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NOTE

Pennicitrinone D, a new citrinin dimer from the halotolerant fungus *Penicillium notatum* B-52

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Halotolerant microorganisms are considered to be extreme microbes because they thrive in high-salt environments, such as marine, salt lake, soda lake, the Dead Sea, salt field, etc.[1,2] It has been postulated that extreme environments, such as high-salt could awaken some silent genes and activate some unique biosynthesis pathways and, thus, make it possible to produce structurally unique and biologically active secondary metabolites.[3,4] In the course of our search for new bioactive compounds from halotolerant fungi,[5–8] a fungal strain, B-52, identified as *Penicillium notatum*, was observed to produce cytotoxic metabolites against the mouse temperature-sensitive *cdc2* mutant cell line, tsFT210. This strain was isolated from salt sediments collected in Qinghai Lake, Qinghai, China. Bioassay-guided fractionation led to the identification of a new citrinin dimer, pennicitrinone D (**1**), along with three known compounds, pennicitrinone A (**2**),[9] citrinin (**3**)[10–12] and mycophenolic acid (**4**) (Figure 1).[13,14]

MATERIALS AND METHODS

General experimental procedures

Optical rotations were obtained on a JASCO P-1020 digital polarimeter (JASCO, Tokyo, Japan). UV spectra were recorded on a Beckmen DU 640 spectrophotometer (Beckman Coulter, Beijing, China). IR spectra were taken on a Nicolet Nexus 470 spectrophotometer (Thermo Scientific, Beijing, China) in KBr disks. 1D- and 2D-NMR spectra were recorded on a JEOL JNM-ECP 600 spectrometer (JEOL Ltd, Beijing, China) using tetramethylsilane as the internal standard, and chemical shifts were recorded as δ-values. Electrospray ionization mass spectrometry was measured on a Q-Tof Ultima Global GAA076 LC mass spectrometer (Waters Asia, Ltd, Singapore). Semi-preparative HPLC was performed on a SHIMAD-ZU LC-6AD liquid chromatograph with an SPD-M10A vp diode array detector (both from Shimadzu International Trading Co. Ltd, Beijing, China).

Strain

The fungus, *P. notatum* B-52, was isolated from salt sediments collected from the Qinghai Lake, Qinghai Province of China. It was identified according to its morphological characteristics and preserved in the China Center for Type Culture Collection (No. CCTCC M205047). The working strain was prepared on potato dextrose agar slants containing 10% NaCl and stored at 4 °C.

Fermentation

The producing strain, *P. notatum* B-52, was inoculated into a 500-ml conical flask containing 100 ml of the liquid medium composed of mannitol (20 g l^{-1}), maltose (20 g l^{-1}), glutamine (10 g l^{-1}), glucose (10 g l^{-1}), yeast extract (3 g l^{-1}), NaCl (80 g l^{-1}), KCl (10 g l^{-1}), $MgSO_4$ (10 g l^{-1}) and seawater (adjusted to pH 6.5 before sterilization) and cultured at 28 °C for 48 h on a rotary shaker at 120 r.p.m. The seed culture was transferred into two-hundred 500-ml conical flasks (150 ml per flask), and fermentation was carried out at 28 °C for 10 days with an agitation rate of 120 r.p.m.

Extraction and isolation

The fermented whole broth (30 l) of *P. notatum* B-52 was filtered through a cheesecloth to separate into filtrate and mycelia. The filtrate was concentrated under reduced pressure to approximately a quarter of the original volume and then extracted thrice with ethyl acetate. The mycelia were extracted thrice with acetone. The acetone solution was concentrated under reduced pressure to afford an aqueous solution. The aqueous solution was extracted thrice with ethyl acetate. The combined ethyl acetate extract was concentrated under reduced pressure to give a crude extract (30 g). The crude extract was separated into five fractions on a flash silica gel column using a step gradient elution of *n*-hexane-EtOAc and then of EtOAc-MeOH. Fractions 2 (1.5 g) and 3 (1.3 g) showed cytotoxicity against tsFT210 cells. Fraction 2 was further separated into four sub-fractions over a silica gel column eluted with *n*-hexane-EtOAc (20:80). Sub-fractions 2-3 (45 mg) and 2-4 (50 mg) were separated by semi-preparative HPLC on a Shin-pak octadecylsilyl column using MeOH-H_2O (50:50 and 70:30, respectively) as an eluting solvent to give **3** (22 mg) and **4** (36 mg), respectively. Fraction 4 (2 g) was further purified into three sub-fractions by vacuum silica gel column chromatography using a step gradient elution of $CHCl_3$-MeOH. Sub-fractions 4-2 (350 mg) were subjected to chromatography over Sephadex LH-20 eluted with $CHCl_3$-MeOH (1:1), and the obtained fraction was further purified by

