Tetrahedron 66 (2010) 9286–9290

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Novel carbon-bridged citrinin dimers from a volcano ash-derived fungus *Penicillium citrinum* and their cytotoxic and cell cycle arrest activities

Lin Du [a], Dehai Li [a], Guojian Zhang [a], Tianjiao Zhu [a], Jing Ai [b], Qianqun Gu [a,*]

[a] Key Laboratory of Marine Drugs, Chinese Ministry of Education, School of Medicine and Pharmacy, Ocean University of China, Qingdao 266003, China
[b] State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, PR China

ARTICLE INFO

Article history:
Received 14 June 2010
Received in revised form 9 September 2010
Accepted 10 September 2010
Available online 17 September 2010

Keywords:
Penicillium citrinum
Citrinin
Polyketide
Cytotoxicity
Cell cycle arrest

ABSTRACT

Three novel carbon-bridged citrinin dimers, dicitrinones A–C (**1**–**3**), were isolated from a volcano ash-derived fungus *Penicillium citrinum*. Their structures were elucidated by spectroscopic methods. Dicitrinone A (**1**) and B (**2**) are two atropisomeric mixtures, which were verified by both NMR and quantum chemical calculations. Their cytotoxicity against four tumor cell lines (HL-60, MOLT-4, A-549, and BEL-7402) was evaluated by SRB and MTT methods. Dicitrinone B (**2**) arrested the HL-60 cell cycle at the G2/M phase, which was analyzed using flow cytometry.

1. Introduction

Mytotoxin citrinin, produced by several fungal species that belong mainly to the genera *Penicillium* and *Monascus*, is widely considered as a hazard contaminant of foods and feeds.[1] It has been reported that this family of compounds possess nephrotoxic activities[2] in addition to a number of other chronic toxic effects.[3] It is thought to exert its toxicity by interfering with the electron transport system of mitochondria[4,5] and inducing cell apoptosis through the intrinsic pathway.[6] Recently, a new class of Diels–Alder coupling citrinin dimers, including pennicitrinones A–C and pennicitrinols A and B, have been isolated from different *Penicillium citrinum* strains.[7–9] Bearing a distinct coupling penta-ring skeleton, the Diels–Alder dimers showed no cytotoxicity on several tumor cell lines while pennicitrinone A and C showed radical-scavenging activities against DPPH.[8] It indicated that citrinin might be a latent precursor of novel active compounds.

In our search for antitumor compounds from microorganisms, a fungus, authenticated as *P. citrinum* (CCTCC M 208,170), was obtained from the volcano ash collected in Guangdong province of China. Three novel 7,7′-carbon-bridged citrinin dimers, dicitrinones A–C (**1**–**3**), were isolated from the lipid extract of this fungus. In this paper, we describe the isolation, structure elucidation, quantum chemical calculations for the atropisomers, and cytotoxicity and cell cycle arrest activities of these new compounds.

2. Results and discussion

Compound **1** was obtained as a yellow solid with molecular formula $C_{28}H_{34}O_6$, which was determined by HRESIMS (*m*/*z* 467.2445 $[M+H]^+$, calcd 467.2434), indicating 12° of unsaturation. The strong IR and UV absorptions at 1636 cm^{-1} and 334 nm, respectively, indicated the existence of conjunct unsaturated ketone systems. The ^{13}C NMR spectrum indicated 47 carbons, many of which appeared in pairs (Table 1). This result indicated that this compound might be a mixture of two stereoisomers, and meanwhile, symmetrical segments consisted in structure (Fig. 1). The ^1H and ^{13}C NMR data (Table 1) were compared with those of citrinin.[2,10] The symmetrical portion could be identified as a 7-substituted citrinin decarboxylate. This result could be further confirmed by analysis of the ^1H–^1H COSY correlations between H3-11, H-2, H-3, and H3-12, and the HMBC correlations from H-2 to C-10, from H-3 to C-4, C-5, and C-9, from H3-13 to C-4, C-5, and C-6, from H-10 to C-2, C-4, C-8, and C-9, from OH-8 to C-7, and from H-1″ to C-6, C-7, and C-8 (Fig. 1B). The ^1H–^1H COSY correlations between H3-4″, H2-3″, H2-2″, and H-1″ helped to depict a propyl chain which attached to two citrinin decarboxylates through a—C-7–C-1″–C-7′—bridge (Fig. 1B). The proposed structure was consistent with the molecular formula obtained by HRESIMS. The propyl chain, which increased the molecular asymmetry, could

