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Article

Four New Citrinin Derivatives from a Marine-Derived *Penicillium* sp. Fungal Strain

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Abstract: Four new citrinin derivatives, including two citrinin dimers and two citrinin monomer derivatives, were isolated and identified from a marine-derived fungal strain *Penicillium* sp. ML226 along with six known related compounds. Their structures were elucidated by spectroscopic and chemical methods. The new compounds showed modest cytotoxic activity against HepG-2 cell line and weak antimicrobial activity against *Staphylococcus aureus*.

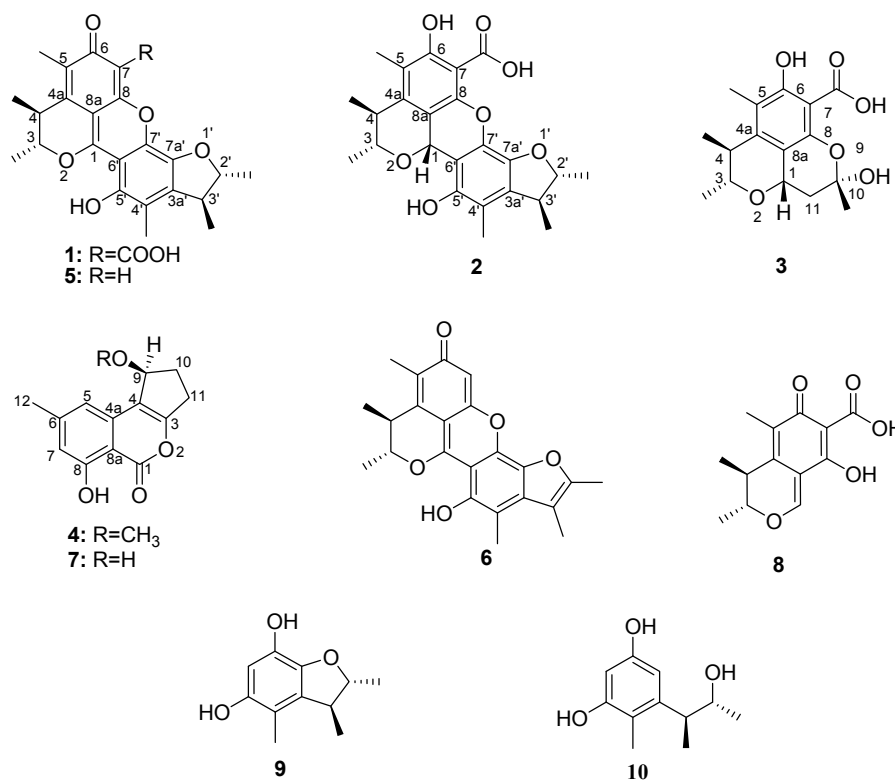
Keywords: citrinin; *Penicillium* sp. ML226; new metabolites; marine fungus

1. Introduction

The search for new bioactive natural products is still the main way of discovering new drugs. Investigating the secondary metabolites of microorganisms isolated from specific ecological environments may increase the chance of finding compounds with novel skeletons and varied and unique bioactivities. It was reported that the specific situations that microorganisms live in might activate some silent genes and induce some unique biosynthetic pathways [1]. Marine microorganisms have attracted extensive attention in this context. Marine fungi are an important resource to find

chemically and biologically diverse compounds due to their special living environment [2,3]. In order to search for new bioactive natural products, a marine-derived fungal strain, ML226, authenticated as *Penicillium* sp., was isolated from the Taiwan Strait, China. The EtOAc extract of *Penicillium* sp. ML226 exhibited cytotoxic and antimicrobial activity. Chemical investigation of the EtOAc extract of *Penicillium* sp. ML226 led to the isolation of two new citrinin dimers—penicitrinone E (**1**) and penicitrinol J (**2**)—two new citrinin monomer derivatives—penicitrinol K (**3**) and citrinolactone D (**4**)—together with six known compounds—penicitrinone A [4] (**5**), penicitrinone B [4] (**6**), citrinolactone B [5] (**7**), citrinin [6] (**8**), 2,3,4-trimethyl-5,7-dihydroxy-2,3-dihydrobenzofuran [7] (**9**) and phenol A [8] (**10**) (Figure 1). In this paper, we report the isolation and structural elucidation of compounds **1–10** and the cytotoxic and antimicrobial activity of **1–4**. They all showed weak cytotoxicity against HepG-2 cell line in the concentration of 10 $\mu\text{g/mL}$ with inhibition rate from 6% to 30%. Compounds **2** and **3** showed weak antimicrobial activity against *Staphylococcus aureus*.

Figure 1. Structures of the isolated compounds **1–10**.