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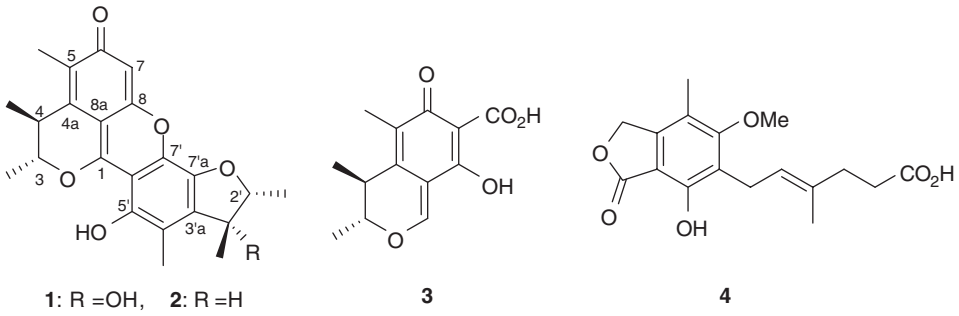


Figure 1 Structures of compounds **1**–**4.**

Table 1 ^{1}H and ^{13}C NMR (600 and 150 MHz) data for **1** and **2**[a]

	1		*2*	
Position	*^{1}H (J in Hz)*	*^{13}C*	*^{1}H (J in Hz)*	*^{13}C*
1	—	156.0 s	—	155.4 s
3	4.89 q (6.0)	82.9 d	4.98 q (6.8)	82.3 d
4	2.97 q (7.3)	34.8 d	3.13 q (7.3)	35.0 d
4a	—	130.0 s	—	130.8 s
5	—	131.8 s	—	131.9 s
6	—	183.8 s	—	184.4 s
7	6.24 s	103.5 d	6.37 s	103.3 d
8	—	157.4 s	—	158.0 s
8a	—	99.6 s	—	100.1 s
3-Me	1.28 d (6.0)	18.7 q	1.45 d (6.8)	18.9 q
4-Me	1.29 d (7.3)	19.1 q	1.32 d (7.3)	19.1 q
5-Me	1.91 s	10.4 q	2.12 s	10.7 q
2	5.07 q (6.0)	89.6 d	4.62 dq (4.1, 6.4)	88.0 d
3	—	80.7 s	3.17 dq (4.1, 6.9)	44.6 d
3a	—	140.5 s	—	139.2 s
4	—	117.0 s	—	116.4 s
5	—	146.8 s	—	147.4 s
6	—	101.7 s	—	102.2 s
7	—	135.8 s	—	135.8 s
7a	—	138.1 s	—	137.8 s
2-Me	1.55 d (6.0)	14.5 q	1.42 d (6.4)	20.9 q
3-Me	1.43 s	21.1 q	1.34 d (6.9)	18.8 q
4-Me	2.32 s	9.4 q	2.21 s	11.5 q
3-OH	4.98	—	—	—
5-OH	7.99	—	8.36	—

Abbreviations: COSY, correlated spectroscopy; ^{13}C NMR, carbon-13 nuclear magnetic resonance; DEPT, distortionless enhancement by polarization transfer; HMQC, heteronuclear multiple quantum coherence; HMBC, heteronuclear multiple bond coherence.
[a]The assignments were based on DEPT, ^{1}H-^{1}H COSY, HMQC and HMBC experiments, and recorded in $CDCl_3$.

preparative HPLC (60% MeOH as the eluant) to yield compound **1** (9 mg). By the same procedure, compound **2** (16 mg) was obtained from the sub-fraction 4-3 (750 mg).