* Corresponding author. Tel.: +86 532 82032065; fax: +86 532 82033054; e-mail address: guqianq@ouc.edu.cn (Q. Gu).

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doi:10.1016/j.tet.2010.09.036

Table 1
^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data for compounds **1**–**3** in DMSO-d_6

No.	**1**		**2**		**3**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
2, 2′	80.0/79.9 79.8/79.7	4.72m 4.69m	80.1/80.0 79.9/79.8	4.73m	80.1	4.74q (6.4)
3, 3′	33.5	2.99m, 2.94m	33.5 33.4	2.99m	33.5	3.02q (7.2)
4, 4′	136.7 136.0		136.7 136.0		136.7	
5, 5′	125.2/125.0 124.5/124.2		125.3/125.0 124.5/124.1		124.4	
6, 6′	186.7/186.6186.4/186.2		186.3 186.1		186.1	
7, 7′	116.8/116.7116.7/116.6		118.1/118.0117.9/117.8		114.0	
8, 8′	162.7/162.6162.4/162.3		162.9/162.4162.2/162.0		162.2	
8-OH 8′OH		13.3s 13.1s		13.4s 13.2s		
9, 9′	107.3/107.2106.9/106.8		107.4/107.2106.9/106.8		106.7	
10, 10′	158.5/158.4157.9/157.7	8.02s/8.01s 7.99s/7.98s	158.7/158.6158.0/157.8	8.04s/8.03s 8.02s/8.01s	158.3	8.03s
11, 11′	17.7 17.5/17.4	1.20d (3H, 6.4) 1.13d (3H, 6.4)	17.7 17.5	1.22 br s (3H) 1.17d (3H, 5.4)	17.5	1.20d (3H, 6.4)
12, 12′	18.4 18.2/18.1	1.09d (3H, 7.2) 1.03d (3H, 7.2)	18.4 18.2	1.09d (3H, 7.3) 1.05d (3H, 5.9)	18.3	1.07d (3H, 7.2)
13, 13′	10.1/10.0 9.7/9.6	1.88s (3H)/1.87s (3H) 1.86s (3H)/1.84s (3H)	10.1/10.0 9.7/9.6	1.89s (3H) 1.87s (3H)	9.8	1.89s (3H)
1″	31.0/30.8	4.29t (7.8)/4.26t (7.8)	25.1/25.0	4.47q (1H, 7.3)	18.1	3.38s (2H)
2″	30.7/30.3	2.10m (2H)/2.05m (2H)	15.4/15.1	1.59d (3H, 6.8) 1.55d (3H, 6.8)		
3″	21.7	1.06m (2H)				
4″	13.7	0.79t (3H, 7.8)				

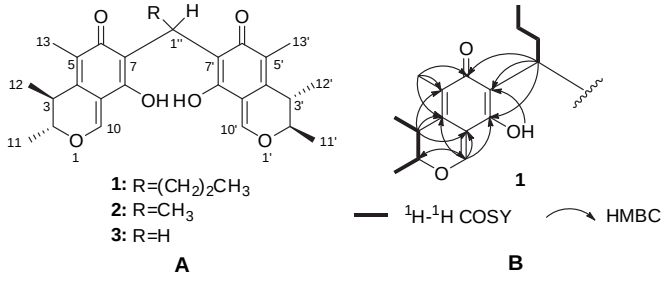


Fig. 1. Structures of compounds **1**–**3** (**A**) and key ^{1}H–^{1}H COSY and HMBC correlations of compound **1** (**B**).

