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Penicidones A–C, three cytotoxic alkaloidal metabolites of an endophytic *Penicillium* sp.

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Abstract

Along with the known secondary metabolites lumichrome, physcion, and emodin-1,6-dimethyl ether, three alkaloids named penicidones A–C (**1–3**) were isolated from the culture of *Penicillium* sp. IFB-E022, an endophytic fungal strain residing in the stem of *Quercus variabilis* (Fagaceae). The structures of penicidones A–C were established by a correlative interpretation of spectroscopic data including IR, UV and HR-ESI-MS, as well as by analysis of a set of 1D and 2D NMR experiments. The stereochemistry of compounds **1** and **2** was obtained by comparison of the optical rotation with those of vermistatin and its analogues. Penicidones A–C were the first group of natural products possessing a penicidone framework. Compounds **1–3** exhibited moderate cytotoxicity against four cancer cell lines.

Keywords: *Penicillium* sp.; *Quercus variabilis*; Fagaceae; Endophyte; Alkaloid; Penicidone A; Penicidone B; Penicidone C; Cytotoxicity

1. Introduction

An endophyte is a bacterial (including actinomycete) or fungal microorganism, which spends the whole or part of its life cycle colonizing inter- and/or intra-cellular healthy tissues of the host plant, typically causing no apparent symptoms of disease. During the long co-evolution of endophytes and their host plants, endophytes have adapted themselves to their special microenvironments by genetic variation, including uptake of some plant DNA into their own genomes. This could have led to the ability of certain endophytes to biosynthesize some 'phytochemicals' originally associated with the host plants. In a coevolutionary view, endophytic microbes improves the resistance of the host plants to adversity by secretion of bioactive secondary metabolites. As a result, endophytes are being accepted as an important source of novel bioactive secondary metabolites that can be excellent new starting points for the development of novel pharmaceuticals and/or agrochemicals (Zhang et al., 2006; Tan and Zou, 2001; Strobel, 2003). In our continuous characterization of biologically potent and structurally new metabolites from endophyte cultures (Shen et al., 2006; Jiao et al., 2006; Ge et al., 2006), the extract of the endophyte fungus *Penicillium* sp. IFB-E022 from the stem of *Quercus variabilis* was shown to be cytotoxic against cancer cell lines. The subsequent bioassay-directed fractionation of the methanol extract of its solid-substrate culture, therefore, led to isolation of three new cytotoxic γ-pyridone alkaloids named penicidones A–C (**1–3**). We hereby wish to present the isolation, structure determination and cytotoxicity assessment of these fungal metabolites.

2. Results and discussion

Compound **1** was obtained as a white amorphous powder. Its positive-ion mode high resolution electrospray ionization mass spectrum gave a quasimolecular ion at *m/z*

