Citrinin Derivatives from the Marine-Derived Fungus *Penicillium citrinum*

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Three new citrinin derivatives, penicitrinols C, D, and E (1—3), along with two known compounds, citrinin (4) and decarboxydihydrocitrinone (5), were isolated from *Penicillium citrinum*. Their structures were determined by spectroscopic methods and X-ray diffraction analysis. Compounds 1 and 3 demonstrated weak cytotoxicity against the HL-60 cell line.

Key words penicitrinol; citrinin derivative; cytotoxicity; *Penicillium citrinum*

Marine microorganisms have attracted considerable attention as important sources of structurally diverse secondary metabolites and as potential leads for drug discovery.[1—4] In an effort to search for new anticancer compounds, more than 300 microbial strains isolated from sediment samples collected from the Min River estuary in China were screened for cytotoxicity against HL-60 cells. Among them, a fungal strain identified as *Penicillium citrinum* exhibited significant cytotoxic activity. We investigated the secondary metabolites of this fungus, and obtained three new citrinin derivatives, penicitrinols C—E (**1**—**3**), and two known compounds, citrinin (**4**) and decarboxydihydrocitrinone (**5**). Herein, we describe the isolation and structural elucidation of these new compounds.

The bioactive ethyl acetate extract of *P. citrinum* was seperated by chromatography on Si gels and Sephadex LH-20 columns and purified by reversed-phase HPLC to yield the five compounds (**1**—**5**).

Results and Discussion

The molecular formula of compound **1** was determined to be $C_{15}H_{20}O_4$ on the basis of high resolution-electrospray ionization-mass spectra (HR-ESI-MS). The infrared (IR) spectrum exhibited absorption bands at 3358 and 1700 cm^{-1}, indicating the presence of OH and CO groups. The ^{1}H-NMR spectrum of **1** indicated the presence of two exchangeable protons (δ 8.94, 9.16), an aromatic proton (δ 6.26), two protons attached to oxygenated carbons (δ 5.07, 3.86), and four CH_3 groups (δ 2.13, 1.94, 1.17, 1.01). The ^{13}C-NMR and distortionless enhancement by polarization transfer (DEPT) data indicated the presence of a carbonyl carbon (δ 207.7), six aromatic carbons (two of which were oxygenated (δ 154.8, 151.4) and one of which was protonated (δ 100.7)), two oxymethine carbons (δ 72.2, 66.1), one methylene carbon (δ 49.8), a methine carbon (δ 35.5), and four methyl carbons (δ 30.6, 20.7, 18.7, 10.4). The connectivities among these groups and carbons were deduced from the correlation spectroscopy (COSY) and heteronuclear multiple bond connectivity (HMBC) spectra (Fig. 2). The nuclear Overhauser effect spectroscopy (NOESY) correlations between H-3/H-12, H-4/H-11, and H-1/H-11 observed in **1** indicated that H-1, H-4, and Me-11 were on the same side while the acetonyl and Me-12 were located on the reverse side. This deduction was further supported by the result of X-ray diffraction (Fig. 3).