hinder the free rotation of the two citrinin decarboxylate segments through the—C-7/7′—C-1″—single bond. The minimum-energy confirmations of **1** were calculated using the Gaussian 09 suite of program.[11–15] Two atropisomers **1a** and **1b**, which had an energy differential of only 1.7 kJ/mol (Fig. 2), were stabilized due to restricted rotation and the intramolecular hydrogen bonds between the two segments. This observation was confirmed by the active hydrogen resonances (δ_H 13.3 and 13.1) in the ^{1}H NMR spectrum. These combined analyses could explain why the two atropisomers are captured simultaneously by NMR in a 1:1 ratio. In order to further investigate the atropisomeric phenomenon, ^{1}H NMR spectra of **1** were performed with different solvents. However, all the ^{1}H NMR spectra of **1** showed resonances for two atropisomers. Methylation, acetylation, and sodium salification reactions were performed to protect the OH-8/8′ groups. Unfortunately all the trials failed due to the weak reaction activity of the phenolic hydroxyl groups.

Compounds **2** and **3** are both analogues of **1** with the same UV and IR spectra as those of **1**, indicating that they all had the same skeleton. Using HRESIMS, the molecular formulas of **2** and **3** were established as $C_{26}H_{30}O_6$ and $C_{25}H_{28}O_6$, respectively. The proposed molecular formulas of **2** and **3** lost a C_2H_4 and a C_3H_6 from the molecular formula proposed for **1**, respectively. Comparison of the ^{1}H and ^{13}C NMR spectra of **1** and **2** (Table 1) also indicated that they both had the same citrinin decarboxylate segments,—C-7—C-1″—C-7′—bridging construction, and stereoisomeric characters. The only difference was that the propyl group in **1** was replaced by a methyl group (δ_H 1.59/1.55, δ_C 15.4/15.1) in **2**. The two atropisomers **2a** and **2b** (Fig. 2), which could both be detected by NMR in a 1:1 ratio (Table 1), were only 1.1 kJ/mol different in energy by the quantum chemical calculations.[11–15] Comparatively, the ^{1}H and ^{13}C NMR spectra of **3** (Table 1) exhibited only one set of signals for one citrinin decarboxylate segment. The carbon-bridged center, C-1″, changed from a methane to a methylene (δ_H 3.28, δ_C 18.1). The removal of the alkyl chain eliminated the asymmetry factor of the molecule and made the latent two atropisomers the same.

The relative configurations of C-2/C-3 and C-2′/C-3′ in **1**–**3** were determined by comparison of the ^{1}H NMR data between **3** and citrinin. In the ^{1}H NMR spectrum of **3**, H-2/2′ and H-3/3′ were exhibited as quartets with coupling constants of 6.4 and 7.2 Hz, respectively (Table 1), which were identical to those of citrinin.[10] So the relative configurations of C-2/C-3 and C-2′/C-3′ were both determined to be *trans*, consistent with those in citrinin.[15] Dicitrinones A–C (**1**–**3**) represent an unprecedented carbon skeleton because of the two citrinin decarboxylates connected through a unique carbon-bridging center with or without a C_1 or C_3 alkyl chain. Biosynthetically, this unprecedented skeleton is proposed to originate from a polyketide pathway (Scheme 1).[16] Condensation of citrinin-CoA with one or two malonyl-CoA units through Claisen reactions followed by decarboxylation would produce the intermediate **a**. The intermediate **a** and citrinin via enzymatic reduction could be transformed into positive charged benzyl intermediates **b** and **c**, respectively, which could further undergo Friedel–Crafts reaction (F–C reaction)[17] with decarbpxycitrinin[7] to provide dicitrinones A–C (**1**–**3**), respectively (Scheme 1). Thus, based on the biogenetic analysis, the absolute configurations of **1**–**3** were deduced as 2*R*, 3*S*, 2′*R*, and 3′*S* (Fig. 1, Table 2).

