariolide E (9). According to the above procedure, pchyclavulariolide E (**9**) (10.5 mg, 0.023 mmol) was acetylated to the compound **10** (EtOAc–hexanes=1:8, 8.3 mg, 0.019 mmol, 84%); physical and spectral data were in full agreement with those of the compound **10**.

3.2.12. Acetylation of pachyclavulariolide (8). According to the above procedure, pachyclavulariolide (**8**) (12.2 mg, 0.034 mmol) was acetylated to the compound **1** (EtOAc–hexanes=1:3, 8.8 mg, 0.025 mmol, 74%); physical and spectral data were in full agreement with those of the natural product **1**.

3.2.13. Acetylation of pachyclavulariolide I (3). According to the above procedure, pachyclavulariolide I (**3**) (9.7 mg, 0.028 mmol) was acetylated to the compound **2** (EtOAc–hexanes=1:3, 6.4 mg, 0.018 mmol, 64%); physical and spectral data were in full agreement with those of the natural product **2**.

3.2.14. Single-crystal X-ray crystallography of 1.[12] Suitable colorless prisms of **1** were obtained from a solution in EtOAc. The crystal (0.20×0.60×0.50 mm) belongs to the orthorhombic system, space group $P2_12_12_1$ (# 19) with *a*=9.386(2), *b*=14.587(4), *c*=17.384(4) Å, *V*=2380.1(8) Å^3, *Z*=4, D_{calcd}=1.213 g/cm^3, λ (Mo Kα)=0.71073 Å. Intensity data were measured on a Rigaku AFC6S diffractometer up to $2\theta_{max}$ of 50.2°. All 2447 unique reflections were collected. The structure was solved by direct method and refined by a full-matrix least-squares procedure. The nonhydrogen atoms were given anisotropic thermal parameters. The refinement converged to a final *R*=0.059, R_w=0.038 for 1121 observed reflections [$I>3.00\sigma(I)$] and 280 variable parameters.

3.2.15. Single-crystal X-ray crystallography of 2.[12] Suitable colorless prisms of **2** were obtained from a solution in EtOAc. The crystal (0.40×0.60×0.60 mm) belongs to the monoclinic system, space group $P2_12_12_1$ (# 19) with *a*=9.150(2), *b*=14.461(4), *c*=17.972(3) Å, *V*=2378.1(8) Å^3, *Z*=4, D_{calcd}=1.214 g/cm^3, λ (Mo Kα)=0.71073 Å. Intensity data were measured on a Rigaku AFC6S diffractometer up to $2\theta_{max}$ of 50.1°. All 2421 unique reflections were collected. The structure was solved by direct method and refined by a full-matrix least-squares procedure. The nonhydrogen atoms were given anisotropic thermal parameters. The refinement converged to a final *R*=0.060, R_w=0.039 for 1422 observed reflections [$I>3.00\sigma(I)$] and 280 variable parameters.

3.2.16. Single-crystal X-ray crystallography of 8.[12] Suitable colourless prisms of **8** were obtained from a solution in EtOAc. The crystal (0.25×0.42×0.59 mm) belongs to the orthorhombic system, space group $P2_1$ (# 4) with *a*=6.169(1), *b*=9.214(2), *c*=17.134(2) Å, *V*=967.5(3) Å^3, *Z*=2, D_{calcd}=1.203 g/cm^3, λ (Mo Kα)=0.71073 Å. Intensity data were measured on a Rigaku AFC6S diffractometer up to $2\theta_{max}$ of 50.0°. Of the 2003 reflections that were collected, 1827 were unique (R_{int}=0.030). The structure was solved by direct method and refined by a full-matrix least-squares procedure. The nonhydrogen atoms were given anisotropic thermal parameters. The refinement converged to a final *R*=0.045, R_w=0.027 for 885 observed reflections [$I>3.00\sigma(I)$] and 226 variable parameters.