2. Results and Discussion

2.1. Structural Elucidation of Compounds

Penicitrinone E (**1**) was obtained as a red, amorphous powder. $[\alpha]_D^{25}$ 0 (0.002, MeOH). A molecular formula of C₂₄H₂₄O₇ was assigned based on the interpretation of HRESIMS data at m/z 425.15965 $[M + H]^+$ (calcd. 425.1600). The ¹H-NMR data of **1** showed four tertiary methyl signals, two aromatic methyl signals, four sp³ methine protons (two oxygenated), and one hydroxyl proton (Table 1). The ¹³C-NMR and DEPT spectra for **1** displayed 24 carbon signals comprising four tertiary methyls, two aromatic methyls, four sp³ methines (two oxygenated), two carbonyl carbons, and 12 sp² quaternary

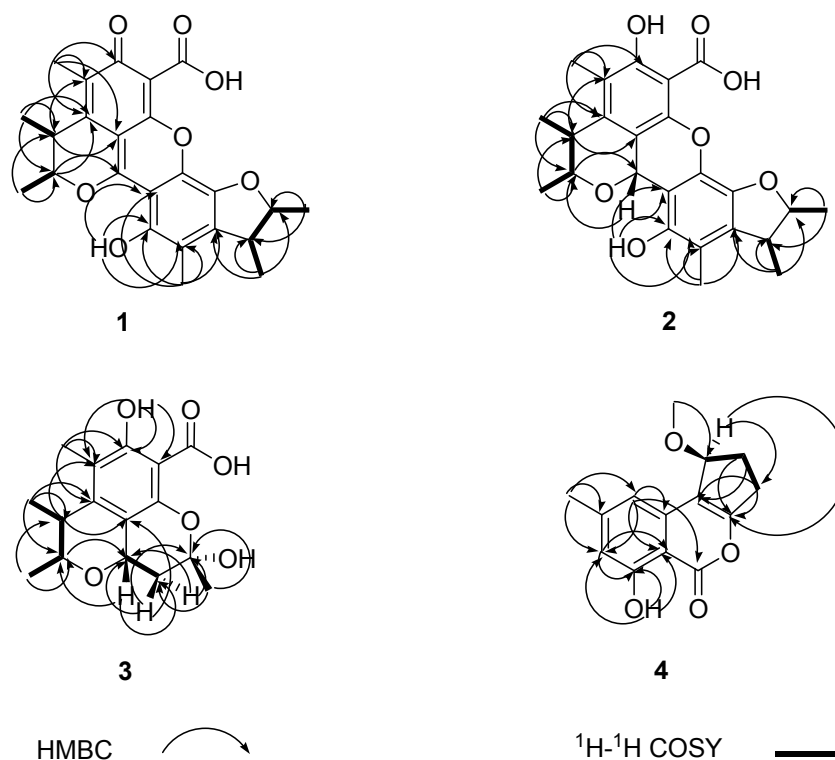
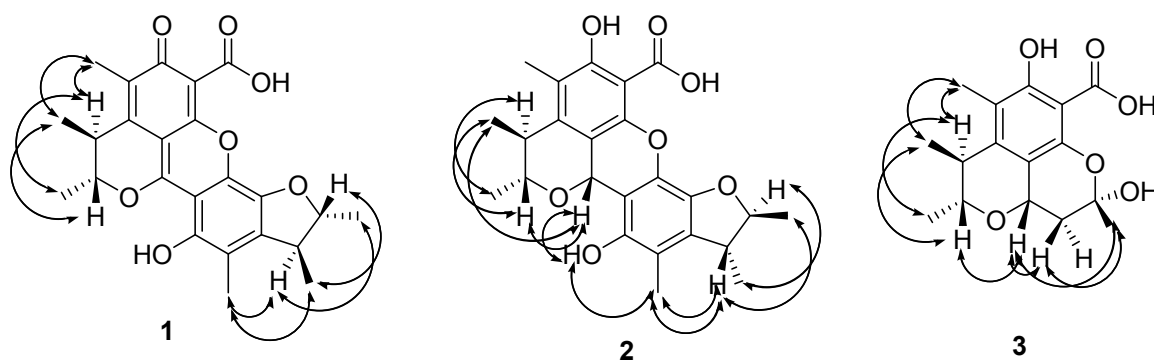
carbons (Table 1). Except for those of the benzopyran moiety, the NMR data were quite similar to those of **5** [4], indicating that they shared the same molecular skeleton. Compared with those of **5**, the NMR spectra of **1** exhibited an additional carboxyl group (δ_c 165.4), two downfield shifts effect of C-1 (+4.6 ppm) and C-8 (+2.7 ppm) because of the inductive effect of the additional carboxyl group. The C-7 of **5** is a sp^2 methine carbon but the C-7 of **1** is a sp^2 quaternary carbon, indicating that the carboxyl group was linked to C-7.

Table 1. 1H - and ^{13}C -NMR (600 and 150 MHz) data for compounds **1** and **2** ($CDCl_3$, δ in ppm).