The cytotoxicity of compounds **1**–**3** were preliminarily evaluated against HL-60 and MOLT-4 cell lines using MTT method,[18] and

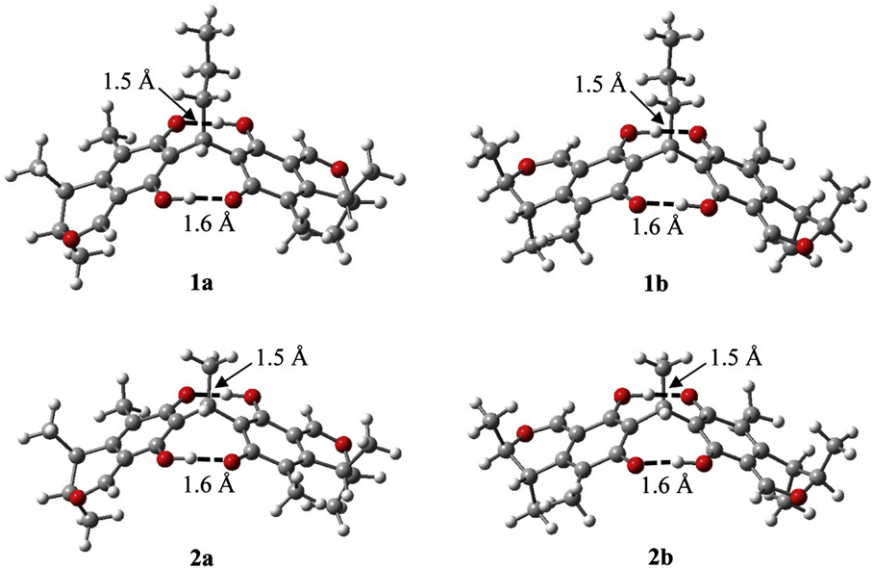


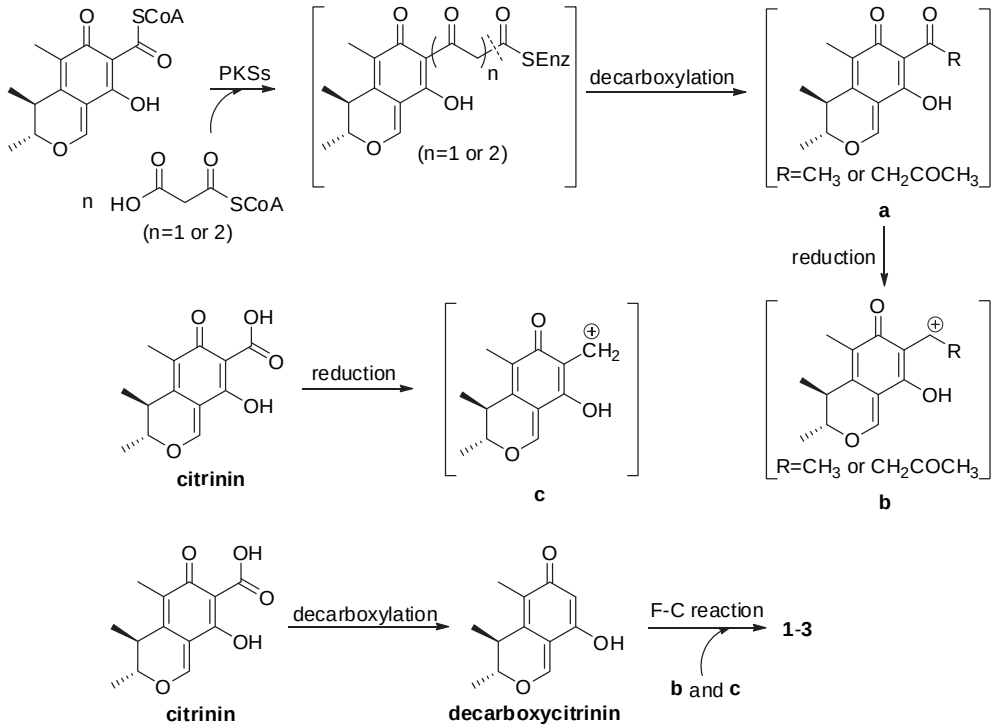
Fig. 2. Molecular modeling of the minimum-energy conformations of the two atropisomers of **1** (**1a** and **1b**) and **2** (**2a** and **2b**) using Gaussview software.[14]