3.2.17. Single-crystal X-ray crystallography of 9.[12] Suitable colourless prisms of **9** were obtained from a solution in EtOAc. The crystal (0.40×0.60×0.70 mm) belongs to the monoclinc system, space group $P2_1$ (# 4) with a=8.871(1), b=9.979(1), c=13.838(2) Å, V=1211.8(3) $Å^3$, Z=2, D_{calcd}=1.235 g/cm^3, λ (Mo Kα)=0.71073 cm^{-1}. Intensity data were measured on a Rigaku AFC6S diffractometer up to $2\theta_{max}$ of 50.0°. Of the 2422 reflections that were collected, 2268 were unique (R_{int}=0.029). The structure was solved by direct method and refined by a full-matrix least-squares procedure. The nonhydrogen atoms were given anisotropic thermal parameters. The refinement converged to a final R=0.038, R_w=0.033 for 1840 observed reflections [$I>3.00\sigma(I)$] and 289 variable parameters.

3.2.18. Cytotoxicity testing. KB and P-388 cells were kindly provided by Prof. J. M. Pezzuto, University of Illinois at Chicago; A549 and HT-29 cells were purchased from the American Type Culture Collection. The cytotoxic activities of tested compounds against the above four cancer cells were assayed with a modification of the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method. Cytotoxicity assays were carried out according to the procedure described previously.[13]

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References

1. Faulkner, D. J. *Nat. Prod. Rep.* **2001**, *18*, 1–49 and previous reports in this series.
2. (a) Sheu, J.-H.; Sung, P.-J.; Wang, G.-H.; Duh, C.-Y.; Shen, Y.-C.; Chiang, M. Y.; Chen, I.-T. *J. Nat. Prod.* **1999**, *62*, 1415–1420. (b) Sung, P.-J.; Su, J.-H.; Wang, G.-H.; Lin, S.-F.; Duh, C.-Y.; Sheu, J.-H. *J. Nat. Prod.* **1999**, *62*, 457–463. (c) Sheu, J.-H.; Sung, P.-J.; Cheng, M.-C.; Liu, H.-Y.; Fang, L.-S.; Duh, C.-Y.; Chiang, M. Y. *J. Nat. Prod.* **1998**, *61*, 602–608.
3. (a) Sung, P.-J.; Su, J.-H.; Duh, C.-Y.; Chiang, M. Y.; Sheu, J.-H. *J. Nat. Prod.* **2001**, *64*, 318–323. (b) Sheu, J.-H.; Sung, P.-J.; Su, J.-H.; Liu, H.-Y.; Duh, C.-Y.; Chiang, M. Y. *Tetrahedron* **1999**, *55*, 14555–14564.
4. Bowden, B. F.; Coll, J. C.; Vasilescu, I. M.; Alderslade, P. M. *Aust. J. Chem.* **1989**, *42*, 1727–1734.
5. Bayer, F. M. *Proc. Biol. Soc. Wash.* **1981**, *94*, 902–947.
6. Wang, G.-H.; Sheu, J.-H.; Chiang, M. Y.; Lee, T.-J. *Tetrahedron Lett.* **2001**, *42*, 2333–2336.
7. Imman, W.; Crews, P. *J. Org. Chem.* **1989**, *54*, 2526–2529.
8. Xu, L.; Patrick, B. O.; Roberge, M.; Allen, T.; Ofwegen, L.; van Andersen, R. J. *Tetrahedron* **2000**, *56*, 9031–9037.
9. Pachyclavulariolide (**8**) was originally reported to be appeared as the colorless oil (see Ref. 7).
10. Bowden, B. F.; Coll, J. C.; Mitchell, S. J.; Ratson, C. L.; Stokie, G. J.; White, A. H. *Aust. J. Chem.* **1979**, *32*, 2265–2274.
11. For significant activity of pure compounds, an ED_{50} value of ≦4.0 μg/mL is required. Geran, R. I.; Greenberg, N. H.; MacDonald, M. M.; Schumacher, A. M.; Abbott, B. J. *Caner Chemother. Rep.* **1972** (3), 1–91.
12. Atomic coordinates for those of structures **1–3** and **9** have been deposited with the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK.
13. Sheu, J.-H.; Liaw, C.-C.; Duh, C.-Y. *J. Nat. Prod.* **1995**, *58*, 1521–1526.