No.	1		2	
	δ_H (mult., J [Hz], int.)	δ_c	δ_H (mult., J [Hz], int.)	δ_c
1	-	161.1s	5.71 (s)	66.3d
3	5.17 (q, 6.7)	83.2d	4.10 (dq, 6.1, 6.8)	79.0d
3-CH ₃	1.49 (d, 6.7, 3H)	19.04q	1.47 (d, 6.8, 3H)	21.9q
4	3.29 (q, 7.2)	34.8d	3.03 (dq, 6.1, 7.0)	37.7d
4-CH ₃	1.38 (d, 7.2, 3H)	18.97q	1.34 (d, 7.0, 3H)	19.6q
4a	-	132.7s	-	144.7s
5	-	130.8s	-	120.6s
5-CH ₃	2.22 (s, 3H)	10.9q	2.21 (s, 3H)	11.2q
6	-	183.8s	-	161.9s
6-OH	-	-	12.52 (s)	-
7	-	103.1s	-	98.0s
7-COOH	-	165.4s	-	170.6s
8	-	160.9s	-	145.3s
8a	-	99.5s	-	108.9s
2'	4.77 (dq, 4.3, 6.4)	88.4d	4.56 (m)	88.3d
2'-CH ₃	1.47 (d, 6.4, 3H)	21.0q	1.36 (d, 6.5, 3H)	20.9q
3'	3.25 (dq, 4.3, 7.1)	45.0d	3.09 (m)	44.3d
3'-CH ₃	1.36 (d, 7.1, 3H)	18.82q	1.35 (d, 7.2, 3H)	19.3q
3a'	-	142.4s	-	133.0s
4'	-	118.2s	-	117.8s
4'-CH ₃	2.28 (s, 3H)	11.6q	2.18 (s, 3H)	11.6q
5'	-	147.2s	-	147.5s
5'-OH	8.25 (s)	-	7.92 (s)	-
6'	-	102.2s	-	105.3s
7'	-	136.2s	-	130.7s
7a'	-	139.2s	-	138.1s

^a The assignments were based on DEPT, 1H - 1H COSY, HMQC, and HMBC experiments, and chemical shift values are in ppm relative to TMS. “-”: no signal.

This deduction was further supported by analyses of the 2D (HMQC, 1H - 1H COSY and HMBC) NMR spectra (Figure 2). The relative configuration of the two methyl residues in the benzopyran moiety was determined as *trans* based on the NOESY correlation of 4-CH₃ with 3-H and $J^{3,4}$ (<0.5 Hz); and the relative configuration of the two methyl residues in the benzofuran moiety was determined as *trans* based on the NOESY correlation of 3'-CH₃ with 2'-H and $J^{2',3'}$ (=4.3 Hz) (Figure 3).

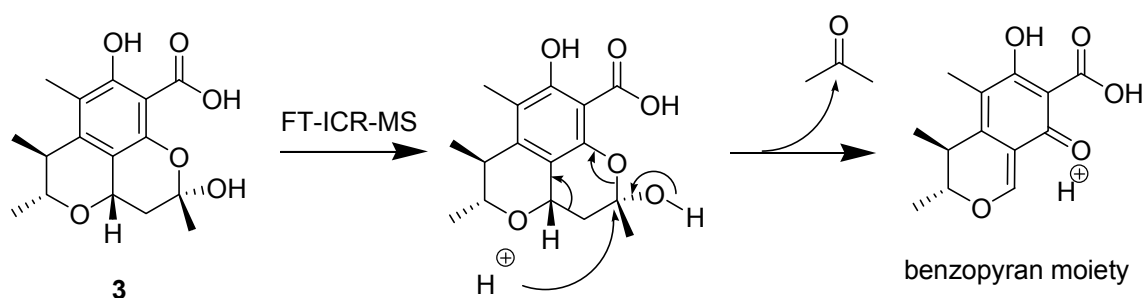
Figure 2. ^1H - ^1H COSY and key HMBC correlations of compounds 1–4.**Figure 3.** Key NOESY effects of compounds 1–3.

Penicitrinol J (**2**) was isolated as a pale yellow, amorphous powder. $[\alpha]_D^{25} -30.0$ (0.001, MeOH). The molecular formula of **2** was determined as $\text{C}_{24}\text{H}_{26}\text{O}_7$ by HRESIMS at m/z 427.17601 $[\text{M} + \text{H}]^+$ (calcd. 427.1757). The ^1H -NMR data of **2** showed four tertiary methyl signals, two aromatic methyl signals, five sp^3 methine protons (three oxygenated), and two hydroxyl protons (Table 1). The ^{13}C -NMR and DEPT spectra for **2** displayed 24 carbon signals including four tertiary methyls, two aromatic methyls, five sp^3 methines (three oxygenated), one carbonyl carbon, and 12 sp^2 quaternary carbons (Table 1). The NMR data were quite similar to those of **1** except for those of the benzopyran moiety. Compared with those of **1**, the NMR spectra of **2** exhibited an additional oxygenated sp^3 methine proton (δ_{H} 5.71) and an additional oxygenated sp^3 methine carbon (δ_{C} 66.3), but missed one carbonyl carbon (δ_{C} 183.8 in **1**). These indicated one of the two additional protons was linked to C-1, the other was the hydroxyl proton of 6-OH, which was further supported by the downfield shift effect of C-4a (+12 ppm) and the high-field shifts effect of H-3 (−1.07 ppm) and H-4 (−0.26 ppm) as a result of the missing of the double

bond between C-1 and C-8a, and the 2D (HMQC, ^1H - ^1H COSY and HMBC) NMR spectra (Figure 2). The NOESY correlation of 3-H with 4-CH₃ and $J^{3,4}$ (=6.1 Hz) established the *trans* of the two methyl residues in the benzopyran moiety; The NOESY correlation of 2'-H with 3'-CH₃ and $J^{2',3}$ (<0.5 Hz) demonstrated the *trans* of the two methyl residues in the benzofuran moiety; and the relative configuration of the 1-H and 3-H was determined as *cis* based on the NOESY correlations of 1-H with 3-H, 5'-OH and 4-CH₃ (Figure 3).

