Two New Citrinin Dimers from a Volcano Ash-Derived Fungus, *Penicillium citrinum* HGY1-5

by **Hong-Chun Liu**[a])[1]), **Lin Du**[a])[1]), **Tian-Jiao Zhu**[a]), **De-Hai Li**[a]), **Mei-Yu Geng**[a])[b]), and **Qian-Qun Gu***[a])

[a]) Key laboratory of Marine Drugs, Chinese Ministry of Education, Institute of Marine Drugs and Food, Ocean University of China, Qingdao 266003, P. R. China
(phone: +86-532-82032065; fax: +86-532-82033054; e-mail: guqianq@ouc.edu.cn)

[b]) State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, P. R. China

Two new citrinin dimers, penidicitrinin A ((2*R*,3*S*,5a*S*,9*R*,10*S*,12a*R*,12b*R*)-2,3,5a,6,9,10,12a,12b-octahydro-7,12a-dihydroxy-12b-methoxy-2,3,4,9,10,11-hexamethyl-5*H*-difuro[2,3-*b* :2′,3′-*h*]xanthen-5-one; **1**) and penidicitrinin B ((1*S*,3*R*,4*S*)-1-{2,6-dihydroxy-4-[(1*S*,2*R*)-2-hydroxy-1-methylpropyl]-3-methylphenyl}-3,4-dihydro-3,4,5-trimethyl-1*H*-2-benzopyran-6,8-diol; **2**), together with three known citrinin monomers were isolated from a volcano ash-derived fungus, *Penicillium citrinum* HGY1-5. Their structures were established by spectroscopic methods, and they showed no cytotoxicity against two tumor cell lines.

Introduction. – *Penicillium citrinum*, a common filamentous fungus found all over the world, is well-known for the production of biologically active metabolites, including the polyketide mycotoxin citrinin [1]. Recently, a new class of citrinin derivatives, *i.e.*, citrinin dimers, have been isolated from different *Penicillium citrinum* strains [2–4]. Some of the dimeric compounds showed potent antioxidant activities [4]. In our search for novel antitumor compounds from microorganisms, a *Penicillium citrinum* strain was obtained from the volcano ash collected in Guangdong Province of China. The AcOEt and BuOH extracts showed cyctoxicity *in vitro* against the HL-60 cell line. The isolation process of the active compounds on this fungus led to two new unprecedented citrinin dimers, penidicitrinol A and B (**1** and **2**, resp.), together with three known monomers including citrinin (**3**) [5], decarboxydihydrocitrinin (**4**) [3], and phenol A (**5**) [6] (*Fig. 1*). In this article, we report the isolation, structure elucidation, and cytotoxicities of the new compounds against HL-60 and A-549 cell lines.

Results and Discussion. – The AcOEt extract was concentrated *in vacuo* and then repeatedly chromatographed by means of the silica gel column chromatography, *Sephadex LH-20*, and semi-preparative HPLC to yield compounds **1** and **3**. Similarly, compounds **2**, **4**, and **5** were isolated from the BuOH extract.

Compound **1**, named penidicitrinin A, was obtained as a pale white powder. Its molecular formula $C_{24}H_{30}O_7$ was determined by HR-ESI-MS (*m/z* 429.1920 ([*M* – H]$^-$;

[1]) These authors equally contributed to this article.

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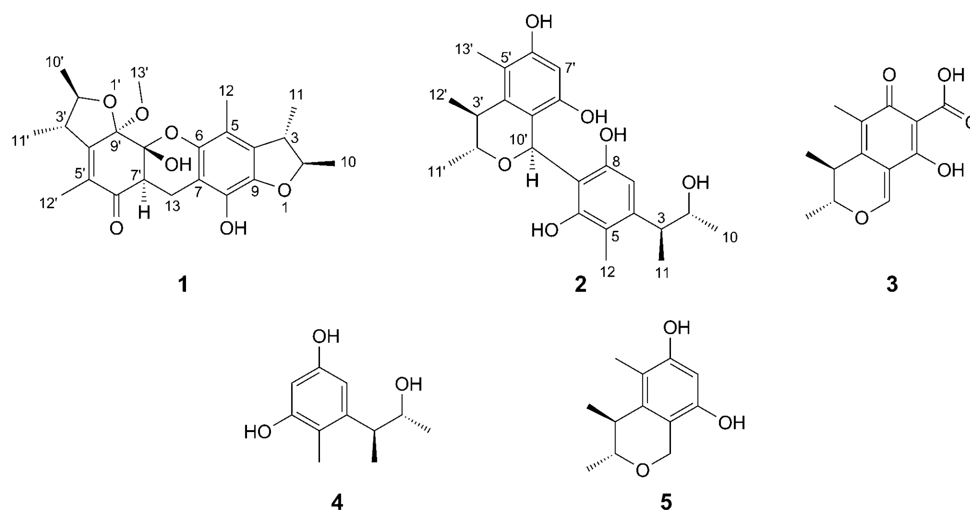