Biological assay

Active fractions were assayed using the methylthiazoletetrazolium (MTT) method[15] with the mouse temperature-sensitive *cdc2* mutant cell line, tsFT210. Compounds **1**–**4** were evaluated for cytotoxic effects on P388 and HL-60 cell lines using the MTT method and on A-549 and BEL-7402 cell lines using the sulforhodamine B (SRB) method.[16]

In the MTT assay, cell lines were grown in RPMI-1640 supplemented with 10% fetal bovine serum under a humidified atmosphere of 5% CO_2 and 95% air at 37 °C (tsFT210 cell line at 32 °C). Cell suspensions of 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 conditions. Next, 2 µl of the test compounds in DMSO at different concentrations was added to each well and further incubated for 72 h under the same conditions. MTT solution (20 µl, 5 mg ml^{-1} in IPMI-1640 medium) was added to each well and incubated for 4 h. Old medium (150 µl) containing MTT was then gently replaced by DMSO and pipetted to dissolve any formazan crystals that had formed. Absorbance was then determined on a Spectra Max Plus plate reader at 540 nm.

In the SRB assay, 200-µl portions of cell suspension were plated in 96-well plates at a density of 2×10^5 cell ml^{-1}. Then, 2 µl of the test solutions (in MeOH) was added to each well and the culture was further incubated for 24 h. The cells were fixed with 12% TCA and the cell layer was stained with 0.4% SRB. The absorbance of the SRB solution was measured at 515 nm. Dose-response curves were generated, and the IC_{50} values, the concentration of compound required to inhibit cell proliferation by 50%, were calculated from the linear portion of log dose-response curves.

Compound **1**: yellow amorphous powder; $[\alpha]_D^{17}$+111. 1 (*c* 0.16, $CHCl_3$); high-resolution electrospray ionization mass spectrometry (HRESI-MS) *m/z* 397.1624 (calcd for $C_{23}H_{25}O_6$, 397.1651); UV ($CHCl_3$) λ_{max} (logε) nm 204 (4.85), 227 (4.93), 272 (4.43), 436 (4.38); UV (MeOH) λ_{max} (logε) nm 202 (4.54), 225 (4.58), 270 (4.23), 274 (4.31), 430 (4.19); IR ν_{max} cm^{-1} (KBr) 3450, 2972, 2930, 1616, 1508, 1447, 1360, 1189, 1136, 1023, 890, 838; ^{1}H ($CDCl_3$, 600 MHz) and ^{13}C ($CDCl_3$, 150 MHz), see Table 1.

Compound **2**: yellow amorphous powder; $[\alpha]_D^{17}$+106.9 (*c* 0.36, $CHCl_3$); HRESI-MS *m/z* 381.1673 (calcd for $C_{23}H_{25}O_5$, 381.1702); UV ($CHCl_3$) λ_{max} (logε) nm 202 (3.46), 226 (3.57), 278 (4.35), 426 (4.24); UV (MeOH) λ_{max} (logε) nm 201 (4.60), 225 (4.50), 267 (4.46), 277 (4.47), 421 (4.40); IR ν_{max} cm^{-1} (KBr) 3440, 2966, 2909, 1617, 1509, 1431, 1324, 1281, 1138, 1042, 984, 884; ^{1}H ($CDCl_3$, 600 MHz) and ^{13}C ($CDCl_3$, 150 MHz), see Table 1.

Pennicitrinone D (**1**) was obtained as a yellow amorphous powder. Its molecular formula was determined as $C_{23}H_{24}O_6$ based on HRESI-MS at *m/z* 397.1624 $[M+H]^+$ (calcd 397.1651). The diagnostic IR peaks were observed for hydroxyl, conjugated carbonyl and benzene ring at 3450, 1616 and 1508 cm^{-1}, respectively. 1D-NMR spectra of **1** displayed six methyls, three sp^3 methines (two oxygenated), one sp^2 methine, one carbonyl, 11 sp^2 quaternary carbons and one sp^3-oxygenated quaternary carbon (Table 1). Except for an oxygenated quaternary carbon signal (δ_{OH} 4.98 and δ_C 80.7) instead of a methine signal (δ_H 3.17 and δ_C 44.6), the ^{1}H- and ^{13}C-NMR spectra of **1** were similar to those of **2**[9], suggesting that they shared the same molecular skeleton. Further comparison of the ^{13}C-NMR spectra of **1** with that of **2** revealed +1.6, +36.1, +1.3, +2.3, –6.4 and –2.1 p.p.m. of chemical shift effects for C-2′, C-3′, C-3′a, C-3′-Me, C-2′-Me and C-4′-Me, respectively, showing that **1** is a 3′-hydroxy derivative of **2**. This conclusion was further confirmed by ^{1}H-^{1}H COSY, heteronuclear

