Citrinin Dimers from the Halotolerant Fungus *Penicillium citrinum* B-57

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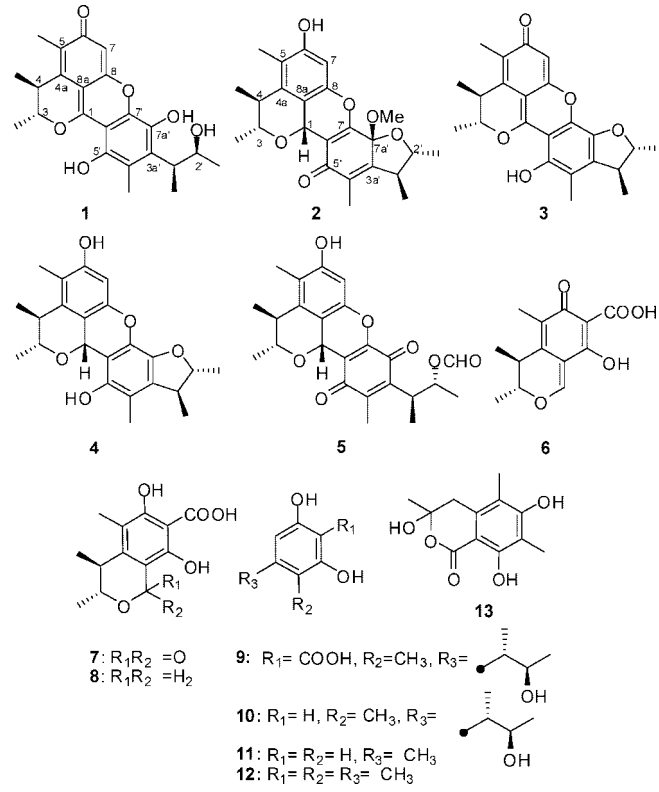
In order to search for structurally novel and bioactive natural compounds from microorganisms, a halotolerant fungal strain, *Penicillium citrinum* B-57, which mainly produces citrinin derivatives, was isolated from sediments collected from the Jilantai salt field. From the ethyl acetate extract of *P. citrinum* B-57, two new citrinin dimers, pennicitrinone C (**1**) and penicitrinol B (**2**), and 11 known related compounds were isolated and identified by spectroscopic and chemical methods. These compounds showed antioxidative activity against DPPH radicals with IC_{50} values ranging from 0.8 to 59 μM.

Investigating the secondary metabolites of microorganisms isolated from unusual or specialized ecological niches may increase the chance of finding structurally novel, bioactive compounds. Halotolerant microorganisms are those microbes that are able to grow well in variable salinity. It was reported that the extreme situations halotolerant microorganisms live in might activate some silent genes and induce some unique biosynthetic pathways.[1] However, besides marine microbes, the halotolerant microorganisms from other salt environments have received little attention as sources of bioactive metabolites. In the course of screening microorganisms for the production of bioactive secondary metabolites,[2,3] a halotolerant fungal strain, B-57, authenticated as *Penicillium citrinum*, was isolated from sediments collected in the Jilantai salt field, Alashan, Inner Mongolia, China. The EtOAc extract of *P. citrinum* B-57 showed obvious cytotoxicity against tsFT210 cells at a concentration of 0.3 mg/mL. HPLC-UV analysis of the crude extract showed a series of compounds with UV absorptions at 213, 237, 303, and 208, 246, 416 nm, similar to those of citrinin dimers.[4] Chemical investigation of the EtOAc extract of *P. citrinum* B-57 resulted in the isolation of two new citrinin dimers, pennicitrinone C (**1**) and penicitrinol B (**2**), together with 11 known citrinin derivates, pennicitrinone A (**3**),[5] penicitrinol A (**4**),[5] citrinin H1 (**5**),[6] citrinin (**6**),[7] dihydrocitrinone (**7**),[8] dihydrocitrinin (**8**),[9] phenol A acid (**9**),[7] phenol A (**10**),[7] 5-methylbenzene-1,3-diol (**11**),[10] 2,4,5-trimethylbenzene-1,3-diol (**12**),[11] and sclerotinin B (**13**).[12] To the best of our knowledge, only eight citrinin dimers have been previously reported: citrinin H1, penicitrinone A, penicitrinol A, penicitrinone B, dicitrinin A (Wakana named it penicitrinone A), dimethyl dicitrinin A, and dicitrinins B and D.[4–6] Citrinin H1 was found to be more toxic than citrinin on HeLa cells,[6] and dicitrinin A was found to be moderately active in the NS-1 cytotoxicity assay with LD_{99} values of 6.3 μg/mL.[4] In this paper, we evaluated the cytotoxicities of the new compounds **1** and **2** and the radical-scavenging activities of all of the isolated compounds against 1,1-diphenyl-2-picrylhydrazyl (DPPH). The new compounds **1** and **2** did not show cytotoxicity against P388, A-549, BEL-7402, and HL-60 cells (IC_{50} >50 μM). Compounds **1**, **3**, **5**, and **10**−**12** showed radical-scavenging activities against DPPH with IC_{50} values from 0.8 to 59 μM.