Fig. 1. *Structures of compounds* **1**–**5**

calc. 429.1913), indicating ten degrees of unsaturation. Its IR spectrum exhibited strong absorptions at 3425 and 1646 cm^{-1}, indicating OH and conjugated C=O groups. A detailed inspection of the ^{1}H- and ^{13}C-NMR data (*Table*) by DEPT, ^{1}H,^{1}H-COSY, and HMQC disclosed the presence of seven Me groups, including a MeO group and two Me groups located on C=C bonds, one CH_2, five CH groups, and eleven quaternary C-atoms, including one C=O C-atom (δ(C) 196.9), and two ketal or hemiketal C-atoms (δ(C) 96.6 and 102.9). An analysis of ^{1}H,^{1}H-COSY and HMBC spectra led to three substructures (*Fig. 2,a*). Substructure **A** (C(2)–C(12)) was assembled on the basis of ^{1}H,^{1}H-COSY correlations (Me(10)/H–C(2)/H–C(3)/Me(11)), HMBC correlations (from Me(12) to C(4), C(5), and C(6), from HO–C(8) to C(7), C(8), and C(9), from CH_2(13) to C(6), C(7), and C(8), and from Me(11) to C(4)) and the comparison of the ^{13}C-NMR data with those of 5,7-dihydroxy-2,3,4-trimethyl-2,3-dihydrobenzofuran [7]. Substructure **B** (C(2′)–C(8′), C(10′)–C(12′), and C(13)) was obtained on the basis of two ^{1}H,^{1}H-COSY spin systems (Me(10′)/H–C(2′)/H–C(3′)/Me(11′) and H–C(7′)/ 2 H–C(13)) and HMBC correlations (from Me(11′) to C(4′), from Me(12′) to C(4′), C(5′), and C(6′), from H–C(7′) to C(6′) and C(8′), from HO–C(8′) to C(7′) and C(8′), and from CH_2(13) to C(6′), C(7′), and C(8′)). Substructure **C** (C(9′) and C(13′)) was established by the only HMBC correlation from Me(13′) to C(9′). Substructures **A** and **B** could be linked through a C(13)–C(7) bond based on the HMBC correlations from CH_2(13) to C(6), C(7), and C(8). Further analysis of the ^{13}C-NMR data and the molecular formula, the ketal C-atom C(9′) (δ(C) 102.9) should be attached to C(2′) (δ(C) 82.3) through an O-bridge, which could be confirmed by the NOE correlation between Me(13′)O and H–C(2′) (*Fig. 2,b*). As the benzo C-atom (C(6), δ(C) 142.6) must be attached to an O-atom on the pyran ring, then, C(9′) could only be attached to C(4′) and C(8′). Accordingly, the hemiketal C-atom (C(8′), δ(C) 96.6) must be attached to C(6) though an O-bridge, which finally established the planar structure of **1** (*Fig. 1*). The relative configurations of **1** were determined by NOE difference

Table. *^{1}H- and ^{13}C-NMR Data* ((D_6)DMSO) *of Compounds* **1** *and* **2**[a]). δ in ppm, J in Hz.