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328.1175 ($[M+H]^+$ calcd. for $C_{18}H_{18}NO_5$: 328.1179, Δ 1.2 ppm) indicative of a molecular formula of $C_{18}H_{17}NO_5$. This was further rationalized by analysis of the ^1H and ^{13}C NMR spectra of **1**, displaying 17 hydrogen integrals and 18 discrete carbon resonance lines, respectively. Its broadened IR absorption bands at 3048 cm^{-1} indicated the presence of NH and/or OH groups, and those at 2944, 1765 and 1627 cm^{-1} of methoxyl, lactone and ketone groups. Overall inspection of the ^1H and ^{13}C NMR spectra of **1** indicated that it contained 2 carbonyls (δ_C 178.0 and 171.2) and 12 sp^2-hybridized carbons which resonated between δ_C 100.1 and 163.4 (Table 1) and consumed altogether eight degrees of unsaturations. The remaining three unsaturations had to be assigned to a tricyclic system in the molecule. Three partial structures ***a–c***, assigned readily from the three coupling systems, were deduced of on the basis of analysis of the COSY and HMBC spectra. Substructure ***a***, 1,3-dimethoxyl-4,5-disubstitutedbenzene ($C_8H_8O_2$), was deduced from two mutually coupled protons H-3 (δ_H 6.91) and H-5 (δ_H 6.83), with the former showing HMBC cross-peaks with C-5 (δ_C 106.1) and C-7 (δ_C 130.0), and the latter with C-3 (δ_C 100.1) and C-7. Two methoxyl groups were positioned at C-4 (δ_C 163.4) and C-6 (δ_C 156.0), respectively, from the HMBC correlations of 4-OMe (δ_H 3.86) with C-4 and 6-OMe (δ_H 3.74) with C-6. The partial structure of ***b***, 2,5-disubstituted pyridin-4(1*H*)-one (C_5H_3NO), was characterized by the HMBC ^3J correlations of a amide proton H-13 (δ_H 11.38) with C-9 (δ_C 122.3) and C-11 (δ_C 115.2), and by correlation of H-11 (δ_H 6.09) with C-9 (δ_C 122.3), together with a typical chemical shift value C-10 ($\delta_C = 178.0$) of an α, β, α, β-unsaturated ketone (Komai et al., 2005). The geometry of the *E*-configured double bond in fragment ***c*** (C_3H_5) was also deduced from the coupling constant ($J = 15.9$ Hz). Substructures ***a*** and ***b*** were connected via C-8 by the HMBC correlations of H-8 (δ_H 6.38) with C-2 (δ_C 130.5), C-9, C-10 and C-14 (δ_C 138.3). The fragments of ***b*** and ***c*** were linked together with the aid of diagnostic HMBC correlations of H-15 (δ_H 6.27) with C-11, and the correlation of H-16 (δ_H 6.52) with C-12. The residual ester group C-1 (δ_C 171.2) and a remaining "suggested ring" were edited in a γ-lactone whose presence was confirmed by both the magnitude of 8-methine shift values (δ_C 77.7, δ_H 6.38) and the HMBC cross-peaks of H-8 with C-1 and C-2, of H-3 with C-1. The stereochemistry of C-8 in compound **1** ($[\alpha]_D^{20} = -98.3$) was deduced as 8*R* by direct comparison of the optical rotation data (Müller and Faulkner, 1997; Lee and Liu, 2003; Tabata et al., 1998) with those of vermistatin ($[\alpha]_D^{20} = -8.3$), dihydrovermistatin ($[\alpha]_D^{19} = -80$), and penisimplicissin ($[\alpha]_D^{19} = -119$) (Fuska et al., 1986; Komai et al., 2005), all of which had the same and only chiral carbon with **1**. In conclusion, the structure of **1** was determined as (*R*,*E*)-5-(5,7-dimethoxy-3-oxo-1,3-dihydroisobenzofuran-1-yl)-2-(prop-1-enyl)pyridin-4(1*H*)-one. For simplicity, it was named penicidone A.

Compound **2** had a pseudomolecular ion at *m/z* 314 in its ESI-MS spectrum, 14 mass units less than that of penicidone A (**1**). The high resolution ESI-MS analysis of **2** indicated the molecular formula to be $C_{17}H_{16}NO_5$ corresponding to the $[M+H]^+$ peak at *m/z* 314.1023 (calcd. for $C_{17}H_{15}NO_5$: 314.1013, Δ 3.1 ppm). The ^1H and ^{13}C NMR spectra (Table 1) of **2** were partially similar to that of penicidone A (**1**). However, the resonances at δ_H 3.90 and δ_C 56.2 ascribable to the 6-methoxy group of **1** were

Table 1
^1H and ^{13}C NMR spectroscopic data of compounds **1**, **2** and **3**[a]

No.	**1**		**2**		**3**	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	171.2		171.3		167.1	
2	130.5		130.4		135.8	
3	100.1	6.91 (br. *s*)	102.8	6.72 (br. *s*)	106.2	6.97 (br. *s*)
4	163.4		161.5		160.9	
5	106.1	6.83 (br. *s*)	106.0	6.70 (br. *s*)	104.0	6.85 (br. *s*)
6	156.0		156.1		158.5	
7	130.0		127.7		130.4	
8	77.7	6.38 (br. *s*)	77.5	6.35 (br. *s*)	193.0	
9	122.3		122.7		123.4	
10	178.0		178.1		177.0	
11	115.2	6.09(*s*)	115.2	6.10 (*s*)	119.1	6.37 (br. *s*)
12	146.7		146.7		146.2	
13-NH		11.38 (br. *s*)		11.34 (br. *s*)		11.42 (br. *s*)
14	138.3	7.39 (br. *s*)	138.1	7.30 (br. *s*)	143.3	8.16 (s)
15	125.6	6.27 (*d*, 15.9)	125.6	6.20 (*d*, 16.0)	125.3	6.31 (*d*, 15.6)
16	133.7	6.52 (dq, 5.9, 15.9)	133.7	6.52 (dq, 6.2, 16.0)	134.2	6.69 (dq, 6.7, 15.6)
17	19.5	1.86 (*d*, 5.9)	19.5	1.85 (*d*, 6.2)	19.6	1.96 (*d*, 6.7)
1-OMe					53.3	3.50
4-OMe	57.24	3.86 (*s*)			57.4	3.85
6-OMe	57.17	3.73 (*s*)	57.0	3.69 (*s*)	56.9	3.67
4-OH				10.34 (*s*)		