against A-549 and BEL-7402 cell lines using SRB method.[19] Compound **2** displayed relatively more potent cytotoxic effects in the leukaemia cell lines HL-60 and MOLT-4 with the IC_{50} of 6.5 and 6.0 μM, respectively (Table 2). Compound **3** inhibited the cell viability of MOLT-4 cell line with the IC_{50} of 6.0 μM, while compound **1** only showed moderate cytotoxicity against the four cell lines (Table 2). We further examined the influence of compound **2** on the HL-60 cell cycle by flow cytometry.[20] Treatment of the HL-60 cells with 20 μM and 25 μM of **2** for 20 h obviously arrested the cell cycle at G_2/M phase, accompanying with moderate cell death (Fig. 3). The cell viability was almost completely inhibited by 30 μM of **2** (Fig. 3). The G_2/M phase cell cycle arrestment could be correlated with many mechanisms including inhibition of tubulin polymerization, one of the hottest targets for cancer chemotherapy.[21] The real molecular targets of the novel class of carbon-bridged citrinin dimers need be further investigated.

Table 2
Cytotoxicity of **1**–**3** against four cancer cell lines

IC_{50} (μM)[a]	HL-60	A-549	BEL-7402	MOLT-4
1	40.1	47.4	39.5	25.9
2	6.5	53.9	39.8	6.0
3	39.5	34.7	32.8	6.0

[a] IC_{50} is defined as the concentration that results in a 50% decrease of viable cell numbers.



Scheme 1. Postulated biosynthetic pathway of compounds **1**–**3**.

