Article

Five New Cytotoxic Metabolites from the Marine Fungus *Neosartorya pseudofischeri*

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Abstract: The marine fungus *Neosartorya pseudofischeri* was isolated from *Acanthaster planci* from the South China Sea. In a preliminary bioactivity screening, the crude methanol extract of the fungal mycelia showed significant inhibitory activity against the Sf9 cell line from the fall armyworm *Spodoptera frugiperda*. Five novel compounds, including 5-olefin phenylpyropene A (**1**), 13-dehydroxylpyripyropene A (**4**), deacetylsesquiterpene (**7**), 5-formyl-6-hydroxy-8-isopropyl-2-naphthoic acid (**9**) and 6,8-dihydroxy-3-((1*E*,3*E*)-penta-1,3-dien-1-yl)isochroman-1-one (**10**), together with eleven known compounds, phenylpyropene A (**2**) and C (**3**), pyripyropene A (**5**), 7-deacetylpyripyropene A (**6**), (1*S*,2*R*,4a*R*,5*R*,8*R*,8a*R*)-1,8a-dihydroxy-2-acetoxy-3,8-dimethyl-5-(prop-1-en-2-yl)-1,2,4a,5,6,7,8,8a-octahydronaphthalene (**8**), isochaetominine C (**11**), trichodermamide A (**12**), indolyl-3-acetic acid methyl ester (**13**), 1-acetyl-β-carboline (**14**), 1,2,3,4-tetrahydro-6-hydroxyl-2-methyl-l,3,4-trioxopyrazino[l,2-a]-indole (**15**) and fumiquinazoline F (**16**), were obtained. The structures of these compounds were determined mainly by MS and NMR data. The absolute configuration of **9** was assigned by the single-crystal X-ray diffraction studies. Compounds **1**–**11** and **15** showed significant cytotoxicity against the Sf9 cells from *S. frugiperda*.

1. Introduction

The fall armyworm *Spodoptera frugiperda* is a pest feeding on the gramineous plants occurring worldwide and causing serious damage to several economically-important crops, such as the maize, sorghum, sugarcane, as well as the cotton, cruciferous and Cucurbitaceae plants. Although other alternative methods have been investigated on the biological control, as well as the development of resistant transgenic plants, the control of *S. frugiperda* still relies mainly on the use of chemical pesticides, easily inducing resistance and causing severe damage to the environment and consumers. However, some natural products with higher selectivity and safety become an important potential source of new biopesticides for the control of *S. frugiperda* [1–5]. Sf9 cells are clonal isolates of *S. frugiperda* Sf21 cells (IPLB-SF21-AE) [6]. A cultured Sf9 cell line is commonly used for primary screening to determine

insecticidal activity [7] and in biomedical research for the purpose of recombinant protein expression using insect-specific viruses called baculoviruses [8].

In recent years, we conducted research on the metabolites of marine fungi and obtained a series of novel and/or bioactive metabolites [9–13]. The marine fungus *Neosartorya pseudofischeri* was isolated from the inner tissue of a starfish *Acanthaster planci* collected from the South China Sea. In the previous metabolites study, we obtained three novel compounds, neosartins A–C, as well as a series of known gliotoxin analogues and diketopiperazines with potent antitumor and antibacterial activities from the culture broth extract of GlyPY (glycerol-peptone-yeast extract) and GluPY (glucose-peptone-yeast extract) media [14]. During our insecticidal activity screening against the fall armyworm *S. frugiperda*, we found that the crude methanol extract of the mycelia of *N. pseudofischeri* cultivated in GluPY showed a 65% cell growth inhibition rate against the Sf9 cell line from *S. frugiperda* at a concentration of 50 mg/L. The bioactivity-guided metabolites isolation afforded five novel compounds, including 5-olefin phenylpyropene A (**1**), 13-dehydroxyl pyripyropene A (**4**), deacetylsesquiterpene (**7**), 5-formyl-6-hydroxy-8-isopropyl-2-naphthoic acid (**9**) and 6,8-dihydroxy-3-((1*E*,3*E*)-penta-1,3-dien-1-yl)isochroman-1-one (**10**), together with eleven known compounds, phenylpyropene A (**2**) and C (**3**), pyripyropene A (**5**), 7-deacetylpyripyropene A (**6**), (1*S*,2*R*,4a*R*,5*R*,8*R*,8a*R*)-1,8a-dihydroxy-2-acetoxy-3,8-dimethyl-5-(prop-1-en-2-yl)-1,2,4a,5,6,7,8,8a-octahydronaphthalene (**8**), isochaetominine C (**11**), trichodermamide A (**12**), indolyl-3-acetic acid methyl ester (**13**), 1-acetyl-β-carboline (**14**), 1,2,3,4-tetrahydro-6-hydroxyl-2-methyl-l,3,4-trioxopyrazino[l,2-a]-indole (**15**) and fumiquinazoline F (**16**) (Figure 1). Compounds **1–11** and **15** showed significant cytotoxicity against the Sf9 cells from *S. frugiperda*. Herein, we report the isolation, structure elucidation and the bioactivity of these compounds.
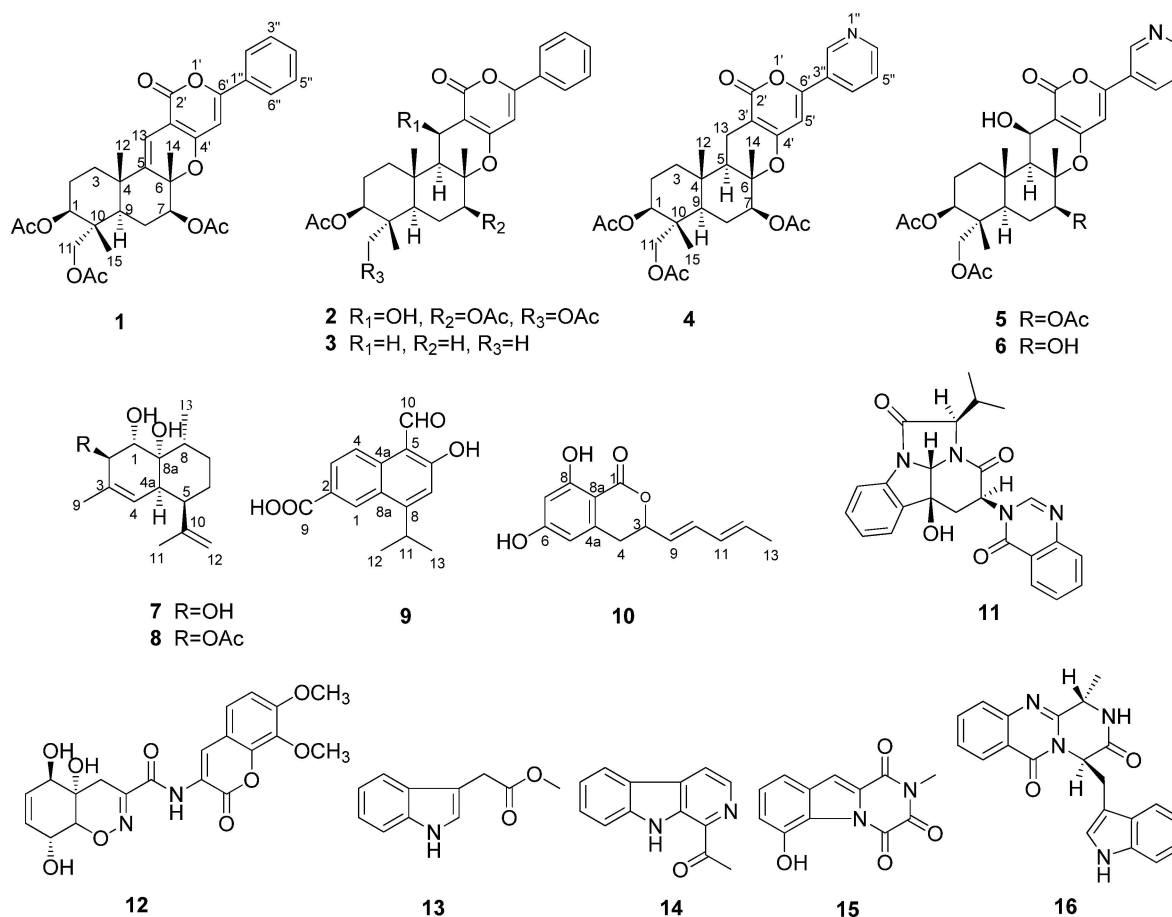