[a] Measured in DMSO-d_6 at 500 MHz (^1H NMR) and 125 MHz (^{13}C NMR).

replaced by a hydroxyl proton singlet at δ_H 10.34 in the ^1H NMR spectrum of **2**. The observation could only be explained by assuming that **2** was a demethylated derivative of **1**. This assumption was reinforced by the HMBC correlations of 4-OH with C-3, C-4 and C-5. The configuration at C-8 was also elucidated to be *R* by its optical rotation ($[\alpha]_D^{20} = -87.4$), with a same sign to that of **1**. Metabolite **2** was named penicidone B.

The molecular formula of compound **3** was established as $C_{19}H_{19}NO_6$ from the $[M+H]^+$ peak at *m/z* 358.1285 in its HRESIMS spectrum (calcd. for $C_{19}H_{20}NO_6$: 358.1291, Δ 1.6 ppm). The ^1H and ^{13}C NMR chemical shifts were similar to those of **1** (Table 1). However, extra methoxyl signals at δ_H 3.50 and δ_C 53.3 appeared in the ^1H and ^{13}C NMR spectra of **3**. The 8-oxygenated methine carbon signal at δ_C 77.7 in **1** was replaced by a ketone carbonyl resonating at δ_C 193.0. In the HMBC spectrum of **3**, this methoxyl proton gave a cross-peaks with C-1 (δ_C 167.1), and the 8-carbonyl (δ_C 193.0) with H-14 (δ_H 8.16). These spectroscopic features suggested that metabolite **3** was derived from **1** by γ-lactone moiety hydrolyzation followed by methyl esterification at C-1 and oxidation at C-8. This proposal was confirmed by the subsequent analyses of its 1D and 2D NMR spectroscopic data to lead to the depicted structure of **3,** named penicidone C.

The *in vitro* cytotoxity assay against four human cancer cell lines SW1116, K562, KB and Hela indicated that compounds **1–3** were moderately cytotoxic with their IC_{50} values between 21.1 and 90.8 μM, and the IC_{50} values of 5-fluorouracil (co-assayed as a positive control) between 17.0 and 122.0 μM against those cell lines (Table 2).

Penicidones A–C (**1–3**) could be biosynthesized through the polyketide pathway (Merlini et al., 1970) together with a set of sequential biotransformations including transamination, cyclization, oxidation, reduction and esterification (Fig. 1). Penicidones A–C (**1–3**) are structurally unique in carrying an identical γ-pyridone nucleus that have so far not been encountered in any reported natural products, although some structurally similar metabolites such as vermistatin and funicone derivatives with a γ-pyrone ring have previously been isolated from fungi belonging to the genera *Penicillium* (Fuska et al., 1986; Komai et al., 2005; Merlini et al., 1970; Murtaza et al., 1997; De Stefano et al., 2002; Kimura et al., 1995), *Mycosphaerella* (Upadhyay et al., 1990), and *Talaromyces* (Komai et al., 2004). From a biochemical viewpoint, the N atom in γ-pyridone ring could originate from sodium glutamate in medium under the catalysis of aminotransferase (Chen et al., 1999).

The three metabolites penicidones A–C (**1–3**) could be re-detected in the acetone extract of the fungal culture by HPLC (mobile phase: CH_3CN–H_2O) and TLC (developed with petroleum ether–acetone mixture), removing any possibility that they could be artifacts generated through methylation where MeOH was used. Furthermore, the methyl-carrying analogs such as funicone, isofunicone, rapicone, vermistatin and its congeners were also reported as natural products from the genus *Penicillium* (Merlini et al., 1970; Kimura et al., 1995; Nozawa et al., 1992; Komai et al., 2005), suggesting that some *Penicillium* strains methylate certain substrates.