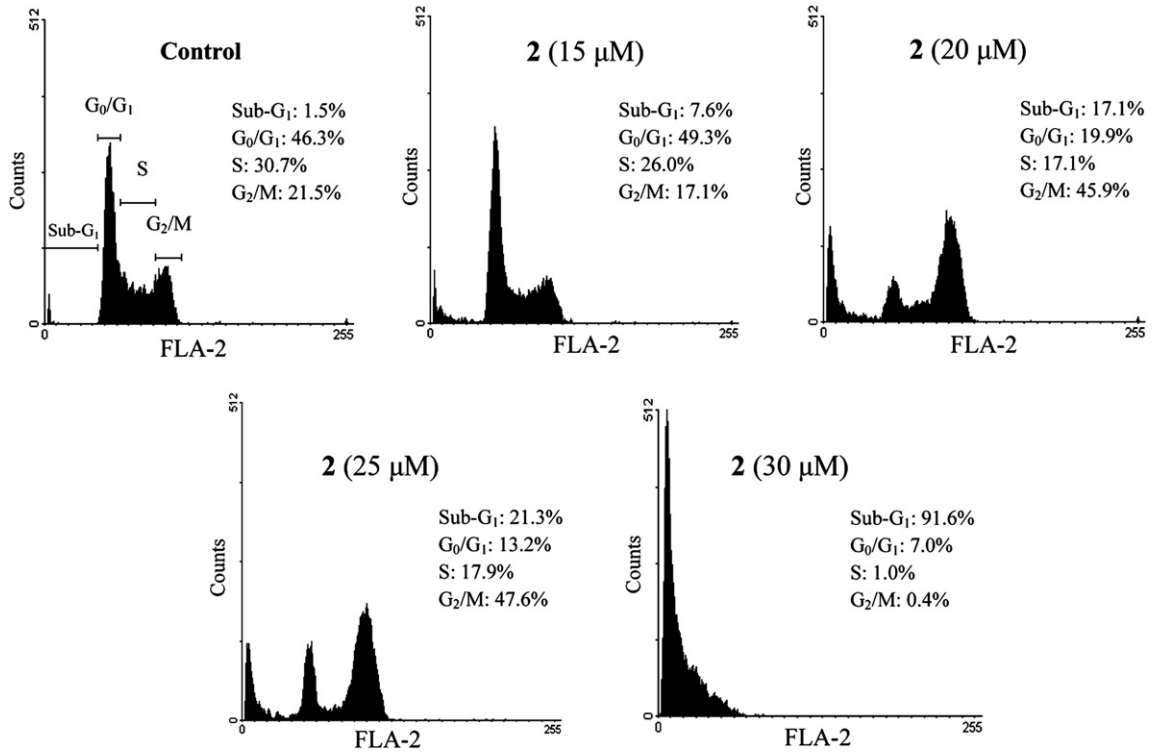


Fig. 3. Effects of compound **2** on HL-60 cell cycle and cytotoxicity. HL-60 cells incubated with **2** (15, 20, 25, and 30 μM) for 20 h were fixed and stained with PI and then analyzed by flow cytometry. The percentage of sub-G_1, G_0/G_1, S, and G_2/M fractions were analyzed using the CellQuest computer software.

3. Experimental

3.1. General

Optical rotations were obtained on a JASCO P-1020 digital polarimeter. UV spectra were recorded on Beckman DU® 640 spectrophotometer. IR spectra were taken on a NICOLET NEXUS 470 spectrophotometer in KBr discs. ^1H, ^{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. ESI-MS was measured on a Q-TOF ULTIMA GLOBAL GAA076 LC mass spectrometer. Semiprepartive HPLC was performed using an ODS column [YMC-pak ODS-A, 10×250 mm, 5 μm, 4 mL/min].

3.2. Culture of *P. citrinum*, strain HGY1–5, extraction and isolation of compounds 1–3

P. citrinum HGY1–5 was isolated from the crater ash collected from the extinct volcano Huguangyan in Guangdong, China. It was identified according to its ribosomal internal transcribed spacers and the 5.8S ribosomal RNA gene (ITS1-5.8S-ITS2). A voucher specimen is preserved in the China Center for Type Culture Collection (patent depositary number: CCTCC M 208,170). The working strain was prepared on Potato Dextrose agar slants and stored at 4 °C. Spores were directly inoculated into 500 mL Erlenmeyer flasks containing 100 mL fermentation media (mannitol 20 g, maltose 20 g, glucose 10 g, monosodium glutamate 10 g, KH_2PO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.3 g, yeast extract 3 g and corn steep liquor 1 g, dissolved in 1 L water, pH 6.5). The culture was incubated on an environmental shaker, which was set at 165 rpm and 28 °C. After 15 days of cultivation, 40 L of whole broth was filtered through cheesecloth to separate the broth supernatant and mycelia. The former was extracted with EtOAc and *n*-butanol in succession. The *n*-BuOH extract (100 g) was concentrated and successively chromatographed over a silica gel column followed by elution with a gradient petroleum ether/Me_2CO (100:1→1:2) to obtain twelve fractions. Fraction 3 was subjected to Sephadex LH-20 CC ($CHCl_3$/MeOH 1:1) and then further purified by HPLC using a reversed-phase C18 column (85/15 MeOH/0.3% THF, 4.0 mL/min) to produce compounds **1** (20 mg), **2** (15 mg), and **3** (10 mg) (Fig. 1A).