Penicitrinol K (**3**) was isolated as a white, amorphous powder. $[\alpha]_D^{25}$ -125.2 (0.002, MeOH). The benzopyran moiety's molecular formula of **3** was determined as C₁₃H₁₄O₅ by HRESIMS at m/z 273.07299 $[\text{M} + \text{Na}]^+$ (calcd. 273.0739) (Figure 4).

Figure 4. The benzopyran moiety of compound **3**.



The molecular formula of **3** was determined to be C₁₆H₂₀O₆ by ESI-MS at m/z 331.2 $[\text{M} + \text{Na}]^+$ and 291.4 $[\text{M} - \text{H}_2\text{O} + \text{H}]^+$. The ^1H -NMR data of **3** showed three tertiary methyl signals, one aromatic methyl signal, one sp^3 methylene signal, three sp^3 methine protons (two being oxygenated), and one hydroxyl proton (Table 2). The ^{13}C -NMR and DEPT spectra of **3** displayed signals for three tertiary methyls, one aromatic methyl, one sp^3 methylene, three sp^3 methines (two being oxygenated) and eight quaternary carbons (Table 2). Compared to those of **2**, compound **3** shared the same benzopyran moiety with **2** which was further supported by the 2D (HMQC, ^1H - ^1H COSY and HMBC) spectra (Figure 2), and the NMR spectra of **3** exhibited a high-field shift effect of C-1 (-1.01 ppm). The ^1H - ^1H COSY correlations between 1-H and 11-Ha, 1-H and 11-Hb, and key HMBC correlations from 1-H, 11-H and 10-CH₃ to corresponding carbons indicated that C-11 was linked to C-1, C-10 was linked to C-11, and 10-CH₃ was linked to C-10 (Figure 2). Finally C-10 was linked to C-8 via O and 10-OH was linked to C-10, which established by the molecular formula of **3**. The NOESY correlation of 3-H with 4-CH₃ and $J^{3,4}$ (=6.3 Hz) determined the *trans* of 3-CH₃ and 4-CH₃. The NOESY correlations between 1-H and 3-H and 1-H and 10-CH₃ established the *cis* configurations of 1-H and 3-H and 1-H and 10-CH₃ (Figure 3).

Table 2. The NMR data for compound **3** (CDCl₃, δ in ppm).

No.	δ_{H} (mult., J [Hz], int.)	δ_{C}
1	4.70 (dd, 6.1, 11.5)	66.2d
3	3.74 (dq, 6.3, 6.2)	78.8d
3-CH ₃	1.39 (d, 6.2, 3H)	21.6q
4	2.88 (dq, 6.3, 6.9)	38.1d
4-CH ₃	1.23 (d, 6.9, 3H)	19.3q

Table 2. *Cont.*

No.	δ_H (mult., J [Hz], int.)	δ_c
4a	-	145.8s
5	-	118.2s
5-CH ₃	2.14 (s, 3H)	11.1q
6	-	161.3s
6OH	12.16 (s)	-
7	-	97.6s
7-COOH	-	171.6s
8	-	146.9s
8a	-	111.7s
10	-	101.4s
10-CH ₃	1.87 (s, 3H)	29.3q
11a	2.53 (dd, 6.1, 12.8)	37.3t
11b	1.84 (dd, 11.5, 12.8)	

Citrinolactone D (**4**) was obtained as a white, amorphous powder. $[\alpha]_D^{25} +6.7$ (0.004, MeOH). The molecular formula of **4** was determined as C₁₄H₁₄O₄ by HRESIMS at m/z 269.07804 [M + Na]⁺ (calcd. 269.0790). The ¹H-NMR data of **4** showed one aromatic methyl signal, one methoxyl group, two sp³ methylene signals, two aromatic protons, one oxygenated sp³ methine proton, and one hydroxyl proton (Table 3). The ¹³C-NMR spectrum of **4** displayed signals for two methyls (one of them oxygenated), two sp³ methylenes, one oxygenated sp³ methine, two sp² methines, one carbonyl carbon, and 6 sp² quaternary carbons (Table 3). The NMR data were quite similar to those of **7** [5]. By comparison with those of **7**, the NMR spectra of **4** exhibited an additional methoxyl group (δ_H 3.50, δ_c 57.3) attached C-9, and which was further supported by HMBC correlations from 9-OCH₃ to C-9 (Figure 2).