Figure 1. Structures of Compounds **1–16**.

2. Results and Discussion

2.1. Structural Elucidation

5-Olefin phenylpyropene A (**1**) was isolated as a yellowish oil. The molecular formula was established as $C_{32}H_{36}O_9$ based on the analysis of ^{13}C-NMR data and HR(+)ESIMS *m/z* 565.2448 [M + H]$^+$ (calcd. for $C_{32}H_{36}O_9$, 565.2432), implying fifteen degrees of unsaturation (Supplementary Figure S1). The IR spectrum indicated the presence of a carbonyl group (1680 cm^{-1}) and a benzene ring (3073, 1574 and 1507 cm^{-1}). UV maxima at 232, 278 and 322 nm supported the long conjugated system containing a benzene ring. The ^{13}C-NMR and DEPT spectra displayed six methyls, four methylenes, ten methines and twelve quaternary carbons. The diagnostic aryl protons at δ_H 7.81 (m, 2H) and 7.44 (m, 3H) revealed a monosubstituted benzene ring. Two alkenyl protons at δ_H 6.46 (s) and 6.36 (s) indicated there were two trisubstituted double bonds in the molecule. Three methyl group singlets at δ_H 2.16, 2.10 and 2.04 showed HMBC correlations with the carbonyl groups at δ_C 169.9, 171.1 and 170.4, respectively, confirming there are three acyloxy groups. The other three methyl group singlets at δ_H 1.57, 1.25 and 0.87 are connected with the quaternary carbons at δ_C 21.2, 24.2 and 13.3, respectively. In the ^{1}H–^{1}H COSY spectrum, the cross peaks of H-1/H-2, H-2/H-3, H-7/H-8, H-8/H-9, H-2″(H-6″)/H-3″(H-5″) and H-3″(H-5″)/H-4″ revealed the partial structure of –CHCH$_2$CH$_2$–, –CHCH$_2$CH– and –CHCHCHCHCH– in the molecule (Figure 2). The key HMBC correlations of H-1/C-10, H-11/C-10, H-15/C-10, H-12/C-3, H-12/C-4, H-12/C-5, H-13/C-4, H-13/C-5, H-13/C-6, H-7/C-6, H-14/C-6, H-12/C-9 and H-15/C-9 deduced the sesquiterpene moiety. The cross peaks of H-13 to C-2′, C-3′ and C-4′, H-5′ to C-4′, C-6′ and C-1″ in the HMBC spectrum confirmed the connectivity of the sesquiterpene fragment, α-pyrone and the monosubstituted phenyl ring (Table 1 and Supplementary Figures S2–S7).
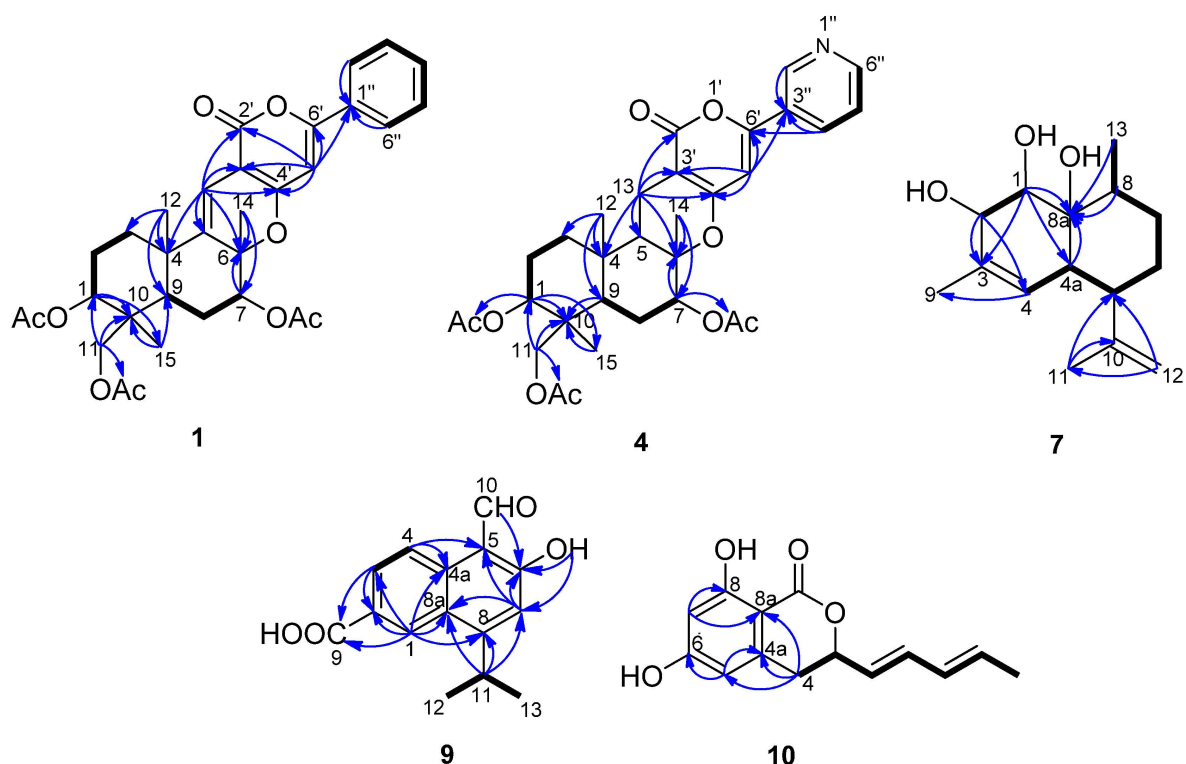