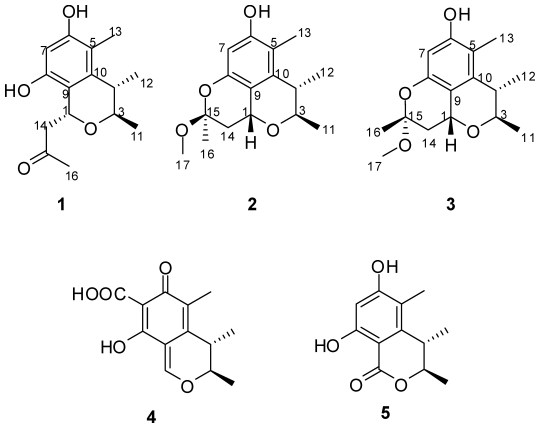

Fig. 1. Structures of Compounds **1**—**5**

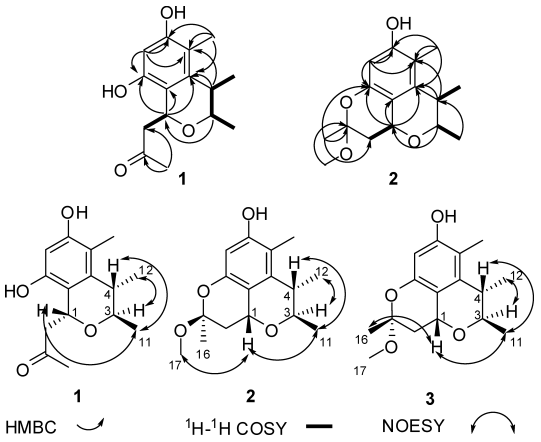

Fig. 2. Key ^{1}H–^{1}H COSY, HMBC, and NOESY Correlations of Compounds **1**—**3**

Compound **2** was isolated as a white crystal with the molecular formula $C_{16}H_{22}O_4$ as determined by HR-ESI-MS (*m*/*z*: 277.1433 $[M-H]^-$, Calcd for $C_{16}H_{21}O_4$, 277.1440). The 1D-NMR data of **2** indicated that it was structurally related to **1**, except for the absence of a ketone carbonyl (δ_C 207.7), the presence of an additional methoxyl group (δ_H 3.34 and δ_C 49.0), and an additional quaternary carbon (δ_C 100.8). A detailed analysis of COSY and HMBC spectra, especially the HMBC correlations of H-16 with C-1, C-8, C-14, and C-15, and H-17 with C-15, revealed that a methoxyl group and a quaternary carbon in **2** replaced a ketone car-

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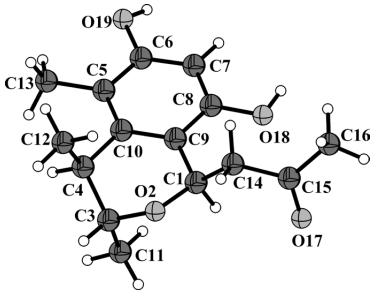

Fig. 3. X-Ray Structure of Penicitrinol C (**1**)

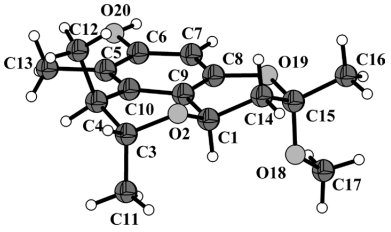

Fig. 4. X-Ray Structure of Penicitrinol D (**2**)

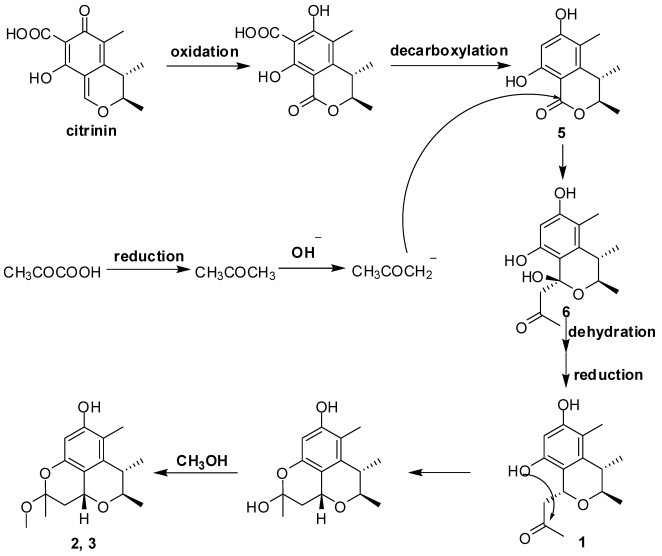

Fig. 5. Plausible Biosynthetic Pathway of Compounds **1**—**3**

bonyl in **1** to form a new acetal cycle, consistent with the molecular formula. Furthermore, NOESY data and the results of X-ray diffraction indicated that the relative configuration of **2** was the same as **1** (Fig. 4).