Results and Discussion

Compound **1** was obtained as a red, amorphous powder, $[\alpha]^{25}_{D}$ +117 (*c* 0.075, $CHCl_3$). The molecular formula of **1** was determined as $C_{23}H_{26}O_6$ by HRESIMS at *m/z* 397.1635 $[M - H]^-$ (calcd 397.1651), 18 amu more than that of compound **3** ($C_{23}H_{24}O_5$). The ^{1}H NMR data of **1** showed four tertiary methyl signals, two aromatic methyl signals, one aromatic proton, and four sp^3 methine protons (two oxygenated) (Table 1). The ^{13}C NMR data of **1** included six methyl carbons, one sp^2 methine carbon, four sp^3 methine carbons (two oxygenated), one carbonyl carbon, and 11 sp^2 quaternary carbons (Table 1). These NMR data were quite similar to those of **3**, except for the downfield shift effect of C-7′a (+4.3 ppm) and high-field shift effect of C-2′ (−17.3 ppm), indicating that **1** was the dihydrofuran-ring cleavage product of **3** formed by hydration. This deduction was further supported by ^{1}H−^{1}H COSY and HMBC spectra (Figure 1) and the observation of two additional exchangeable protons (δ_H 6.33, 2′-OH and 10.16, 7a′-OH) in the ^{1}H NMR spectra (DMSO-d_6 as solvent) (Table 1). The configuration of the two methyl residues in the benzopyran moiety was determined as *trans* based on the NOESY correlation of 4-CH_3 with 3-H and $^3J_{3,4}$ (<0.5 Hz). [4] In order to determine the relative configuration of the 3-hydroxy-2-butyl moiety in **1**, we carefully compared the chemical shifts of 3′-CH_3 (15.8 ppm) and 2′-CH_3 (21.7 ppm) of compound

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Table 1. ^{1}H and ^{13}C NMR (600 and 150 MHz) Data[a] for Pennicitrinone C (**1**) and Penicitrinol B (**2**)