Figure 2. ^{1}H–^{1}H COSY (bold lines) and the main HMBC (arrows) correlations of Compounds **1**, **4**, **7**, **9** and **10**.

Table 1. ^{1}H and ^{13}C-NMR data of **1**, **4** and **6** at 400/100 MHz, respectively, δ in ppm.

Position	1 [a]		4 [a]		6 [b]	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	73.3, CH	4.79, dd (11.6, 4.4)	73.5, CH	4.79, dd (11.6, 4.8)	74.4, CH	4.79, dd (11.2, 5.6)
2	23.2, CH$_2$	1.98, m; 1.75, m	22.8, CH$_2$	1.88, m; 1.70, m	23.6, CH$_2$	1.84, m; 1.88, m
3	35.4, CH$_2$	2.08, m; 1.62, m	36.6, CH$_2$	1.83, m; 1.20, ddd (13.2, 13.2, 3.2)	36.8, CH$_2$	1.43, td (12.8, 4.8); 2.15, td (12.8, 4.8)
4	38.7, C		36.6, C		38.8, C	
5	143.7, C		50.3, CH	1.60, dd (12.4, 4.8)	55.0, CH	1.66, d (3.6)
6	83.5, C		81.9, C		86.2, C	
7	77.7, CH	5.22, d (12.0, 5.2)	77.5, CH	5.02, dd (11.6, 4.8)	77.6, CH	4.97, dd (12.4, 5.2)
8	24.3, CH$_2$	1.81, m; 1.63, m	24.9, CH$_2$	1.78, m; 1.50, m	29.0, CH$_2$	1.60, d (12.4); 1.82, m
9	41.0, CH	1.73, m	45.1, CH	1.66, d (12.0)	46.2, CH	1.53, d (3.6)
10	40.5, C		40.2, C		41.3, C	
11	64.6, CH$_2$	3.78, d (12.0); 3.74, d (12.0)	64.7, CH$_2$	3.78, d (12.0); 3.73, d (12.0)	64.6, CH$_2$	3.83, d (11.6); 3.77, d (11.6)
12	24.2, CH$_3$	1.25, s	15.5, CH$_3$	1.00, s	17.9, CH$_3$	1.48, s
13	111.4, C	6.36, s	16.9, CH$_2$	2.57, dd (17.2, 4.8); 2.33, dd (17.2, 12.4)	60.6, CH	4.95, d (3.6)
14	21.2, CH$_3$	1.57, s	15.4, CH$_3$	1.32, s	15.9, CH$_3$	1.76, s
15	13.3, CH$_3$	0.87, s	13.3, CH$_3$	0.86, s	13.3, CH$_3$	0.92, s
2′	162.1, C		163.7, C		163.5, C	
3′	100.3, C		99.8, C		100.1, C	
4′	160.2, C		162.2, C		157.9, C	
5′	97.4, CH	6.46, s	99.2, CH	6.43, s	147.6, CH	
6′	154.9, C		155.9, C		128.4, C	
1″	131.0, C					
2″	125.6, CH	7.81, m	146.7, CH	8.99, s	147.6, CH	9.07, dd (2.4, 0.8)
3″	128.9, CH	7.44, m	127.3, C		127.5, C	
4″	131.0, CH	7.44, m	132.8, CH	8.09, brd (8.0)	133.6, CH	8.22, ddd (8.4, 2.4, 1.6)
5″	128.9, CH	7.44, m	123.6, CH	7.39, dd (8.0, 4.8)	124.6, CH	7.53, ddd (8.4, 4.8, 0.8)
6″	125.6, CH	7.81, m	151.2, CH	8.66, d (4.8)	152.3, CH	8.69, dd (4.8, 1.6)
1-OCO*CH$_3$*	21.1, CH$_3$	2.04, s	21.1, CH$_3$	2.04, s	21.0, CH$_3$	2.00, s
1-O*CO*CH$_3$	170.0, C		170.5, C		170.6, C	
7-OCO*CH$_3$*	21.1, CH$_3$	2.16, s	21.3, CH$_3$	2.15, s		
7-O*CO*CH$_3$	169.9, C		170.1, C			
11-OCO*CH$_3$*	20.8, CH$_3$	2.10, s	20.8, CH$_3$	2.11, s	20.7, CH$_3$	1.99, s
11-O*CO*CH$_3$	171.1, C		171.0, C		170.8, C	
13-OH						4.28, brs
7-OH						4.10, brs

[a] ^{1}H and ^{13}C-NMR data were measured in CDCl$_3$; [b] ^{1}H and ^{13}C-NMR data were measured in acetone-d_6.