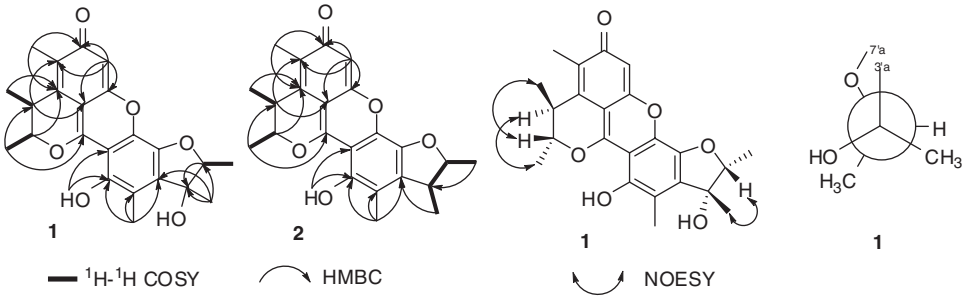
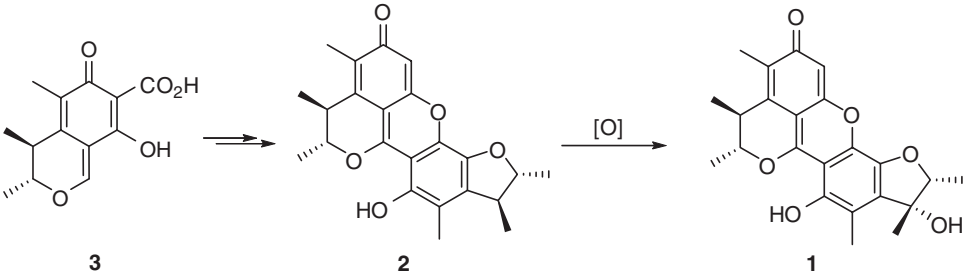


Figure 2 The key HMBC, ^{1}H-^{1}H COSY and NOESY correlations of **1** and **2** and *Gauche* conformation along the $C_{3'}$~$C_{2'}$ of **1.** COSY, correlated spectroscopy; NOESY, nuclear overhauser enhanced and exchange spectroscopy; HMBC, heteronuclear multiple bond coherence.



Scheme 1 The postulated biosynthesis of **1**.

multiple quantum coherence (HMQC), NOESY and heteronuclear multiple bond coherence (HMBC) experiments (Figure 2). The upfield shifts of C-2′-Me and C-4′-Me were caused by the γ-*gauche* effect and by the stereospecific blockade of 3′α-OH, respectively. The absolute configuration of **1** was determined as shown by the NOE correlations and further by comparing the $[\alpha]_D$ value, +111.1, with that of **2**, +106.9 and a possible biogenic pathway (Scheme 1). Compound **1** is most likely produced from the oxidation of **2** that is produced by a Diels–Alder reaction of **3**.[5,17] Thus, the structure of **1** was elucidated as (3′*R*)-3′-hydroxypennicitrinone A.

According to ^{1}H-^{1}H COSY, HMQC and HMBC experiments, ^{13}C NMR data of 3-Me, 2′-Me and 3′-Me of **2** in the literature[9] should be interchanged as 19.1, 21.0 and 18.8 p.p.m., respectively.

The cytotoxicity of compounds **1–4** was evaluated against P388 and HL-60 cell lines by the MTT method,[15] and against BEL-7402 and A-549 cell lines by the SRB method.[16] Compound **2** showed weak cytotoxicity against P388 and BEL-7402 cells with IC_{50} values of 25 and 16 μM, respectively. Compound **4** exhibited moderate cytotoxicity against A-549 cells with an IC_{50} value of 0.95 μM, whereas compounds **1** and **3** were inactive.

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