3.3. Computational methods

All calculations were carried out using the Gaussian 09[11] suite of program. Geometry optimizations were performed using the B3LYP[12]–15 method in conjunction with the 6-31G(d,p) basis set. Frequency calculations were performed at the same level of theory in order to confirm that the obtained geometries are minima and the zero-point vibrational energy (ZPVE) was corrected. Relative energies were obtained by performing single point calculations at the B3LYP/6-311+G(2df,p) level of theory based on the above optimized geometries with ZPVE corrected, i.e., B3LYP/6-311+G (2df,p)//B3LYP/6-31G(d,p)+ZPVE.

3.4. Biological assays

Cytotoxicity assay: cytotoxicity assays were performed in HL-60 and MOLT-4 cell lines using MTT method,[18] and in A-549 and BEL-7402 cell lines using SRB method.[19] Dose response curves were generated and the IC_{50} values were calculated from the linear portion of log dose response curves.

Flow cytometric analysis:[20] the cells were seeded in 24-well cell culture plates (2×10^5 cells/well), in triplicate, and then treated with MeOH (Vehicle) or compound **2** for 24 h at the concentrations of 15,

20, 25, and 30 μM. The cells were washed with PBS twice. The cellular DNA was stained with 200 μL of propidium iodide for 20 min at 4 °C (PI, 50 μg/mL, RNase 1 μg/mL, Triton X-100 0.1%). After incubation, the cells were analyzed using flow cytometry (Becton–Dickinson, Vantage, San Diego, CA).

*3.4.1. Dicitrinone A (**1**).* Yellow solid; $[\alpha]_D^{25}$ −79.7 (*c* 0.243, MeOH); UV λ_{max} (MeOH) nm (log ε) 206 (4.75), 334 (4.30); IR (KBr) ν_{max} 3447, 2965, 2929, 2870, 2671, 2580, 1636, 1591, 1558, 1506, 1470, 1375, 1343, 1300, 1279, 1213, 1167, 1133,1058, 1021, 990, 927, 861, 796 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) and ^{13}C NMR (DMSO-d_6, 150 MHz), see Table 1; HRESIMS *m*/*z* 467.2445 $[M+H]^+$ (calcd for $C_{28}H_{35}O_6$, 467.2434).

*3.4.2. Dicitrinone B (**2**).* Yellow solid; $[\alpha]_D^{25}$ −65.5 (*c* 0.163, MeOH); UV λ_{max} (MeOH) nm (log ε) 205 (4.71), 333 (4.20); IR (KBr) ν_{max} 3452, 2969, 2929, 2872, 2660, 2582, 1636, 1591, 1511, 1457, 1375, 1338, 1282, 1216, 1169, 1128, 1005, 800 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) and ^{13}C NMR (DMSO-d_6, 150 MHz), see Table 1; HRESIMS *m*/*z* 439.2103 $[M+H]^+$ (calcd for $C_{26}H_{31}O_6$, 439.2121).

*3.4.3. Dicitrinone C (**3**).* Yellow solid; $[\alpha]_D^{25}$ −54.9 (*c* 0.165, MeOH); UV λ_{max} (MeOH) nm (log ε) 205 (4.72), 327 (4.21); IR (KBr) ν_{max} 3443, 2971, 2928, 2869, 2667, 2593, 1638, 1591, 1562, 1509, 1473, 1374, 1300, 1276, 1218, 1173, 1131,1027, 989, 848, 811 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) and ^{13}C NMR (DMSO-d_6, 150 MHz), see Table 1; HRESIMS *m*/*z* 425.1979 $[M+H]^+$ (calcd for $C_{25}H_{29}O_6$, 425.1964).

Acknowledgements

This work was financially supported by the Natural Science Foundation of China (Nos. 30973627 and 30772640) and the State Key Laboratory of Drug Research Fund (SIMM0901KF-05). The authors thank Dr. Haining Liu for the quantum chemical calculations.

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