The relative configuration of **1** was determined by the analysis of the NOESY spectrum. The NOE correlations between H-12/H-14 and H-12/H-15, H-1/H-9, H-7/H-9 and H-7/H-11 were observed. Consequently, H-12, H-14 and H-15 are β-oriented, while H-1, H-7 and H-9 are α-oriented (Figure 3).
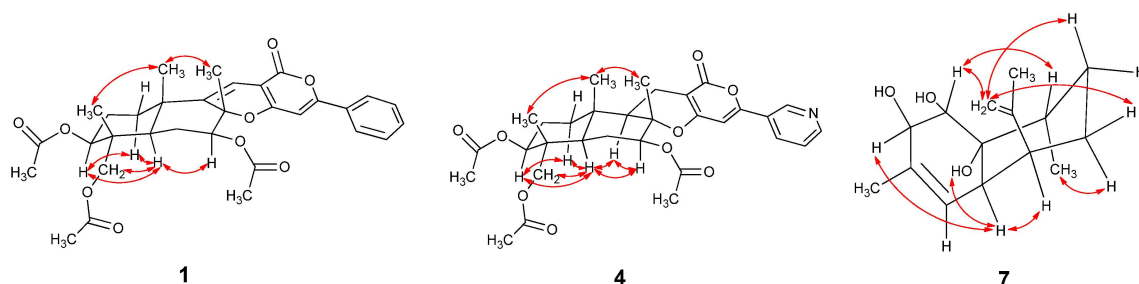
Figure 3. Key NOESY correlations of Compounds **1**, **4** and **7**.

Compounds **2** and **3** were identified as phenylpyripyropene A and C [15,16], by comparing their NMR data to the reference values. The double bond between the C-5 and C-13 positions in **1** was replaced by the saturated carbon-carbon bond in **2**. Compounds **1** and **2** have triacyloxy groups at the C-1, C-7 and C-11 positions; however, Compound **3** has only one acyloxy group at the C-1 position (Supplementary Figures S8–S11).

13-Dehydroxylpyripyropene A (**4**) was obtained as a white powder. It showed a molecular formula of $C_{31}H_{37}NO_9$ determined by the ^{13}C-NMR data and HR(+)ESIMS peak at *m/z* 568.2549 [M + H]$^+$ (calcd. for $C_{31}H_{37}NO_9$, 568.2541) (Supplementary Figure S12). The ^{13}C-NMR and DEPT spectra (Table 1) displayed six methyls, five methylenes, nine methines and eleven quaternary carbons. By comparing the NMR data with pyripyropene A (**5**) (Supplementary Figures S13–S20) [17], a quick identification was made revealing that C-13 of **4** was a methylene (δ_C 16.9, δ_H 2.57, 2.33), corresponding to one methine group (δ_C 60.1, δ_H 4.99) connected to one hydroxyl group in pyripyropene A (Figure 2). The relative configuration of **4** was determined on the basis of NOESY data. The NOE correlations of H-12/H-15, H-14/H-15 supported these methyl groups on the β-position of the ring system; while those correlations of H-1/H-9, H-5/H-7, H-5/H-9, H-7/H-9 and H-9/H-11 were used to place these protons on the α-position (Figure 3).

Compound **6** was elucidated as 7-deacetyl pyripyropene A (Supplementary Figures S21 and S22). **6** was once obtained by hydrolysis of pyripyropene A with 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU) in 80% methanol [18]. However, this is the first report including the detailed NMR data.

Deacetylsesquiterpene (**7**) was isolated as a yellowish oil. The molecular formula was determined as $C_{15}H_{24}O_3$ from the ^{13}C-NMR data and the HR(+)ESIMS peak at *m/z* 275.1603 [M + Na]$^+$ (calcd. for $C_{15}H_{24}O_3Na$, 275.1618), indicating four degrees of unsaturation (Supplementary Figure S23). The strong IR absorption at 3443 cm^{-1} indicated the presence of the hydroxyl groups. The ^{13}C-NMR and DEPT spectra displayed three methyls, three methylenes, six methines and three quaternary carbons (Table 2). The quaternary carbon (δ_C 132.3) and the methine (δ_C 124.4, δ_H 5.28) formed a trisubstituted double bond. The quaternary carbon (δ_C 147.2) and the methylene (δ_C 111.0, δ_H 4.94 and 4.72) constructed a terminal double bond. Thus, Compound **7** must be bicyclic to account for the four double bond equivalents required by the molecular formula. The ^{1}H–^{1}H COSY cross peaks of H-1/H-2, H-4/H-4a, H-4a/H-5, H-5/H-6, H-6/H-7, H-7/H-8 and H-8/H-13 established two partial structures, –CHCH– and –CHCHCHCH$_2$CH$_2$CHCH$_3$ (Figure 2). The HMBC correlations of H-1/C-8, H-2/C-3, H-5/C-10, H-8/C-8a, H-9/C-3 and H-11/C-10 established the skeleton; three hydroxyl groups were attached to C-1 (δ_C 74.6), C-2 (δ_C 74.2) and C-8a (δ_C 73.8). The NOESY correlations of H-1/H-12, H-7a/H-12, H-6e/H-12, H-2/H-4a, H-4a/H-5, H-4a/8a-OH and H-6a/H-13 revealing 1-OH, 8a-OH, H-5 and H-13 were α-oriented; however, 2-OH was β-oriented (Figure 3 and Supplementary Figures S24–S29).

Table 2. ^{1}H and ^{13}C-NMR data of **7**, **9** and **10**, δ in ppm.

Position	7 [a]		9 [b]		10 [c]	
	δ_C, Type	δ_H, Mult., (*J* in Hz)	δ_C, Type	δ_H, Mult., (*J* in Hz)	δ_C, Type	δ_H, Mult., (*J* in Hz)
1	74.6, CH	3.98, d (1.2)	127.9, CH	8.92, d (1.5)	169.1, C	
2	74.2, CH	4.05, s	137.4, C			
3	132.3, C		129.2, CH	8.22, dd (9.0,1.5)	78.4, CH	5.13, ddd (10.0, 6.8, 4.0)
4	124.4, CH	5.28, s	121.6, CH	8.77, d (9.0)	32.5, CH_2	3.00, dd (16.4, 4.0); 2.93, dd (16.4, 10.0)
4a	38.1, CH	2.67, s	127.3, C		141.7, C	
5	41.4, CH	2.38, brd (12.0)	111.4, C		107.1, CH	6.24, d (2.0)
6	25.9, CH_2	1.56, m; 1.33, m	167.1, C		163.4, C	
7	30.7, CH_2	1.48, m; 1.40, m	117.0, CH	7.21, s	101.0, CH	6.18, d (2.0)
8	31.2, CH	1.99, m	159.5, C		164.8, C	
8a	73.8, C		126.3, C		100.1, C	
9	20.1, CH_3	1.78, s	167.6, C		127.0, CH	5.71, dd (15.2, 6.8)
10	147.2, C		195.3, CH	11.42, brs	133.3, CH	6.35, dd (15.2, 10.0)
11	22.4, CH_3	1.74, brs	30.1, CH	3.90, heptet (7.0)	130.4, CH	6.10, ddd (15.2, 10.0, 1.2)
12	111.0, CH_2	4.94, q (1.2); 4.72, s	23.4, CH_3	1.45, d (7.0)	131.7, CH	5.81, dq (15.2, 6.8)
13	15.0, CH_3	0.93, d (6.8)	23.4, CH_3	1.45, d (7.0)	18.0, CH_3	1.74, dd (6.8, 1.2)
1-OH		2.00, s				
2-OH		2.00, s				
2-O*CO*CH$_3$						
2-OCOC*H*$_3$						
6-OH				13.42, s		11.06, brs
8-OH						11.06, brs
8a-OH		2.00, s				
CO*OH*				10.94, brs		