Compound **3** had the same molecular formula as **2** ($C_{16}H_{22}O_4$) as revealed by HR-ESI-MS data (*m/z*: 277.1460 $[M-H]^-$, Calcd for $C_{16}H_{21}O_4$, 277.1440). The NMR (^{1}H-NMR, ^{13}C-NMR, COSY, ^{1}H-detected heteronuclear multiple quantum coherence (HMQC), and HMBC) data revealed that both **3** and **2** possessed the same planar structure. However, the NOESY correlations of H-1 with H-11 and H-16, and H-4 with H-11 suggested **3** and **2** were stereoisomers that differed in the configuration of C-15.

To explain the biogenetic origin of penicitrinols C, D, and E (**1**—**3**), a plausible biosynthetic pathway is proposed in Fig. 5. Oxidation and decarboxylation of citrinin originated from acetyl coenzyme A forms compound **5**. Subsequent aldol condensation of the carbonyl group at C-1 of **5** with an acetone anion, which is biosynthesized from pyruvic acid *via* enzymatic reduction, leads to an intermediate structure **6**. Further dehydration and the following reduction of **6** yields penicitrinol C (**1**). Finally, acetalization between the carbonyl group on the acetonyl group at C-1 and the hydroxyl group at C-8 of **1** produces penicitrinol D (**2**) and penicitrinol E (**3**). Based on this biosynthetic scheme, the absolute configurations were deduced as 1*R*, 3*R*, 4*S*- for **1**, 1*R*, 3*R*, 4*S*, 15*S*- for **2**, and 1*R*, 3*R*, 4*S*, 15*R*- for **3** (Fig. 1).

Compounds **1**—**3** were evaluated for cytotoxicity against the P388 and HL-60 cell lines by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.[5] Compounds **1** and **3** showed weak cytotoxicity against HL-60 cells, with IC_{50} values of 52.8 and 41.2 μmol/l (Table 2).

Mytotoxin citrinin, produced by several fungal species that belong mainly to the genera *Penicillium* and *Monascus*, is widely considered a hazardous contaminant of foods and animal feeds.[6] Until now, a large number of citrinin derivatives have been isolated.[7] From the degree of polymerization, they can be seperated into two large groups, monomeric and dimeric derivatives.[8—13] The former usually contain one or two rings, while the latter usually contain four or five rings. To the best of our knowledge, the monomeric derivatives with tricycle skeletons are rare in nature and plausible biosynthetic pathways are postulated above.

Experimental

General Experimental Procedures Optical rotations were obtained from a Shenguang SGW-1 digital polarimeter. UV spectra were recorded on a Shimadzu UV-2450 spectrophotometer. IR spectra were recorded on a Nicolet Nexus spectrophotometer. ^{1}H-NMR, ^{13}C-NMR, DEPT spectra and 2D-NMR were recorded on a BRUKER BIOSPIN AVANCE III spectrometer using tetramethylsilane (TMS) as the internal standard. ESI-MS were obtained by an AGILENT 1200/Q-TOF 6510 LC mass spectrometer. Semi-preparative HPLC was performed using an ODS column (ODS-A, 10×250 mm, 5 μm) at 5 ml/min.

Fungal Material The fungus *P. citrinum* was isolated from marine sediments collected from Langqi Island, Fujian, China. It was identified according to its morphological characteristics and ITS by Beijing Sunbiotech Co., Ltd., and preserved in our laboratory at −80 °C. The producing strain was prepared on Martin medium and stored at 4 °C.

Fermentation and Extraction The fungus was cultured under static conditions at 28 °C for 30 d in 1000-ml conical flasks containing the liquid medium (400 ml/flask) composed of glucose (10 g/l), maltose (20 g/l), mannitol (20 g/l), monosodium glutamate (10 g/l), KH_2PO_4 (0.5 g/l), $MgSO_4\cdot 7H_2O$ (0.3 g/l), yeast extract (3 g/l), and seawater. The fermented whole broth (60 l) was filtered through cheese cloth to separate supernatant from mycelia. The former was extracted two times with ethyl acetate to yield an ethyl acetate solution that was concentrated under reduced pressure to give a crude extract (32.0 g).