	1				**2**			
position	H (*J* in Hz)[b]	C[b]	H (*J* in Hz)[c]	C[c]	H (*J* in Hz)[b]	C[b]	H (*J* in Hz)[c]	C[c]
1		157.9qC		156.3qC	5.36 s	61.0CH	5.21 s	60.5CH
3	5.02 q (6.9)	82.8CH	5.07 q (6.9)	81.4CH	4.11 dq (4.1, 6.4)	79.1CH	3.88 dq (4.1, 6.4)	78.6CH
3-CH_3	1.42 d (6.9)	18.9CH_3	1.30 d (6.9)	18.1CH_3	1.27 d (6.4)	22.9CH_3	1.12 d (6.4)	22.5CH_3
4	3.16 q (7.3)	35.0CH	3.17 q (7.3)	33.9CH	2.90 dq (4.1, 7.3)	37.9CH	2.82 dq (4.1, 6.9)	37.3CH
4-CH_3	1.33 d (7.3)	19.1CH_3	1.20 d (7.3)	18.6CH_3	1.33 d (7.3)	21.3CH_3	1.23 d (6.9)	20.9CH_3
4a		131.2qC		132.1qC		140.5qC		140.1qC
5		131.2qC		129.9qC		119.3qC		119.3qC
5-CH_3	2.15 s	10.9CH_3	1.97 s	10.3 CH_3	2.16 s	10.5CH_3	2.04 s	10.5CH_3
6		184.1qC		182.2qC		154.4qC		155.8qC
6-OH					5.65 brs		9.76 brs	
7	6.72 s	102.0CH	6.00 s	101.0CH	6.62 s	100.5CH	6.49 s	99.0CH
8		159.1qC		157.6qC		145.3qC		144.2qC
8a		100.2qC		98.8qC		112.1qC		111.1qC
2′	4.47 dq (4.1, 6.4)	70.7CH	4.21 m	69.3CH	4.30 dq (4.1, 6.4)	83.5CH	4.14 dq (4.1, 5.9)	82.6CH
2′-CH_3	1.18 d (6.4)	21.7CH_3	1.06 d (5.8)	21.8CH_3	1.33 d (6.4)	20.4CH_3	1.30 d (5.9)	20.0CH_3
2′-OH			6.33 brs					
3′	3.38 dq (4.1, 6.9)	41.8CH	3.20 m	41.5CH	2.54 m	41.6CH	2.63 m	40.8CH
3′-CH_3	1.41 d (6.9)	15.8CH_3	1.27 d (7.3)	15.4CH_3	1.33 d (7.3)	17.2CH_3	1.23 d (6.8)	16.7CH_3
3a′		137.5qC		137.1qC		150.8qC		151.4qC
4′		118.8qC		119.2qC		129.5qC		127.9qC
4′-CH_3	2.26 s	11.6CH_3	2.19 s	11.8CH_3	1.93 d (0.9)	11.1CH_3	1.82 d (0.9)	10.7CH_3
5′		144.6qC		144.3qC		186.5qC		185.4qC
5′-OH	8.20 s		8.73 s					
6′		102.4qC		102.0qC		111.6qC		110.5qC
7′		138.0qC		136.4qC		156.7qC		156.2qC
7a′		142.1qC		140.4qC		98.4qC		97.5qC
7′a-OCH_3					3.22 s	51.1CH_3	3.12 s	50.5CH_3
7a′-OH			10.16 s					

[a] The assignments were based on DEPT, ^{1}H–^{1}H COSY, HMQC, and HMBC experiments. [b] NMR data were measured in $CDCl_3$. [c] NMR data were measured in DMSO-d_6.

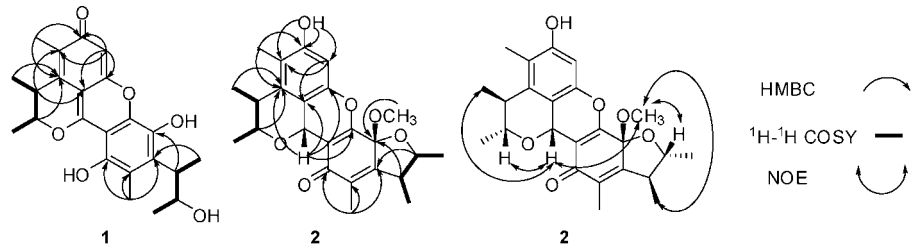

Figure 1. ^{1}H−^{1}H COSY and key HMBC correlations of compounds **1** and **2** and NOE effects of **2**.

1 with those of 3-arylbutan-2-ol analogues in the literature.[7,13] The chemical shifts of the two CH_3 groups of *erythro*- and *threo*-3-(*m*-tolyl)-2-butanol were 16.1 and 21.0, and 17.6 and 20.4 ppm, respectively.[13] Further, the chemical shifts of the two CH_3 groups of (2*S*,3*S*)-*erythro*- and (2*R*,3*S*)-*threo*-(−)-3-[3,5-bis(benzyloxy)-2-methylphenyl]butan-2-ol were 15.7 and 21.2, and 17.8 and 20.4 ppm, respectively.[7] These data indicated that the chemical shift of the 3′-CH_3 of the *erythro*-isomer of 3-arylbutan-2-ol was more upfield (∼16 ppm in $CDCl_3$) than that of the *threo*-isomer (∼18 ppm in $CDCl_3$). This phenomenon was also seen in **9** and **10** (17.6 and 17.8 ppm, respectively). Thus, the relative configuration of the 3-hydroxy-2-butyl chain in **1** was determined as *erythro*.