[a] ^{1}H and ^{13}C-NMR data were measured at 400/100 MHz, in CDCl$_3$; [b] ^{1}H and ^{13}C-NMR data were measured at 500/125 MHz, in acetone-d_6; [c] ^{1}H and ^{13}C-NMR data were measured at 400/100 MHz, in DMSO-d_6.

The formula of Compound **8** was identified as $C_{17}H_{26}O_4$, based on the analysis of ^{13}C-NMR data and HREIMS peak at *m/z* 294.1828 [M]$^+$ (calcd. for $C_{17}H_{26}O_4$, 294.1826). The NMR spectroscopic data of Compound **8** were very similar to those of Compound **7**, except that the C-2 hydroxyl group in **7** was replaced by an acetoxy group in **8** (Supplementary Figures S30 and S31). Fortunately, we obtained a single crystal of **8** from the MeOH solution. In the crystal, the adjacent molecules are interlinked by a pair of strong O1-H...O4 (hydroxyl) and O4-H...O3 (carbonyl) hydrogen bonds to form a zigzag chain running parallel to the b-axis. Neighboring chains of molecules are packed closely together with the hydrophobic methyl groups pointing towards each other. The absolute configuration of **8** was determined as 1*S*, 2*R*, 4a*R*, 5*R*, 8*R*, 8a*R* with the Flack parameter value −0.16(17) by single-crystal X-ray diffraction analysis (Figure 4 and Supplementary Crystallographic Information Framework (CIF)) using Cu K*α* radiation. Compound **8**, named (1*S*,2*R*,4a*R*,5*R*,8*R*,8a*R*)-1,8a-dihydroxy-2-acetoxy-3,8-dimethyl-5-(prop-1-en-2-yl)-1,2,4a,5,6,7,8,8a-octahydronaphthalene, was previously obtained as the degradation product of the natural product CJ-12662, which was obtained from the fermentation broth of *Aspergillus fischeri* var. *thermomutatus* ATCC 18,618 [19] and later also isolated from fungi *Neosartorya pseudofischeri* and *Eurotium chevalieri* [20,21]. However, the single-crystal X-ray diffraction data were never reported.
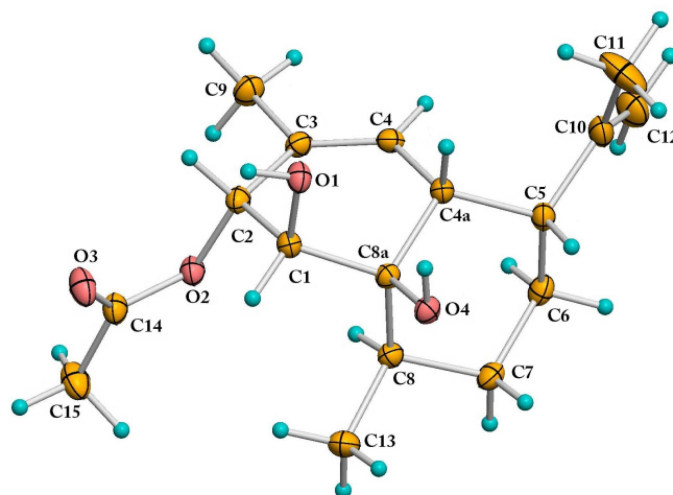
Figure 4. ORTEP (Oak Ridge Thermal Ellipsoid Plot) drawing of Compound **8**.

Compound **9** was obtained as a white powder. Its molecular formula was deduced to be C15H14O4 by analysis of the ^{13}C-NMR data and HR(-)ESIMS ion at m/z 257.0817 [M − H]$^-$ (calcd. for $C_{15}H_{13}O_4$, 257.0819), which required nine degrees of unsaturation (Supplementary Figure S32). The IR spectrum revealed the presence of a carboxyl group (3283 and 1671 cm^{-1}) and an aldehyde group (2812 cm^{-1}). Two methyl groups at δ_H 1.45 (d, J = 7.0 Hz, 6H) showed ^{1}H–^{1}H COSY correlations with the methine group at δ_H 3.90 (seven peaks, J = 7.0 Hz) and formed an isopropyl fragment (Table 2, Figure 2 and Supplementary Figures S33–S36). According to the HMBC correlations of H-1/C-2, H-1/C-8a, H-3/C-2 and H-4/C-4a, the protons at δ_H 8.92 (d, J = 1.5 Hz, H-1), 8.22 (dd, J = 9.0, 1.5 Hz, H-3) and 8.77 (d, J = 9.0 Hz, H-4) were located at the 1,3,4-position on a phenyl ring. HMBC correlations of H-1/C-2, H-1/C-8a, H-3/C-2, H-4/C-4a, H-4/C-5, H-7/C-5, H-7/C-6, H-11/C-7 and H-11/C-8 indicated the existence of a naphthyl ring, accounting for seven degrees of unsaturation. The remaining carboxyl group (δ_C 167.6, δ_H 10.94), the aldehyde (δ_C 195.3, δ_H 11.42), phenolic hydroxyl group (δ_H 13.42, and the isopropyl fragment were located on C-2, C-5, C-6 and C-8, respectively, based on the HMBC cross peaks of H-1/C-9, H-3/C-9, H-10/C-6, 6-OH/C-6, 6-OH/C-7 and H-11/C-8. The aldehyde and hydroxyl groups formed an intramolecular hydrogen bond, which accounted for the downfield resonance of the phenolic hydroxyl group. Therefore, the structure of **9** was elucidated as 5-formyl-6-hydroxy-8-isopropyl-2-naphthoic acid.