	1		**2**	
	δ(C)	δ(H)	δ(C)	δ(H)
H–C(2)	85.6 (*d*)	4.36 (*qd*, J = 6.4, 4.1)	69.1 (*d*)	3.65–3.67 (*m*)
HO–C(2)				4.39 (*d*, J = 4.6)
H–C(3)	43.4 (*d*)	3.00 (*qd*, J = 6.8, 4.1)	41.1 (*d*)	2.92 (*dq*, J = 7.0, 5.5)
C(4)	129.0 (*s*)		142.5 (*s*)	
C(5)	111.2 (*s*)		112.9 (*s*)	
C(6)	142.6 (*s*)		153.6 (*s*)	
HO–C(6)				8.71 (*s*)
C(7)	109.1 (*s*)		112.4 (*s*)	
C(8)	135.8 (*s*)		153.6 (*s*)	
HO–C(8)		8.63 (*s*)		8.55 (*s*)
C(9) or H–C(9)	139.1 (*s*)		105.5 (*d*)	6.25 (*s*)
Me(10)	20.8 (*q*)	1.25 (*d*, J = 6.4)	19.0 (*q*)	0.95 (*d*, J = 6.8)
Me(11)	19.3 (*q*)	1.17 (*d*, J = 6.8)	14.9 (*q*)	1.05 (*d*, J = 7.0)
Me(12)	11.5 (*q*)	2.03 (*s*)	10.8 (*q*)	1.92 (*s*)
CH_2(13)	16.7 (*t*)	2.93 (*dd*, J = 6.4, 16.9), 2.28 (*dd*, J = 12.3, 16.9)		
H–C(2′)	82.3 (*d*)	3.97–4.02 (*m*)	72.5 (*d*)	3.85 (*qd*, J = 6.4, 2.3)
H–C(3′)	42.1 (*d*)	2.56–2.60 (*m*)	35.3 (*d*)	2.62 (*qd*, J = 6.4, 2.3)
C(4′)	159.2 (*s*)		138.1 (*s*)	
C(5′)	126.4 (*s*)		112.4 (*s*)	
C(6′)	196.9 (*s*)		154.9 (*s*)	
HO–C(6′)				9.03 (*s*)
H–C(7′)	43.3 (*d*)	2.85 (*ddd*, J = 2.2, 6.4, 12.3)	100.5 (*d*)	6.15 (*s*)
C(8′)	96.6 (*s*)		151.8 (*s*)	
HO–C(8′)		6.23 (*d*, J = 2.2)		8.71 (*s*)
C(9′)	102.9 (*s*)		112.3 (*s*)	
Me(10′) or H–C(10′)	19.6 (*q*)	1.39 (*d*, J = 5.9)	63.0 (*d*)	6.07 (*s*)
Me(11′)	17.0 (*q*)	1.24 (*d*, J = 6.9)	18.1 (*q*)	1.15 (*d*, J = 6.4)
Me(12′)	10.4 (*q*)	1.70 (*d*, J = 1.0)	19.4 (*q*)	1.28 (*d*, J = 6.8)
Me(13′)	50.1 (*q*)	3.45 (*s*)	10.5 (*q*)	1.99 (*s*)

[a]) Spectra were recorded at 600 MHz for ^{1}H-NMR and 150 MHz for ^{13}C-NMR with Me_4Si as internal standard.

experiments (*Fig. 2,b*). NOEs of Me(13′)O with H–C(2′), H–C(7′), and Me(11′) indicated that they had the same orientation on the ring systems. The NOE correlation between Me(13′)O and Me(12) and the lack of NOE correlation between Me(13′)O and HO–C(8′) indicated that HO–C(8′) must have the orientation opposite to that of Me(13′)O. That Me(10) correlated with H–C(3) but not with Me(11) established the *trans*-configuration of the Me groups at C(2) and C(3). The absolute configuration of **1** was proposed based on the biogenic analysis and the CD spectra. Citrinin (**3**) has been established as the biosynthetic precursor of many monomeric and dimeric derivatives [2–4][6]. Consequently, the absolute configurations at C(2)/C(2′) and C(3)/C(3′) should be consistent with those of citrinin [8], and accordingly the absolute configuration of **1** was postulated as (2*R*,3*S*,2′*R*,3′*S*,7′*S*,8′*R*,9′*R*). The minimum-energy conformation corresponding to the proposed configuration was simulated using the