Table 2
In vitro cytotoxicity (IC_{50} μM) of **1**, **2** and **3** against four cancer cell lines

	SW1116	K562	KB	Hela
1	60.1 ± 3.2	54.0 ± 4.3	46.5 ± 4.5	41.5 ± 1.6
2	54.2 ± 2.1	21.1 ± 0.8	29.6 ± 1.5	35.1 ± 2.1
3	80.8 ± 5.5	54.3 ± 1.4	44.3 ± 2.4	54.7 ± 2.3
5-Fluorouracil[a]	47.1 ± 3.1	43.1 ± 1.7	17.0 ± 1.4	122.0 ± 5.9

[a] Used as a positive control.

3. Experimental

3.1. General procedures

Optical rotations were taken on a Perkin–Elmer 341 digital polarimeter, whether UV spectra were recorded on a Hitachi U-3000 spectrophotometer. The IR spectra were measured on a Nexus 870 FT-IR spectrometer. ESIMS spectra were recorded on a Mariner System 5304 mass spectrometer. NMR data were acquired in DMSO-d_6 on a Bruker DRX500 NMR spectrometer with ^1H and ^{13}C NMR spectra observed at 500 and 125 MHz with solvent signals (DMSO-d_6, δ_H: 2.50 and δ_C: 40.8 ppm) as internal references. Silica gel (200–300 mesh) for CC was produced by Qingdao Marine Chemical Factory, Qingdao, China. Sephadex LH-20 was purchased from Pharmacia Biotech, Sweden. HPLC analyses were performed by using a column of Allsphere ODS-2.5 mm (250 × 4.6 mm), Hitachi pump L-7100, and a UV detector L-7400. All other chemicals used in this study were of analytical grade.

3.2. Fungus identification

Penicillium sp. IFB-E022 was isolated from a healthy stem of *Q. variabilis* collected in April 2002 from the Zijin Mountain in Nanjing, China. The specimen of *Q. variabilis* was authenticated by Prof. L.X. Zhang (School of Life Sciences, Nanjing University). The strain was identified by Dr. Y.C. Song through its morphological characteristics with additional confirmation from its 18S rDNA sequence that gave a 99% sequence similarity to those accessible at the BLASTN of *Penicillium* sp. The sequences of the strain have been deposited to the GenBank, as EF211128 (http://www.ncbi.nlm.nih.gov/Genbank/update.html). Colonies of the title strain showed shades of yellow, consisting of a dense felt of conidiophores after 4-day growth on PDA Petri dish. Chains of conidia were produced in basipetal succession from the phialide. Phialides were produced in groups from branched metulae and had a brush-like appearance. The branched metulae showed a two-stage pattern. The conidiophores were cylindrical and rough-walled. These are typical characteristics of *Penicillium*