Compound **10** was isolated as a white powder. The ^{13}C-NMR data and HR(-)ESIMS peak at m/z 245.0815 [M − H]$^+$ (calcd. for $C_{14}H_{13}O_4$, 245.0819), established the molecular formula as $C_{14}H_{14}O_4$ (Supplementary Figure S37). The ^{13}C-NMR and DEPT spectra revealed fourteen carbon resonances, including one methyl group, one methylene group, seven methine groups and five quaternary carbons. The ^{1}H-NMR spectrum exhibited two meta-aromatic protons at δ_H 6.24 (d, J = 2.0 Hz) and 6.18 (d, J = 2.0 Hz), indicating the presence of a tetrasubstituted aromatic ring. Two phenolic hydroxyl groups at δ_H 11.06 (brs) were connected to C-6 (δ_C 163.4) and C-8 (δ_C 164.8). Additionally, two pairs of trans-oriented olefinic protons at δ_H 5.71 (dd, J = 15.2, 6.8 Hz), 6.35 (dd, J = 15.2, 10.0 Hz), 6.10 (ddd, J = 15.2, 10.0, 1.2 Hz), 5.81 (dq, J = 15.2, 6.8 Hz), one oxymethine at δ_H 5.13 (ddd, J = 10.0, 6.8, 4.0 Hz), one methylene at δ_H 3.00 (dd, J = 16.4, 4.0 Hz), 2.93 (dd, J = 16.4, 10.0 Hz) and one methyl group at δ_H 1.74 (dd, J = 6.8, 1.2 Hz) were connected in sequence and formed a pentadienyl group based on their ^{1}H–^{1}H COSY correlations. The remnant quaternary carbon (δ_C 169.1) connected with C-8a (δ_C 100.1) and the oxymethine and formed a lactone. The HMBC correlations of H-4/C-4a, H-4/C-8a, H-5/C-6, H-7/C-8 and H-7/C-8a revealed a 3,6,8-trisubstituted isocoumarin ring system (Table 2, Figure 2 and Supplementary Figures S38–S43). The absolute configuration at C-3 remains undetermined. Therefore, Compound **10** was elucidated as 6,8-dihydroxy-3-((1*E*,3*E*)-penta-1,3-dien-1-yl)isochroman-1-one.

The other known compounds, isochaetominine C (**11**) [22], trichodermamide A (**12**) [23], indolyl-3-acetic acid methyl ester (**13**) [24], 1-acetyl-β-carboline (**14**) [25], 1,2,3,4-tetrahydro-6-hydroxy-2-methyl-l,3,4-trioxopyrazino[l,2-a]-indole (**15**) [26] and fumiquinazoline F (**16**) [27], were identified by comparing their spectroscopic data with the literature values (Supplementary Figures S44–S55).

2.2. Proposed Biosynthetic Pathways

Tomoda *et al.* described the biosynthetic origin of pyripyropene A (**5**), which was established by feeding experiments using various [^{13}C] and [^{14}C] precursors. Pyripyropene A (**5**) is derived from three mevalonates, five acetates and one nicotinic acid. The pyridino-α-pyrone moiety is produced via condensation of a primer nicotinic acid with two acetates in a "head-to-tail" fashion; an all-trans farnesyl pyrophosphate is produced via the mevalonate pathway; the two parts are linked and cyclized to form the core skeleton, and then three acetyl residues from the acetates are introduced into the skeleton to yield **5** [28]. Based on the above finding, we proposed that the phenyl-α-pyrones (**1**–**3**) shared a similar biosynthetic pathway (Figure 5). Benzoic acid, instead of nicotinic acid, is one of the precursors. It is very interesting that Compound **2** was observed slowly changing into Compound **1** when stored in DMSO-d_6. The change was recorded by ^{1}H-NMR spectra. After 18 days, Compound **2** was changed into Compound **1** thoroughly. However, the pure Compounds **1** and **2** are stable at the temperature range from −20 °C–90 °C.
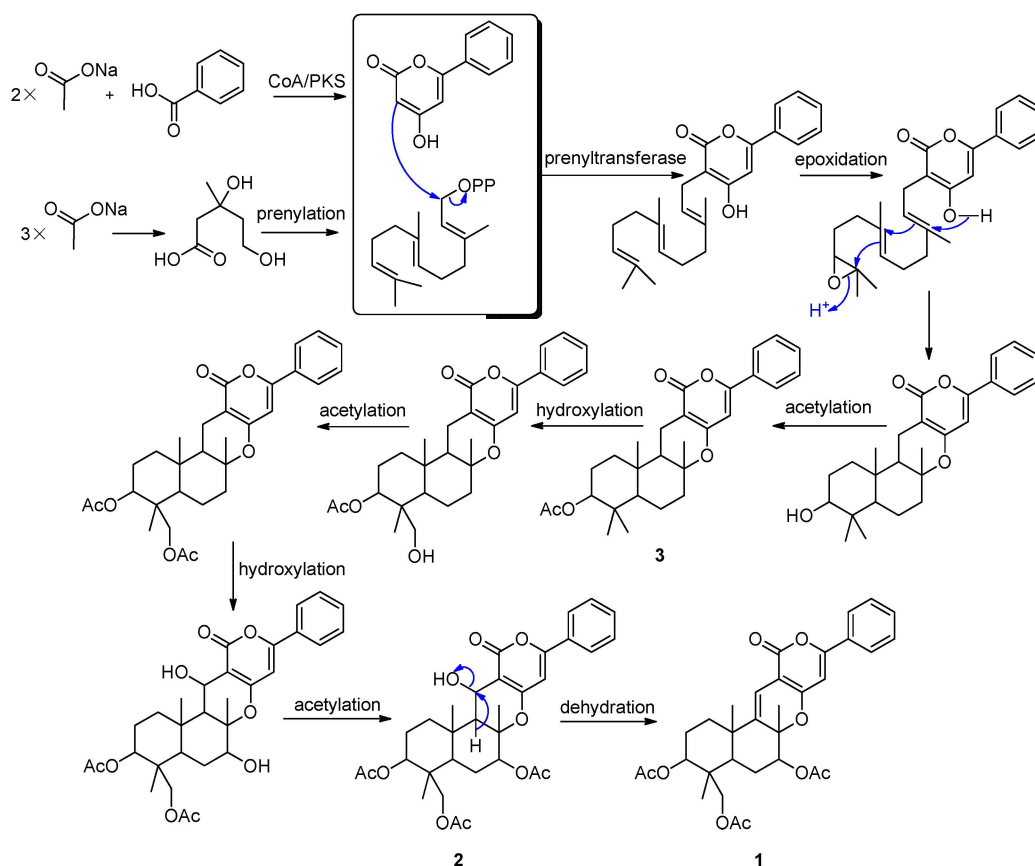

Figure 5. Proposed biosynthetic pathways of **1**–**3**.