Purification The crude extract (32.0 g) was separated into 11 fractions on a Si gel column using a step gradient elution of petroleum ether, CH_2Cl_2, and MeOH. Fraction 4 (4.8 g) eluted with petroleum ether/CH_2Cl_2 (1 : 1) was further purified on a Sephadex LH-20 (CH_2Cl_2 : MeOH, 1 : 1) and a reversed-phase column (MeOH : H_2O, 3 : 2) to give compound **4** (58.4 mg). Fraction 9 (5.6 g) eluted with CH_2Cl_2/MeOH (50 : 1) was further separated on a Sephadex LH-20 (CH_2Cl_2 : MeOH, 1 : 1). Subfraction 9-8 (650 mg) was purified by a reversed-phase column (MeOH : H_2O, 3 : 2) and semipreparative HPLC (45% CH_3CN), yielding compounds **1** (37.7 mg), **2** (52.3 mg), **3** (29.5 mg), and **5** (17.9 mg).

X-Ray Crystallography of 1 $C_{15}H_{20}O_4$, molecular weight (MW)= 264.31, space group $P4_3$, a=7.9954(11) Å, b=7.9954(11) Å, c=22.341(5) Å, V=1428.2(4) Å^3, Z=4. The X-ray diffraction intensity data of **1** were collected on a Rigaku Saturn 724 CCD diffractometer with graphite-monochromater Mo$K\alpha$ radiation (λ=0.71073 Å) by the ω scan technique [$2\theta \leq 51°$]. A total of 2634 reflections were collected, of which 2502 with $|F|^2 \geq 2\sigma|F|^2$ were observed. The structure was solved by direct methods and refined by full-matrix least-squares procedure to R_1=0.0628 and wR_2=0.1436. H atoms bonded to C atoms were refined in idealized positions using the rid-

Table 1. ^{1}H- and ^{13}C-NMR Data (400, 100 MHz, *J* in Hz and δ in ppm) of Compounds **1**—**3**

Position	**1**[a]		**2**[b]		**3**[b]	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	5.07 (H, dd, 9.6, 2.5)	66.1 d	4.90 (H, dd, 12.2, 4.8)	59.4 d	4.58 (H, dd, 12.3, 5.0)	61.4 d
3	3.86 (H, dq, 1.9, 6.4)	72.2 d	4.13 (H, q, 6.8)	75.0 d	4.13 (H, q, 6.8)	74.8 d
4	2.54 (H, dq, 1.9, 6.7)	35.5 d	2.65 (H, q, 6.9)	35.1 d	2.62 (H, q, 7.0)	34.6 d
5		112.4 s		114.4 s		114.9 s
6		154.8 s		154.0 s		153.8 s
7	6.26 (H, s)	100.7 d	6.20 (H, s)	100.3 d	6.28 (H, s)	101.7 d
8		151.4 s		149.3 s		150.2 s
9		113.3 s		111.8 s		115.3 s
10		138.0 s		136.9 s		135.8 s
11	1.01 (3H, d, 6.4)	18.7 q	1.38 (3H, d, 6.8)	18.6 q	1.32 (3H, d, 6.8)	18.1 q
12	1.17 (3H, d, 6.7)	20.7 q	1.21 (3H, d, 6.9)	22.4 q	1.24 (3H, d, 7.0)	21.5 q
13	1.94 (3H, s)	10.4 q	2.09 (3H, s)	10.0 q	2.09 (3H, s)	9.9 q
14	2.45 (H, dd, 14.5, 9.6)	49.8 t	1.82 (H, t, 12.2)	38.6 t	1.99 (H, t, 12.3)	41.2 t
	3.14 (H, dd, 14.5, 2.5)		2.30 (H, dd, 12.2, 4.8)		2.39 (H, dd, 12.3, 5.0)	
15		207.7 s		100.8 s		102.9 s
16	2.13 (3H, s)	30.6 q	1.54 (3H, s)	23.8 q	1.62 (3H, s)	25.5 q
17			3.34 (3H, s)	49.0 q	3.34 (3H, s)	49.0 q
OH-6	8.94 (H, br s)		5.53 (H, br s)		5.18 (H, br s)	
OH-8	9.16 (H, br s)					

a) DMSO-d_6 as solvent. *b*) $CDCl_3$ as solvent.