Compound **2** was obtained as a pale yellow, amorphous powder, $[\alpha]^{25}_D$ +16.7 (*c* 0.215, MeOH). The molecular formula of **2** was determined as $C_{24}H_{28}O_6$ by HRESIMS at *m*/*z* 413.1970 $[M + H]^+$ (calcd 413.1964). The ^{1}H NMR data of **2** showed four tertiary methyl signals, two aromatic methyl signals, one methoxyl group, one aromatic proton, and five sp^3 methine protons (three oxygenated) (Table 1). The ^{13}C NMR data of **2** exhibited six methyl carbons, one methoxy carbon, one sp^2 methine carbon, five sp^3 methine carbons (three oxygenated), one carbonyl carbon, and 10 sp^2 quaternary carbons (Table 1). Except for those of the benzofuran moiety, these data were similar to those of **4**,[5] indicating that they had the same molecular skeleton. In comparison to those of **4**, the NMR spectrum of **2** exhibited an additional methoxyl group (δ_H 3.22, δ_C 51.1), a high-field shift for C-7′a (−39.4 ppm), and downfield shifts for C-5′, C-7′, and C-3′a (+38.4, +23.4, and +18.5 ppm, respectively). A detailed analysis of ^{1}H−^{1}H COSY and HMBC spectra revealed that the methoxyl group was linked to C-7′a and the hydroxyl on C-5′ in **4** was oxidized to a carbonyl in **2** (Figure 1). In the NOE difference experiment, irradiation on H-1 caused a nuclear Overhauser effect of H-3, 4-CH_3, and 7′a-OCH_3, while irradiation of the 7′a-OCH_3 affected H-1, H-2′, and 3′-CH_3, which indicated the relative configuration of **2** was *trans*-3,4-diCH_3, *trans*-2′,3′-diCH_3, *trans*-3-CH_3 and 7′a-OCH_3, *cis*-1-H and 4-CH_3, and *cis*-1-H and 7′a-OCH_3 (Figure 1).

These compounds likely have a biogenesis via the polyketide pathway. Citrinin (**6**),[14] phenol A acid (**9**), and sclerotinin B (**13**)[14] are biosynthesized from acetyl coenzyme A, which then forms dihydrocitrinone (**7**),[15] dihydrocitrinin (**8**),[16] phenol A (**10**), and 2,4,5-trimethylbenzene-1,3-diol (**12**) after undergoing hydration−oxidation, reduction, and decarboxylation. Pennicitrinone C (**1**) is postulated to be produced from the hydration of **3**, which results from the Diels–Alder reaction of **6**.[4] Penicitrinol B (**2**) is postulated to derive from the hydrolysis–condensation of **5** resulting from the Diels–Alder reaction of **6**.[4,6] Compound **2** may also be formed from the Diels–Alder reaction of **6** with **10** and then undergo dehydration, oxidation, and aldol condensation (Scheme 1). Thus, the absolute configuration of **1** and **2** is deduced as 3*R*, 4*S*, 2′*S*, 3′*S*- and 3*R*, 4*S*, 2′*R*, 3′*S*, 7′a*R*-, respectively.