2.3. Cytotoxicity

Compounds **1**–**11** and **15** showed significant cytotoxicity against the Sf9 cells (Table 3). After 48 h of treatment at the concentration of 50 mg/L, the cell growth inhibition rates of compounds **2**, **3**,

7–9, **11** and **15** were greater than 90%. Therefore, further in-depth studies to identify the insecticidal activities *in vivo* and the mechanism are warranted for their development as biorational pesticides.

Table 3. The death rate (%) of Compounds **1–11** and **15** against insect cell line Sf9 ($n = 3$, $p \leqslant 0.05$).

Compounds	6 h	12 h	24 h	48 h
1	65.79	68.26	76.16	85.24
2	60.75	68.86	81.21	95.01
3	72.55	72.15	73.08	93.73
4	47.53	57.41	73.21	85.37
5	41.05	54.60	58.04	71.77
6	25.58	29.46	35.30	56.03
7	79.58	81.89	90.46	98.68
8	64.52	69.33	77.98	91.87
9	57.96	65.32	69.92	90.97
10	36.47	38.25	52.36	61.67
11	63.70	69.16	71.65	94.53
15	56.97	61.43	80.33	92.52
Rotenone	69.21	79.43	90.93	98.54

3. Experimental Section

3.1. General Experimental Procedures

Preparative HPLC was performed using a Shimadzu LC-20AT HPLC pump (Shimadzu Corporation, Nakagyo-ku, Kyoto, Japan) equipped with an SPD-20A dual λ absorbance detector (Shimadzu Corporation, Nakagyo-ku, Kyoto, Japan) and a Shim-pack PRC-ODS HPLC column (250 mm × 20 mm, Shimadzu Corporation, Nakagyo-ku, Kyoto, Japan). Optical rotations were measured using a Schmidt and Haensch Polartronic HNQW5 optical rotation spectrometer (SCHMIDT + HAENSCH GmbH & Co., Berlin, Germany). UV spectra were recorded on a Shimadzu UV-VIS-NIR spectrophotometer (Shimadzu Corporation, Nakagyo-ku, Kyoto, Japan). IR spectra were recorded on a PerkinElmer Frontier FT-IR spectrophotometer (PerkinElmer Inc., Waltham, MA, USA). 1D and 2D NMR spectra were recorded on Bruker Avance II 400 spectrometers (Bruker BioSpin AG, Industriestrasse 26, Fällanden, Switzerland) and a Varian INOVA500NB spectrometer (Varian Medical Systems In., Salt Lake City, UT, USA). The chemical shifts are relative to the residual solvent signals (CDCl$_3$: δ_H 7.26 and δ_C 77.0; acetone-d_6: δ_H 2.05 and δ_C 29.92; DMSO-d_6: δ_H 2.50 and δ_C 39.51). The low- and high-resolution EI mass spectra were obtained on Thermo DSQ and Thermo MAT95XP mass spectrometers (Thermo Fisher Scientific, Waltham, MA, USA), respectively. The low- and high-resolution ESI-MS analyses were performed with a Thermo LCQ DECA XP liquid chromatography-mass spectrometry (Thermo Fisher Scientific, Waltham, MA, USA) and a Thermo Fisher LTQ Orbitrap Elite High Resolution liquid chromatography-mass spectrometry (Thermo Fisher Scientific, Waltham, MA, USA). X-ray diffraction data were acquired on a Bruker SMART APEX CCD X-ray single-crystal diffractometer (Bruker AXS GmbH, Karlsruhe, Germany).

3.2. Fungal Material

The marine fungus *N. pseudofischeri* was isolated from the inner tissue of the starfish *A. planci* collected from the Hainan Sanya National Coral Reef Reserve, China. The fungus was identified on the basis of the DNA sequences of the ITS1-5.8S-ITS2 (Internal Transcribed Spacer, ITS) regions of their rRNA gene. The ITS gene sequence showed 100% homology with that of the fungus *N. pseudofischeri* in GenBank (Accession Number KF999816).

3.3. Fermentation, Extraction and Isolation

The 20 L scale-up fermentation of *N. pseudofischeri* was carried out in 1000 mL Erlenmeyer flasks containing 500 mL liquid culture media, which was composed of glucose 10 g, peptone 5 g, yeast extract 2 g, $CaCO_3$ 1 g and sea water 1 L. After 30 days of growth, the mycelia of *N. pseudofischeri* were extracted with methanol, and the organic solvent was evaporated to dryness under vacuum to afford the crude extract (5.36 g). Then, the extract was separated by flash silica gel column chromatography using petroleum ether-ethyl acetate in a gradient elution (100:0–0:100, *v/v*), followed by ethyl acetate-methanol in a gradient elution (100:0–0:100, *v/v*) to give 10 fractions (F1–F10). Subfractions F4.1–F4.6 were obtained from F4 (610 mg) by reverse phase silica gel using methanol-water (30:70–100:0, *v/v*) gradient elution. Then, F4.3 (44 mg) was further separated by RP-HPLC using methanol-water (65:35, *v/v*) as the eluent to obtain Compounds **1** (6.2 mg), **4** (4.1 mg) and **5** (3.4 mg). F4.4 (32 mg) was further separated by RP-HPLC eluted with methanol-water (68:32, *v/v*) to obtain Compounds **2** (10.1 mg), **3** (3.5 mg), **6** (1.7 mg) and **12** (2.4 mg). F5 (157 mg) was further separated by RP-HPLC eluted with methanol-water (70:30, *v/v*) to obtain Compound **8** (32.6 mg). F6 (113 mg) was further separated by RP-HPLC eluted with methanol-water (60:40, *v/v*) to obtain **7** (12.1 mg) and **9** (2.8 mg). F7 (201 mg) was separated by RP-HPLC eluted with MeCN-water (60:40, *v/v*) to get Compounds **10** (4.5 mg), **11** (3.1 mg) and **16** (2.3 mg). F8 (97 mg) was further separated by RP-HPLC eluted with MeCN-water (50:50, *v/v*) to obtain Compounds **13** (2.0 mg), **14** (2.9 mg) and **15** (23.2 mg).