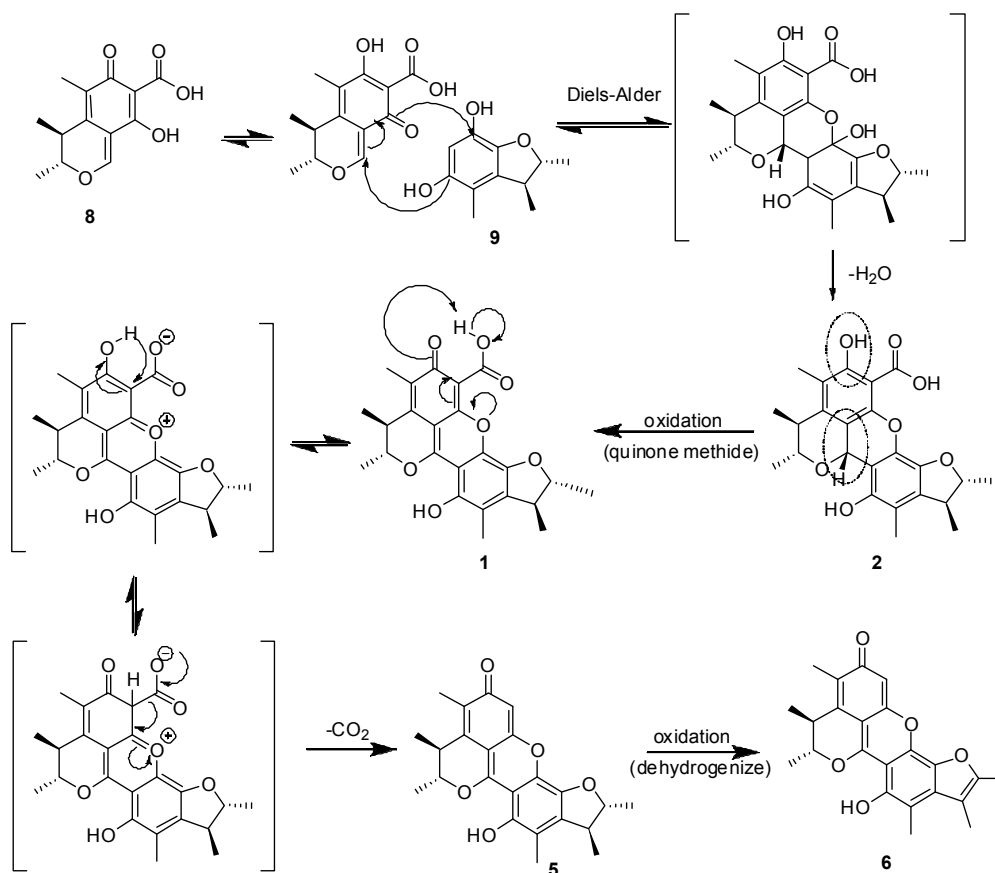
Table 3. The NMR data of compound **4** (CDCl₃, δ in ppm).

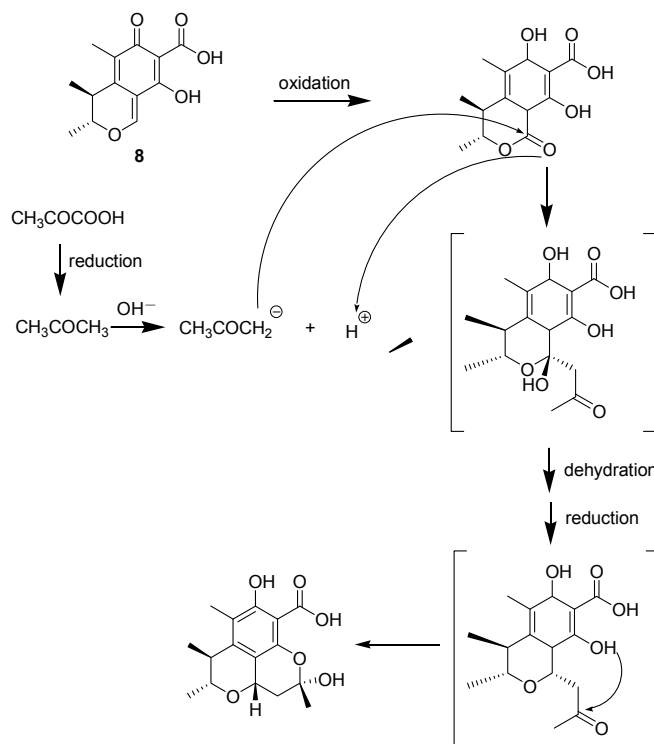
No.	δ_H (mult., J [Hz], int.)	δ_c
1	-	181.1s
3	-	173.9s
4	-	119.9s
4a	-	157.3s
5	6.71 (s)	107.6d
6	-	146.7s
7	6.63 (s)	112.6d
8	-	161.0s
8-OH	12.56 (s)	-
8a	-	109.0s
9	4.95 (d, 6.8)	79.4d
10a	2.35 (m)	27.6t
10b	2.17 (m)	
11a	3.22 (m)	30.2t
11b	2.81 (m)	
12	2.40 (s, 3H)	22.3q
9-OCH ₃	3.50 (s, 3H)	57.3q

2.2. Biosynthesis

These compounds likely have the same biogenetic origin via the polyketide pathway [9]. citrinolactone B (7), citrinin (8), 2,3,4-trimethyl-5,7-dihydroxy-2,3-dihydrobenzofuran (9), phenol A (10) are biosynthesized from acetyl coenzyme A. Citrinolactone D (4) is the result of the methylation of citrinolactone B at 9-OH (7). To explain the biogenetic origin of penicitrinone E (1), penicitrinol J (2) and penicitrinol K (3), a postulated biosynthetic pathway is proposed in Schemes 1,2. Penicitrinone E (1) is postulated to derive from the oxidation of penicitrinol J (2), which results from the Diels-Alder reaction of 8 with 9 and then undergoes dehydration. Subsequent decarboxylation and the following dehydrogenation of penicitrinol J (2), leads successively to penicitrinone A (5) and penicitrinone B (6) [10] (Scheme 1). Oxidation of citrinin (8) forms dihydrocitrinone [11]. After undergoing aldol condensation of the carbonyl group at C-1 of dihydrocitrinone with an acetone anion, which is biosynthesized from pyruvic acid via enzymatic reduction [12], dihydrocitrinone changes to an intermediate structure. Further dehydration, reduction and the following aldol condensation of the intermediate structure yields penicitrinol K (3) (Scheme 2). Based on this biosynthetic scheme, the absolute configurations were deduced as 3*R*, 4*S*, 2'*R*, 3'*S* - for 1, 1*R*, 3*R*, 4*S*, 2'*R*, 3'*S* - for 2, 1*S*, 3*R*, 4*S*, 10*S* - for 3, 9*S* - for 4.