Scheme 1. Postulated Biosynthesis of **1** and **2**

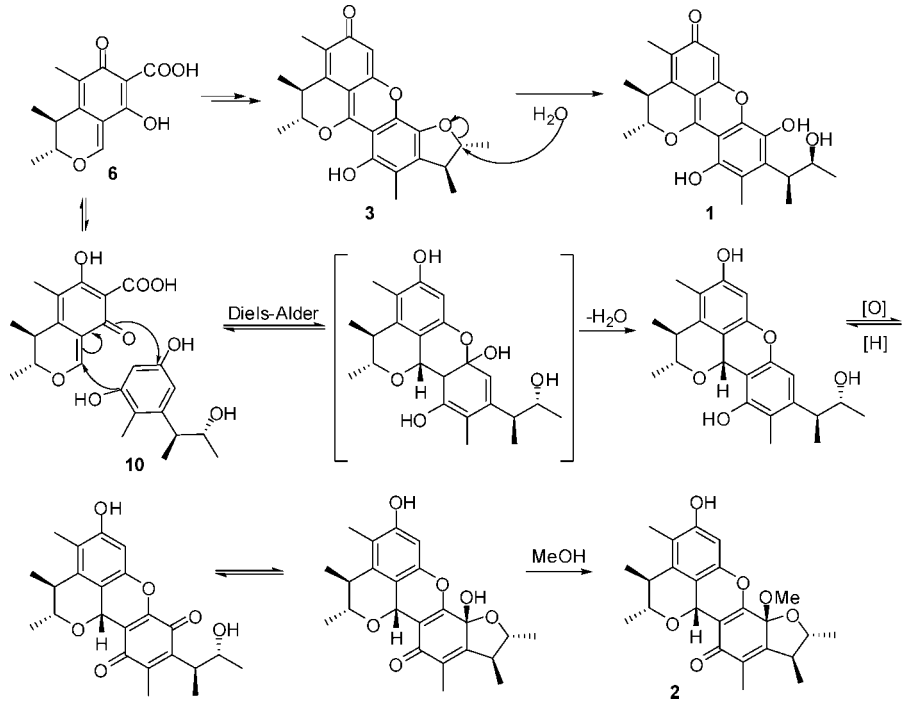

Table 2. DPPH-Scavenging Activities of Compounds **1–13**

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	L-ascorbic acid
IC_{50} (μM)	55.3	291.3	10.6	26.8	58.7	160.0	>1000	123.1	795.8	22.5	0.8	3.8	>1000	22.7

The new compounds **1** and **2** were tested for cytotoxic effects against the P388 and HL-60 cell lines using the MTT method[17] and the BEL-7402 and A-549 cell lines using the SRB method;[18] however, none were cytotoxic against any of the cell lines (IC_{50} >50 μM) (paclitaxel as positive control, IC_{50} 0.93 μM).

Compounds **1**−**13** were also evaluated for their radical-scavenging activities against DPPH.[19] Compounds **1**, **3**, **5**, and **10**−**12** displayed scavenging activity with IC_{50} values of 55.3, 10.6, 58.7, 22.5, 0.8, and 3.8 μM, respectively, while compounds **2**, **4**, **6**−**9**, and **13** were inactive (IC_{50} >100 μM) (L-ascorbic acid as positive control, IC_{50} 22.7 μM) (Table 2).

In this paper, we isolated two new citrinin dimers along with five known dimers and evaluated the two new compounds for scavenging of DPPH radicals and cytotoxicity against P388, A-549, BEL-7402, and HL-60 cells. The new compounds enrich the structural diversity of citrinin dimers, and the scavenging activities of these compounds of DPPH radicals are reported here for the first time. The possible biosynthetic pathways of these citrinin derivatives are also discussed.

Experimental Section

General Experimental Procedures. Optical rotations were obtained on a JASCO P-1020 digital polarimeter. UV spectra were recorded on a Beckmen DU 640 spectrophotometer. ^{1}H NMR, ^{13}C NMR, and DEPT spectra and 2D NMR were recorded on a JEOL JNM-ECP 600 spectrometer using TMS as internal standard, and chemical shifts were recorded as δ values. ESIMS was measured on a Q-TOF Ultima Global GAA076 LC mass spectrometer. Semipreparative HPLC was performed using an ODS column [YMC-pack ODS-A, 10 × 250 mm, 5 μM, 4 mL/min].

Fungal Material. The fungal strain *P. citrinum* B-57 was isolated from the sediments collected in Jilantai salt field, Alashan, Inner Mongolia, China. It was identified according to its morphological characteristics and ITS by Prof. Kui Hong, Institute of Tropical Biological Sciences and Biotechnology, Chinese Academy of Tropical Agricultural Science, Haikou, China. The voucher specimen is deposited in our laboratory at −80 °C. The producing strain was prepared on potato dextrose agar slants containing 10% NaCl and stored at 4 °C.

Fermentation and Extraction. The fungus *P. citrinum* B-57 was grown under static conditions at 30 °C for 28 days in one hundred 1000 mL conical flasks containing liquid medium (300 mL/flask) composed of glucose (10 g/L), maltose (20 g/L), mannitol (20 g/L), malt extract (3 g/L), monosodium glutamate (10 g/L), NaCl (120 g/L), NH_4Cl (10 g/L), $MgSO_4$ (5 g/L), and KCl (5 g/L) after adjusting its pH to 7.0. The fermented whole broth (30 L) was filtered through cheesecloth to separate the supernatant from the mycelia. The former was concentrated under reduced pressure to about a quarter of the original volume and then extracted three times with EtOAc to give an EtOAc solution, while the latter was extracted three times with acetone. The acetone solution was concentrated under reduced pressure to afford an aqueous solution. The aqueous solution was extracted three times with EtOAc to give another EtOAc solution. Both EtOAc solutions were combined and concentrated under reduced pressure to give a crude extract (18.0 g).