Table 2. Cytotoxicity of Compounds **1**—**3** in Two Cancer Cell Lines

Compounds	Cytotoxicity (IC_{50}, μmol/l)	
	P388 cells	HL-60 cells
1	>100	52.8
2	>100	>100
3	>100	41.2

ing-model. All calculations were carried out on a personal computer using the SHELX-97 program system.

X-Ray Crystallography of 2 $C_{16}H_{22}O_4$, MW=278.34, space group $P2_1$, a=8.1789(16) Å, b=6.2628(13) Å, c=14.945(3) Å, V=745.7(3) Å^3, Z=2. The X-ray diffraction intensity data of **2** were collected on a Rigaku Saturn 724 CCD diffractometer with graphite-monochromater Mo$K\alpha$ radiation (λ=0.71073 Å) by the ω scan technique [$2\theta \leq 51°$]. A total of 2737 reflections were collected, of which 2582 with $|F|^2 \geq 2\sigma|F|^2$ were observed. The structure was solved by direct methods and refined by full-matrix least-squares procedure to R_1=0.0448 and wR_2=0.1005. H atoms bonded to C atoms were refined in idealized positions using the riding-model. All calculations were carried out on a personal computer using the SHELX-97 program system.

Supporting Information Available The X-ray crystallographic data for the structures of **1** and **2** have been deposited at the Cambridge Crystallographic Data Centre (CCDC 797919 and 797920). Copies of the data can be obtained free of charge by applying to CCDC, 12 Union Road, Cambridge CB2 1EZ, U.K. (fax: +44 1223 336033; e-mail: deposit@ccdc.cam.ac.uk).

Penicitrinol C (**1**): Obtained as white crystals (H_2O/MeOH); $[\alpha]_D^{25}$ +33.2° (c=1.2, CH_3CN); UV λ_{max} (CH_3CN) nm (log ε): 286 (2.26); IR (KBr) ν_{max} 3358, 2966, 2925, 1700, 1606, 1460, 1375, 1249, 1161, 1107, 1051 cm^{-1}; ^{1}H- and ^{13}C-NMR (see Table 1); HR-ESI-MS *m/z*: 263.1276 $[M-H]^-$ (Calcd for $C_{15}H_{19}O_4$, 263.1283).

Penicitrinol D (**2**): Obtained as white crystals (H_2O/MeOH); $[\alpha]_D^{25}$ +25.6° (c=1.6, CH_3CN); UV λ_{max} (CH_3CN) nm (log ε): 287 (2.98); IR (KBr) ν_{max} 3315, 2968, 2932, 1607, 1381, 1159, 1140, 1106, 1078, 1046, 1009 cm^{-1}; ^{1}H- and ^{13}C-NMR (see Table 1); HR-ESI-MS *m/z*: 277.1433 $[M-H]^-$ (Calcd for $C_{16}H_{21}O_4$, 277.1440).

Penicitrinol E (**3**): Obtained as white solid; $[\alpha]_D^{25}$ −6.9° (c=1.1, CH_3CN); UV λ_{max} (CH_3CN) nm (log ε): 283 (3.31); IR (KBr) ν_{max} 3299, 2968, 2928, 1605, 1455, 1383, 1277, 1159, 1106, 1078, 1052 cm^{-1}; ^{1}H- and ^{13}C-NMR (see Table 1); HR-ESI-MS *m/z*: 277.1460 $[M-H]^-$ (Calcd for $C_{16}H_{21}O_4$, 277.1440).

Biological Assays Cytotoxic activity was evaluated by the MTT method using P388 and HL-60 cell lines. The cell lines were grown in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) under a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Those cell suspensions (200 μl) at a density of 5×10^4 cell ml^{-1} were plated in 96-well microtiter plates and incubated for 24 h at the above condition. The test compound solution (2 μl in dimethyl sulfoxide (DMSO)) at different concentrations was added to each well and further incubated for 72 h in the same condition. Then 20 μl of the MTT solution (5 mg/ml in RPMI-1640 medium) was added to each well and incubated for 4 h. The old medium containing MTT (150 μl) was then gently replaced by DMSO and pipetted to dissolve any formazan crystals formed. Absorbance was then determined on a Spectra Max Plus plate reader at 540 nm.

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