Scheme 1. Postulated Biosynthesis of 1, 2, 5 and 6 resulting from 8 and 9.



Scheme 2. Postulated Biosynthetic Pathway of **3**.

To best of our knowledge, four penicitrinones have been reported: penicitrinones A–B [4], pennicitrinone C [9] and pennicitrinone D [13] and nine penicitrinols have been reported: Penicitrinol A [4], pennicitrinol B [9], penicitrinols C–E [12] and penicitrinols F–I [14]; Three citrinolactones have been reported: citrinolactones A–C [5]. Thus, the four new citrinin derivatives—penicitrinone E (**1**), penicitrinol J (**2**), penicitrinol K (**3**) and citrinolactone D (**4**) enrich the number of penicitrinones, penicitrinols and citrinolactones respectively. Markedly, the 7-COOH of penicitrinone E (**1**) and penicitrinol J–K (**2–3**) were reported in both penicitrinones and penicitrinols for the first time, which complemented the Diels-Alder reaction of citrinin [10,12].

2.3. Cytotoxic and Antimicrobial Activity

The new compounds **1–4** were tested for cytotoxic effects against the HeLa and HepG-2 cell lines using the MTT method [15]. However, they exhibited no remarkable cytotoxic activity against any of the cell lines in the concentration of 10 $\mu\text{g}/\text{mL}$ (100% of cis-platinum as positive control). The results of cytotoxic tests of compounds **1–4** are shown in Table 4.

Table 4. Biological Activities of Compounds **1–4**.

Compound	Inhibitory ratio (%)	
	HeLa	HepG-2
1	-	6.3
2	-	25.1
3	-	9.2
4	4.0	16.1

The antimicrobial activity of compounds 1–4 against *Staphylococcus aureus* (CMCC26003), *Escherichia coli* (CMCC44103), *Candida albicans* (AS2.538), and *Aspergillus niger* (ACCC30005) were also evaluated by paper diffusion method with concentration of 20 µg/6 mm paper disk. Only compounds 2 and 3 showed weak antimicrobial activity against *Staphylococcus aureus* CMCC26003 with inhibition zones of 10 and 9 mm diameter, respectively (18 mm of gentamicin as positive control).

3. Experimental

3.1. General Procedures

Optical rotations were obtained on a PerkinElmer 341 automatic polarimeter. UV spectra were recorded on a Persee TU-1901 spectrophotometer. IR spectra were recorded on a Nicolet Avatar 330FT spectrometer. ¹H-NMR, ¹³C-NMR, and DEPT spectra and 2D NMR spectra were recorded on a Bruker Avance III-600 NMR spectrometer using TMS as internal standard, and chemical shifts were recorded as values. HRESIMS data were measured on a Bruker FT-ICR-MS mass spectrometer. ESIMS was measured on a Finnigan mass spectrometer. TLC was carried out using glass-precoated silica gel GF₂₅₄ (Qingdao Marine Chemical, Inc., Qingdao, China) and visualized under UV light or by spraying with vanillin (contains H₂SO₄) ethanol reagent. Sephadex LH-20 (40–70 µm, Amersham Pharmacia Biotech AB, Uppsala, Sweden), silica gel (200–300mesh, Qingdao Marine Chemical, Inc., Qingdao, China), and Lichroprep reversed-phase RP-18 silica gel (40–63 µm, Merck, Darmstadt, Germany) were used for column chromatography (CC).

3.2. Fungal Material

The fungal strain *Penicillium* sp. ML226 was isolated from the sediment of Fu Gong mangrove region, Long Hai, Taiwan Strait, China. It was identified according to its morphological characteristics and ITS sequence. It was identified as a sporulating fungus by traditional morphology. A BLAST search result showed that the internal transcribed spaces (ITS) sequence of ML226 was highly homologous (96% percent similarity) to that of a *Penicillium* species (JX192960), indicating that ML226 belongs to this genus. The voucher specimen is deposited in our laboratory at –80 °C. The producing strain was prepared on potato dextrose agar slants and stored at 4 °C.

3.3. Fermentation and Extraction

The fungus *Penicillium* sp. ML226 was inoculated on slope of YMG media (glucose 4.0, malt extract 10.0, yeast extract 4.0, pH 7.2) in a 250 mL solanad type flask containing solid media (25 mL/flask) at 28 °C for 4 days to afford spores. Then the spores were obtained by scraping and agitating from the slope of YMG media using 120 mL ddH₂O. Solid media fermentation was performed with YMG media (12 L) at 28 °C for 7 days, and the spores was inoculated with inoculating loop. The cultured agar was chopped, diced and extracted with EtOAc-MeOH-AcOH (80:15:5, 3.5 liters) at room temperature overnight. The organic solution was collected through filtration, and the remaining agar residue was extracted several times more as described above until the filtrate was colourless. The combined filtrates were concentrated under vacuum to remove organic solvents. The aqueous solution was extracted five times with EtOAc to give an EtOAc solution, which was

concentrated under vacuum to give a crude EtOAc extract. Then the EtOAc extract was dissolved with MeOH to give a MeOH solution. The MeOH solution was concentrated under vacuum to give a crude extract (5.00 g).