Purification. The crude extract (18.0 g) was separated into 15 fractions on a Si gel column using a step gradient elution of petroleum ether, $CHCl_3$, and MeOH. Fraction 4 (3.6 g) eluted with $CHCl_3$ and then was recrystallized from MeOH, yielding compound **6** as a yellow crystal (1.1 g). Fraction 7 (1.2 g) eluted with $CHCl_3$/MeOH (98:2) and was further chromatographed on Si gel using petroleum ether/EtOAc (80:20). Subfraction 7-3 (130 mg) was purified by semipreparative HPLC (80% MeOH), yielding compound **1** (6.2 mg, t_R 6.2 min) and compound **3** (4.3 mg, t_R 8.9 min). Subfraction 7-5 (85 mg) was purified by semipreparative HPLC (80% MeOH), yielding compound **2** (15.2 mg, t_R 14.5 min), and by semipreparative HPLC (60% MeOH), yielding compound **12** (6.0 mg, t_R 7.5 min). Subfraction 7-6 (97 mg) was purified by semipreparative HPLC (60% MeOH), yielding compound **11** (18.2 mg, t_R 8.8 min). Fraction 8 (1.7 g) eluted with $CHCl_3$/MeOH (95:5) and was further chromatographed on Si gel using petroleum ether/$CHCl_3$ (50:50). Subfraction 8-3 (342 mg) was purified by semipreparative HPLC (80% MeOH), yielding compound **4** (18.0 mg, t_R 23 min) and compound **5** (3.5 mg, t_R 18 min). Subfraction 8-4 (95 mg) was purified by semipreparative HPLC (70% MeOH), yielding compound **10** (6.5 mg, t_R 10.2 min) and compound **13** (4.5 mg, t_R 7.8 min). Subfraction 8-5 (67 mg) was purified by semipreparative HPLC (60% MeOH), yielding compound **7** (10.6 mg, t_R 6.9 min) and compound **8** (8.0 mg, t_R 8.1 min). Subfraction 8-7 (38 mg) was purified by semipreparative HPLC (35% MeOH), yielding compound **9** (4.0 mg, t_R 6.5 min).

Biological Assays. In the MTT assay, cell lines were grown in RPMI-1640 supplemented with 10% FBS under a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. 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. Then, 2 μL of the test solutions (in MeOH) was added to each well and further incubated for 72 h. The MTT solution (20 μL, 5 mg/mL in IPMI-1640 medium) was then added to each well and incubated for 4 h. 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.

In the SRB assay, 200 μL of the cell suspensions 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% trichloroacetic acid, 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.

In the DPPH-scavenging assay, 160 μL of reaction mixtures containing test samples and 40 μL of DPPH (Sigma) dissolved in MeOH were plated in 96-cell plates incubated in the dark for 30 min. After the reaction, absorbance was measured at 520 nm, and percent inhibition was calculated. The antioxidant activity of each sample was expressed in terms of IC_{50} (μM required to inhibit DPPH radical formation by 50%) and calculated from the log-dose inhibition curve.

Penicitrinone C (1): red, amorphous powder; $[\alpha]^{25}_D$ +117 (*c* 0.075, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 398 (3.14), 309 (2.62), 280 (3.08), 266 (3.10) nm; IR (KBr) ν_{max} 3467, 3440, 2963, 2928, 2869, 1650, 1611, 1553, 1530, 1495, 1452, 1401, 1297, 1141, 1052, 834 cm^{-1}; ^1H and ^{13}C NMR (see Table 1); HRESIMS *m/z* 397.1635 $[M - H]^-$ (calcd for $C_{23}H_{25}O_6$, 397.1651).

Penicitrinol B (2): pale yellow, amorphous powder; $[\alpha]^{25}_D$ +16.7 (*c* 0.215, MeOH); UV ($CHCl_3$) λ_{max} (log ϵ) 325 (3.65) nm; IR (KBr) ν_{max} 3409, 2970, 2928, 2866, 1697, 1650, 1596, 1488, 1405, 1374, 1442, 1110, 1060, 1013, 920, 900 cm^{-1}; ^1H and ^{13}C NMR (see Table 1); HRESIMS *m/z* 413.1970 $[M + H]^+$ (calcd for $C_{24}H_{29}O_6$, 413.1964).

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