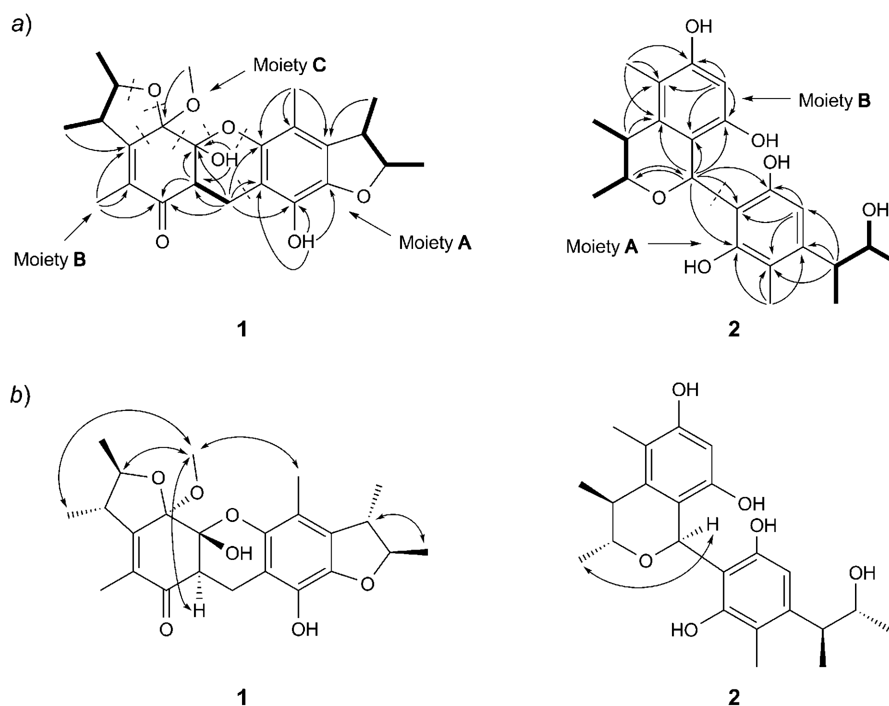

Fig. 2. *Key $^1H,^1H$-COSY* (—), *HMBC* (*a*; H → C), *and NOE* (*b*; H ↔ H) *correlations of compounds* **1** *and* **2**

MM2 calculation in ChemBio3D Ultra 11.0 (*Fig. 3*). According to the classical CD rule of α,β-unsaturated ketones [9], the predicted *Cotton* effects (*Fig. 3*) were consistent with the observed *Cotton* effects (λ_{max} ($\Delta\varepsilon$): 375.0 (1.0), 258.8 (– 0.5)). So, the structure of **1** was determined to be (2*R*,3*S*,5a*S*,9*R*,10*S*,12a*R*,12b*R*)-2,3,5a,6,9,10,12a,12b-octahydro-7,12a-dihydroxy-12b-methoxy-2,3,4,9,10,11-hexamethyl-5*H*-difuro[2,3-*b* :2′,3′-*h*] xanthen-5-one.

Compound **2** was obtained as a yellowish powder. Its molecular formula was determined as $C_{23}H_{30}O_6$ based on HR-ESI-MS (*m*/*z* 401.1961 ([*M* – H]$^-$; calc. 401.1964). The molecular formula indicated nine degrees of unsaturation. IR Absorptions implied the presence of OH groups (3445, 3384, and 3305 cm^{-1}), and UV absorptions (210 (4.42), 287 (3.68)) indicated the existence of phenyl rings. Detailed comparison of ^{1}H- and ^{13}C-NMR data (*Table*) with those of the known compounds decarboxydihydrocitrinin (**4**) [3] and phenol A (**5**) [6], together with comprehensive analysis of the 2D-NMR (^{1}H,^{1}H-COSY, HMBC, and HMQC) correlations (*Fig. 2,a*), indicated two moieties, a phenol A moiety (**A**) and a decarboxydihydrocitrinin moiety (**B**). The two moieties were linked through a C(7)–C(10′) bond on the basis of analysis of the key HMBC correlations from H–C(10′) to C(6), C(7), and C(8) (*Fig. 2,a*). The relative configuration at C(2′) and C(3′) was determined to be *trans* by comparison of the *J*(2′,3′) value (2.3 Hz) with that in **4** (2.3 Hz; [3]: 2.4 Hz). The NOEs of H–C(10′) with Me(11′) (*Fig. 2,a*), indicated

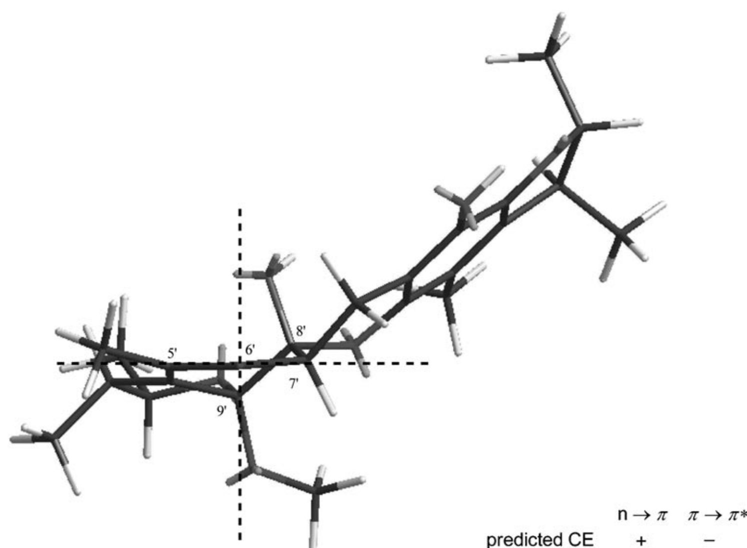

Fig. 3. *The minimum-energy three-dimensional structure and the predicted* Cotton *effect signs of* **1**