3.4. Purification

The crude MeOH extract (5.00 g) was subjected to MPLC over RP-18 silica gel (170 g) using a stepwise gradient of 30, 50, 70 and 100% (v/v) MeOH in H₂O. Then we achieved Fr.B (244 mg) and Fr.C (251 mg) obtained from 30% MeOH and Fr.D (560 mg) and Fr.E (362 mg) obtained from 50% MeOH. These fractions were further purified by repeated column chromatography (CC) on Sephadex LH-20 and silica gel.

Fr.B (244 mg) was fractionated by Sephadex LH-20 CC (140 g, eluted with MeOH) to provide five fractions (Ba–Be). Fr.Bb (50.9 mg) was fractionated by Sephadex LH-20 CC (80 g, eluted with acetone) to provide four fractions (Bb1–Bb4). Fr.Bb2 (8.0 mg) was further purified by silica gel CC [step gradient, 0–3% EtOAc in petroleum ether (PE)] to yield **9** (3.4 mg). Fr.Bb4 (3.5 mg) was further purified by silica gel CC (step gradient, 0–10% EtOAc in PE) to afford **10** (1.8 mg).

Fr.C (251 mg) was fractionated by Sephadex LH-20 CC (140 g, eluted with MeOH) to provide seven fractions (Ca–Cg). Fr.Cb (36.6 mg) was further purified by silica gel CC (step gradient, 0–12.5% acetone in PE) to yield **1** (8.5 mg). Fr.Cd (60.0 mg) was fractionated by Sephadex LH-20 CC (80 g, eluted with acetone) to provide four fractions (Cd1–Cd4). Fr.Cd2 (10.0 mg) was further purified by silica gel CC (step gradient, 0–2.5% acetone in PE) to obtain **9** (3.2 mg). Fr.Cg (34, 2 mg) was further purified by silica gel CC (step gradient, 0–1.5% EtOAc in PE with 0.5% HCOOH) to produce **2** (4.0 mg) and **1** (2.2 mg).

After settling, some crystals of **8** (80.7 mg) appeared in Fr.D (560 mg) and the remaining mother liquor solution (463 mg) was fractionated by Sephadex LH-20 CC (140 g, eluted with MeOH) to provide seven fractions (Da–Dg). Fr.Dc (73.6 mg) was fractionated by Sephadex LH-20 CC [80 g, eluted with MeOH/acetone (1:4)] to provide four fractions (Dc1–Dc4). Fr.Dc3 (26.0 mg) was further purified by silica gel CC (step gradient, 0–50% EtOAc in PE) to yield **6** (3.7 mg).

Fr.E (362 mg) was fractionated by Sephadex LH-20 CC (140g, eluted with MeOH) to provide five fractions (Ec–Ee). Fr.Ec (55.1 mg) was fractionated by Sephadex LH-20 CC [80 g, eluted with MeOH/acetone (1:4)] to provide three fractions (Ec1–Ec3). Fr.Ec2 (24.0 mg) was further purified by silica gel CC (step gradient, 0–25% acetone in PE) to yield **5** (3.0 mg). Fr.Ee (121.9 mg) was fractionated by Sephadex LH-20 CC (140g, eluted with acetone) to provide four fractions (Ee1–Ee4). Fr.Ee2 (12.9 mg) was further purified by silica gel CC (step gradient, 0–2.5% acetone in PE) to yield **3** (3.2 mg). Fr.Ee3 (43.0 mg) was further purified by silica gel CC (step gradient, 0–3.3% acetone in PE) to yield **4** (7.1 mg). Fr.Ee4 (73.0 mg) was fractionated by Sephadex LH-20 CC [80 g, eluted with MeOH/acetone (1:4)] to provide three fractions ((Ee41–Ee43). Fr.Ee43 (36.9 mg) was further purified by silica gel CC (step gradient, 0–3.3% acetone in PE) to obtain **7** (2.6 mg).

3.5. Biological Assays

Cancer cell lines were derived from the cell bank of the Chinese Academy of Sciences. The cytotoxicities of the compounds **1–5** were measured by the MTT (Sigma) assay [15]. The cells in 100 μL of culture medium were plated in each well of 96-well plates (Falcon, CA). After 24 h of incubation for a density of $5 \times 10^3/100 \mu\text{L}$ medium, the cells were treated in triplicate with the concentration of 10 $\mu\text{g/mL}$ of every compound for 72 h at 37 $^\circ\text{C}$. A 20 μL aliquot of MTT solution (5 mg/mL) was added directly to all wells and incubated for 4 h at 37 $^\circ\text{C}$. To quench the reaction, 100 μL of triplex solution (10% SDS, 5% isobutanol, 12 mM HCl) was added to each well and incubated overnight at 37 $^\circ\text{C}$. The optical density of each well was measured with a microplate reader (M-3350, Bio-Rad) at 595nm (excitation). Growth inhibition rates were calculated with the following equation:

$$\text{Inhibition rate} = \frac{OD_{\text{control}} - OD_{\text{treated}}}{OD_{\text{control}} - OD_{\text{blank}}} \times 100 \%$$