5-Olefin phenylpyropene A (**1**): yellowish oil; $[\alpha]_D^{20}$: + 129° (*c* = 0.1, $CHCl_3$); UV (MeOH) λ_{max} (ε) 234 (13,810), 322 (10,108) nm; IR ν_{max} 2960, 2925, 1735, 1602, 1566, 1451, 1378, 1241, 1043, 758 cm^{-1}; for ^{1}H and ^{13}C-NMR data, see Table 1; LR(+)ESIMS *m/z* 565.3 [M + H]$^+$. HR(+)ESIMS *m/z* 565.2448 [M + H]$^+$ (calcd. for $C_{32}H_{37}O_9$, 565.2432).

13-Dehydroxylpyripyropene A (**4**): white powder; $[\alpha]_D^{20}$: +20° (*c* = 0.1, $CHCl_3$); white powder; UV (MeOH) λ_{max} (ε) 216 (18,120), 246 (12,977), 278 (4038) nm; IR ν_{max} 2960, 2921, 2851, 1713, 1464, 1377, 1260, 1035, 810, 721 cm^{-1}. For ^{1}H and ^{13}C-NMR data, see Table 1; LR(+)ESIMS *m/z* 568.2 [M + H]$^+$. HR(+)ESIMS *m/z* 568.2549 [M + H]$^+$ (calcd. for $C_{31}H_{38}NO_9$, 568.2541).

7-deacetylpyripyropene A (**6**): white powder; for ^{1}H and ^{13}C-NMR data, see Table 1.

Deacetylsesquiterpene (**7**): yellowish oil; $[\alpha]_D^{20}$: +38° (*c* = 0.058, MeOH); UV (MeOH) λ_{max} (ε) 212 (136) nm; IR ν_{max} 3443, 2959, 2926, 2856 1721, 1675, 1609, 1455, 1376, 1260, 1095, 1029, 992, 887, 756 cm^{-1}. For ^{1}H and ^{13}C-NMR data, see Table 2; HR(+)ESIMS: *m/z* 275.1603 [M + Na]$^+$ (calcd. for $C_{15}H_{24}O_3Na$, 275.1618).

(1S,2R,4aR,5R,8R,8aR)-1,8a-dihydroxy-2-acetoxy-3,8-dimethyl-5-(prop-1-en-2-yl)-1,2,4a,5,6,7,8, 8a-octahydronaphthalene (**8**): white crystal. $[\alpha]_D^{20}$: −68° (*c* = 0.079, MeOH); IR (MeOH) ν_{max} 3677, 3436, 3317, 2928, 2862, 1731, 1642, 1444, 1405, 1369, 1238, 1030, 967, 945, 927, 885, 720, 609 cm^{-1}. For ^{1}H and ^{13}C-NMR spectra, see Supplementary Figures S30 and S31; HREIMS: *m/z* 294.1828 [M]$^+$ (calcd. for $C_{17}H_{26}O_4$, 294.1826). The crystal of **8** was obtained from MeOH solution. $C_{17}H_{26}O_4$, *M* = 294.38, colorless block, Orthorhombic system, P 2$_1$ 2$_1$ 2$_1$ (19), a = 8.3364 (1), b = 9.3709 (2), c = 21.3150 (5) Å. V = 1665.12 (6) Å^3, Z = 4. D_{calcd} = 1.174 g/cm^3, crystal size 0.41 mm × 0.45 mm × 0.43 mm, F(000) = 640, T = 173(2) K.

5-formyl-6-hydroxy-8-isopropyl-2-naphthoic acid (**9**): white powder; IR ν_{max} 3283, 3020, 2953, 2928, 2867, 2812, 2720, 1671, 1616, 1601, 1512, 1458, 1422, 1393, 1289, 1026, 1208, 1148, 1109, 1080, 1008, 985, 921, 796 cm^{-1}; for ^{1}H and ^{13}C-NMR data, see Table 2; HR(-)ESIMS *m/z* 257.0817 [M − H]$^-$ (calcd. for $C_{15}H_{13}O_4$, 257.0819).

6,8-dihydroxy-3-((1E,3E)-penta-1,3-dien-1-yl)isochroman-1-one (**10**): white powder; $[\alpha]_D^{20}$: +40° (*c* = 0.1, MeOH); UV (MeOH) λ_{max} (ε) 232 (6312), 268 (3427), 302 (2060) nm; IR ν_{max} 3528, 3400, 2953, 2926, 2855, 1656, 1622, 1460, 1374, 1247, 1110, 1003, 772 cm^{-1}. For ^{1}H and ^{13}C-NMR data, see Table 2; HR(-)ESIMS *m/z* 245.0815 [M − H]$^-$ (calcd. for $C_{14}H_{13}O_4$, 245.0819).

3.4. Bioassay

To evaluate the biological activities of these compounds, cytotoxic assays were carried out with insect cultured cell line Sf9 from *S. frugiperda*. Sf9 cells were maintained at 27 °C in TC-199-MK medium supplemented with 10% FCS (*v/v*), 1% L-glutamine 200 mmol/L and penicillin-streptomycin-neomycin solutions (*v/v*). The cell growth inhibition was measured by the MTT method. Cells were seeded in 96-well microtitration plates at the exponential growth phase. Different concentrations of compounds diluted with medium were added at their log-phase growth stage. Additionally, 0.2 μL of solvent (DMSO) only was added as the control (CK). The final concentration of solvent in the cultures assayed was 1%. Compounds were solubilized with DMSO at concentrations of 10 and 50 mg/L. The rotenone was used as the positive control. After 6 h, 12 h, 24 h and 48 h of treatment, 5 mg/mL MTT were dissolved in PBS, and 20 μL of this stock solution were added to the culture cells. After an additional 3 h of incubation, the medium was discarded, and the 96-well plates were dried in the air. Then, 100 μL of DMSO were added to dissolve the formazan crystals, and the absorbance was measured at 570 nm by a microplate reader (Spectramex, 190 Molecular Devices Inc., Sunnyvale, CA, USA).

The cytotoxic effect was expressed as the relative percentage of inhibition calculated as follows: cell growth inhibition rate (%) = [(A control − A treatment)/A control] × 100 [29].

4. Conclusions

In summary, sixteen compounds, including five new compounds (**1**, **4**, **7**, **9** and **10**), were obtained from the mycelium of marine fungus *N. pseudofischeri*. Their structure types include phenylpyripyropenes, pyripyropenes, sesquiterpenoids, isocoumarin and alkaloids. Our findings provide further evidence that the marine fungus *N. pseudofischeri* has the tremendous potential of biosynthesis. The potent cytotoxicity of these compounds against the Sf9 cells revealed the prospect to develop biorational pesticides.

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