that they had the same orientation on the pyran ring. By comparison of the J(2,10), J(2,3), and J(3,11) values of **2** (6.8, 5.5, and 7.0 Hz, resp.) with those of *erythro*-3-(*o*-tolyl)butan-2-ol (6.8, 6.6, and 6.8 Hz, resp.) and *threo*-3-(*o*-tolyl)butan-2-ol (6.7, 7.9, and 5.9 Hz, resp.) [10], the relative configuration at C(2) and C(3) was determined to be *erythro*. The absolute configurations of naturally occurring decarboxydihydrocitrinin (**4**) and phenol A (**5**) from the same species have been established by synthesis, and they were both established to be derived from citrinin (**3**) [3][6][11]. Based on the obvious biogenetic relationship of **2** with **3**–**5**, which were also isolated from this strain, the absolute configuration of **2** was proposed as (2*R*,3*S*,2′*R*,3′*S*,10′*S*), and the structure of **2** was established as (1*S*,3*R*,4*S*)-1-{2,6-dihydroxy-4-[(1*S*,2*R*)-2-hydroxy-1-methylpropyl]-3-methylphenyl}-3,4-dihydro-3,4,5-trimethyl-1*H*-2-benzopyran-6,8-diol, named penidicitrinin B (*Fig. 1*).

The cytotoxicity of compounds **1** and **2** was evaluated *in vitro* on the HL-60 cell line by the MTT method [12] and on the A-549 cell line by the SRB method [13]. They all showed no obvious cytotoxicity on either cell line with IC_{50} values > 50 μM.

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Experimental Part

General. Column chromatography (CC): silica gel (SiO_2; 200–300 mesh, 10–40 μm, *Qingdao Marine Chemical Inc.*, P. R. China), *RP-18* (40–63 mm, *YMC Co.*, Japan), *Sephadex LH-20* (*GE Healthcare*, Sweden). TLC: SiO_2 *GF254* (10–40 μm, *Qingdao Marine Chemical Inc.*, P. R. China). Semi-prep. HPLC: *ODS* column (*YMC-Pack ODS-A*, 10 × 250mm, 5 μm). UV Spectra: *Beckman DU® 640* spectrophotometer; λ_{max} (log ε) in nm. CD Spectra: *JASCO J-810* circular dichroism chiroptical spectrometer; λ_{max} ($\Delta\varepsilon$) in nm. IR Spectra: *NICOLET NEXUS470* spectrophotometer; KBr discs; $\tilde{\nu}$ in

cm^{-1}. ^{1}H-, ^{13}C-NMR, DEPT, and 2D-NMR spectra: *JEOL Eclipse-600* spectrometer; δ in ppm rel. to Me_4Si as internal standard, J in Hz. ESI-MS: *Micromass Q-TOF ULTIMA GLOBAL GAA076 LC* mass spectrometer; in m/z (rel. %).

Fungal Material. 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), and the percentage of similarity was 99% compared to those of a *P. citrinum* strain NRRL 31486 (NCBI/Blast, No. AF484404.1). A voucher specimen is deposited with the China Center for Type Culture Collection (patent depository No.: CCTCC M 208170). The working strain was prepared on potato dextrose agar slants and stored at 4°.

Fermentation and Extraction. 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), $MgSO_4 \cdot 7\ H_2O$ (0.3 g), yeast extract (3 g), and corn steep liquor (1 g), dissolved in H_2O (1 l), pH 6.5). The flasks were incubated on a rotatory shaker at 165 rpm at 28°. After 15 d of cultivation, 40 l of whole broth was filtered through cheesecloth to separate the broth supernatant and mycelia. The former was successively extracted with AcOEt (40 l) and BuOH (40 l). The two extracts were concentrated *in vacuo* to give an AcOEt gum (88 g) and a BuOH gum (100 g), resp.