3.6. Spectral Data

Penicitrinone E (1): red, amorphous powder; $[\alpha]_D^{25}$ 0 (0.002, MeOH); UV (MeOH) λ_{max} (log ϵ) 204 (2.74), 214 (2.76), 232 (2.58), 258 (2.37), 278 (2.49), 315 (2.34), 400 (2.49) nm; IR (KBr) ν_{max} 3453, 2967, 2928, 2873, 2358, 2332, 1687, 1641, 1611, 1527, 1506, 1451, 1408, 1380, 1325, 1269, 1154, 1101, 1060, 1026, 993, 933, 899, 858, 822, 748, 700, 675, 652, 552, 505 cm^{-1} ; $R_f = 0.318$ (PE : acetone = 1:1), $R_f = 0.611$ (PE : acetone = 1:1, with formic acid); ^1H - and ^{13}C -NMR (see Table 1); HRESIMS m/z 425.15965 $[\text{M} + \text{H}]^+$ (calcd. for $\text{C}_{24}\text{H}_{25}\text{O}_7$, 425.1600).

Penicitrinol J (2): pale yellow, amorphous powder; $[\alpha]_D^{25}$ -30.0 (0.001, MeOH); UV (MeOH) λ_{max} (log ϵ) 203 (3.05), 215 (3.12), 257 (2.61), 277 (2.56), 315 (2.55), 400 (2.51) nm; IR (KBr) ν_{max} 3343, 2967, 2925, 2869, 2364, 2338, 1684, 1636, 1586, 1527, 1504, 1451, 1421, 1385, 1328, 1274, 1239, 1165, 1136, 1074, 1022, 996, 974, 929, 907, 858, 815, 778, 754, 729, 704, 636, 550 cm^{-1} ; $R_f = 0.591$ (PE : acetone = 1:1), $R_f = 1$ (PE:acetone = 1:1, with formic acid); ^1H - and ^{13}C -NMR (see Table 1); HRESIMS m/z 427.17601 $[\text{M} + \text{H}]^+$ (calcd. for $\text{C}_{24}\text{H}_{27}\text{O}_7$, 427.1757).

Penicitrinol K (3): white, amorphous powder; $[\alpha]_D^{25}$ -125.2 (0.002, MeOH); UV (MeOH) λ_{max} (log ϵ) 213(2.68), 257 (2.34), 325 (1.90) nm; IR (KBr) ν_{max} 3433, 3230, 2970, 2930, 2872, 2360, 2334, 1687, 1623, 1589, 1445, 1419, 1386, 1353, 1298, 1270, 1232, 1200, 1169, 1105, 1079, 1025, 905, 876, 809, 753, 729, 683, 582, 549 cm^{-1} ; $R_f = 0.524$ (CHCl_3 : MeOH = 10:1); ^1H - and ^{13}C -NMR (see Table 2); The citrinin moiety's HRESIMS m/z 273.07299 $[\text{M} + \text{Na}]^+$ (calcd. for $\text{C}_{13}\text{H}_{14}\text{O}_5 \text{Na}$, 273.0739); ESIMS m/z 331.2 $[\text{M} + \text{Na}]^+$ and 291.4 $[\text{M} - \text{H}_2\text{O} + \text{H}]^+$.

Citrinolactone D (4): white, amorphous powder; $[\alpha]_D^{25}$ $+6.7$ (0.004, MeOH); UV (MeOH) λ_{max} (log ϵ) 204 (2.35), 229 (2.45), 237 (2.49), 257 (2.31), 325 (1.80) nm; IR (KBr) ν_{max} 3437, 2965, 2924, 2824, 2365, 2337, 1653, 1622, 1599, 1490, 1451, 1364, 1296, 1253, 1206, 1141, 1083, 1031, 1011, 941, 922, 869, 822, 784, 757, 698, 656, 619, 570, 513, 471 cm^{-1} ; $R_f = 0.675$ (PE : acetone = 2:1); ^1H - and ^{13}C -NMR (see Table 3); HRESIMS m/z 269.07804 $[\text{M} + \text{Na}]^+$ (calcd. for $\text{C}_{14}\text{H}_{14}\text{O}_4 \text{Na}$, 269.0790).

4. Conclusions

Four new compounds, penicitrinone E (**1**), penicitrinol J (**2**), penicitrinol K (**3**) and citrinolactone D (**4**), were isolated together with six known compounds from the marine-derived *Penicillium* sp. ML226. Penicitrinone E (**1**), penicitrinol J (**2**) and penicitrinol K (**3**) showed modest selective cytotoxicity against HepG-2 cell line. Citrinolactone D (**4**) showed weak cytotoxicity against HepG-2 and HeLa cell lines. penicitrinol J (**2**) and penicitrinol K (**3**) showed mild antimicrobial activity against *Staphylococcus aureus*.

Supplementary Materials

The NMR spectra and HRESIMS spectra of compounds **1–4** can be accessed at: <http://www.mdpi.com/1420-3049/18/5/5723/s1>.

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Conflict of Interest

The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds **1–10** are available from the authors.

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