Purification. The AcOEt gum (88 g) was subjected to CC (SiO_2; petroleum ether (PE)/acetone, gradient) to give eleven fractions, *Frs. 1–11. Fr. 6* (5.6 g) was recrystallized in MeOH to afford compound **3** (1.1 g). *Fr. 7* (4.2 g) was subjected to repeated CC (*Sephadex LH-20*; $CHCl_3$/MeOH 1:1). *Subfr. 7-2-1* (120 mg) was further purified by HPLC (reversed-phase (RP) C_{18} column MeOH/H_2O 75:25; 4.0 ml/min) to give compound **1** (t_R 11.5 min; 12 mg).

The BuOH gum (100 g) was subjected to CC (SiO_2; PE/acetone, gradient) to give twelve fractions, *Frs. 1–12. Fr. 6* (7.2 g) was subjected to CC (*Sephadex LH-20*; $CHCl_3$/MeOH 1:1) and CC (SiO_2; PE/acetone, $CHCl_3$/MeOH, gradient) successively. *Subfr. 6-2-1* (210 mg) was further purified by HPLC (RP-C_{18} column; MeOH/H_2O 45:55; 4.0 ml/min) to give compound **5** (t_R 10.2 min; 35 mg). Similarly, compounds **2** (t_R 13.2 min; 15 mg) and **4** (t_R 7.4 min; 65 mg) were isolated from *Subfr. 6-2-2* (231 mg) by HPLC (*RP18*; MeOH/H_2O 60:40; 4.0 ml/min).

Penidicitrinin A (=*(2*R*,3*S*,5a*S*,9*R*,10*S*,12a*R*,12b*R*)-2,3,5a,6,9,10,12a,12b-Octahydro-7,12a-dihydroxy-12b-methoxy-2,3,4,9,10,11-hexamethyl-5*H*-difuro[2,3-*b*:2',3'-*h*]xanthen-5-one*; **1**). Pale white powder. $[\alpha]_D^{20} = +69.4$ ($c = 0.06$, MeOH). CD (MeOH): 375.0 (1.0), 295.8 (0.5), 258.8 (−0.5). UV (MeOH): 226 (4.29), 294 (3.66), 373 (3.29). IR (film): 3425, 2951, 2924, 2854, 1646, 1561, 1537, 1518, 1452, 1401, 1370, 1254, 1165, 1106, 1060, 1029. ^{1}H- and ^{13}C-NMR: *Table.* HR-ESI-MS: 429.1920 ($[M-H]^-$, $C_{24}H_{29}O_7^-$; calc. 429.1913).

Penidicitrinin B (=*(1*S*,3*R*,4*S*)-1-{2,6-Dihydroxy-4-[(1*S*,2*R*)-2-hydroxy-1-methylpropyl]-3-methylphenyl}-3,4-dihydro-3,4,5-trimethyl-1*H*-2-benzopyran-6,8-diol*; **2**). Yellowish powder. $[\alpha]_D^{20} = -103.9$ ($c = 0.15$, MeOH). UV (MeOH): 210 (4.42), 287 (3.68). IR (film): 3445, 3384, 3305, 3163, 2976, 2927, 2869, 1616, 1592, 1558, 1456, 1435, 1372, 1318, 1275, 1249, 1196, 1155, 1098, 1051, 1021, 957, 898, 838. ^{1}H- and ^{13}C-NMR: *Table.* HR-ESI-MS: 401.1961 ($[M-H]^-$, $C_{23}H_{29}O_6^-$; calc. 401.1964).

Biological Assay. In the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (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°. Cell suspensions (200 μl) at a density of 5×10^4 cell/ml were plated in 96-well microtiter plates and incubated for 24 h. Then, 2 μl of the tested solns. (in MeOH) were added to each well and further incubated for 72 h. Then, 20 μl of the MTT soln. (5 mg/ml in IPMI-1640 medium) was 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 sulforhodamine-B colorimetric (SRB) assay, 200 μl of the cell suspensions were plated in 96-cell plates at a density of 2×10^5 cell/ml. Then, 2 μl of the tested solns. (in MeOH) was added to each well, and the culture was further incubated for 24 h. The cells were fixed with 12% Cl_3CCOOH, and the cell layer was strained with 0.4% SRB. The absorbance of SRB soln. was measured at 515 nm. Dose–response curves were generated, and the IC_{50} (the concentration of compound required to inhibit cell proliferation by 50%) values were calculated from the linear portion of log dose–response curves.

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