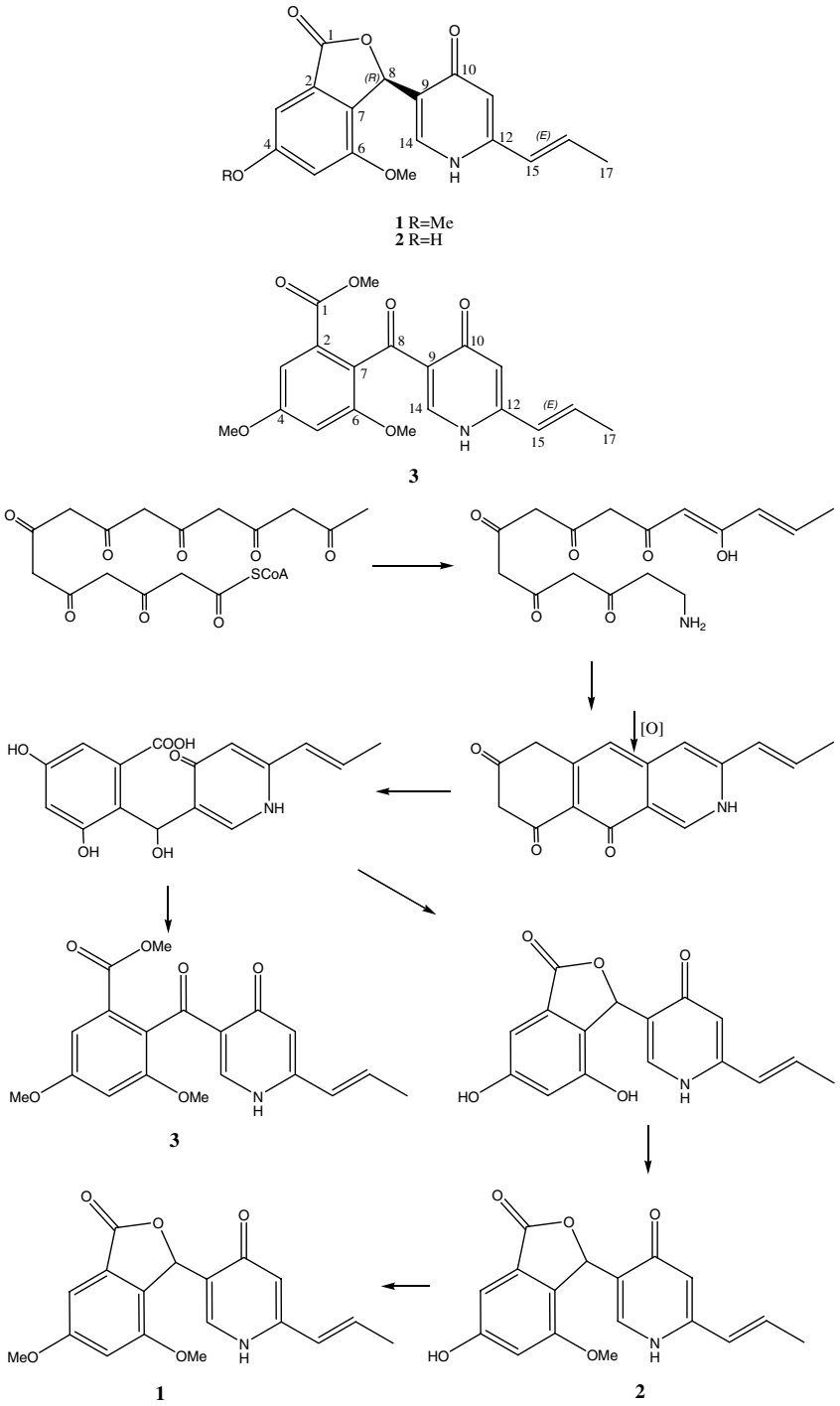

Fig. 1. Proposed biosynthetic pathway for compounds **1**, **2** and **3**.

(Wei, 1979). A living culture, together with a voucher specimen of the host plant, was preserved under the number IFB-E022 in the Herbarium of the Institute of Functional Biomolecules, Nanjing University, Nanjing, China.

3.3. Fermentation and isolation

After growing on PDA (potato dextrose agar) medium at 28 °C for 5 days, the title endophyte, *Penicillium* sp. IFB-E022, was inoculated into Erlenmeyer flasks (1000 mL) containing 300 mL of PDA medium. After incubation for 4 days at 28 °C on a rotary shaker at 150 rpm, culture liquid (20 mL) was transferred as the "seed" into 250 mL flasks, each preloaded with evenly mingled medium composed of grain (7.5 g) bran (7.5 g) yeast extract (0.5 g) sodium tartrate (0.1 g) $FeSO_4 \cdot 7H_2O$, (0.01 g) sodium glutamate, (0.1 g) pure corn oil, (0.1 mL) and H_2O, (30 mL) and grew for 20 days at 28 °C with the relative humidity

in the range 60–70%. The harvested solid culture was dehydrated to a residue (2.7 kg) which was extracted at room temperature with MeOH (4 L × 4). Evaporation of the solvent under reduced pressure gave a black oil (225 g) which was diluted with H_2O (700 mL) to give an aqueous suspension. After defatting with petroleum ether (700 mL × 3), the suspension was successively extracted with EtOAc (700 mL × 3) and *n*-butanol (700 mL × 3). The EtOAc fraction, shown to be cytotoxic, was concentrated *in vacuo* to give a residue (24.2 g) which was subjected to silica gel CC eluted with a $CHCl_3$–MeOH mixture (1:0; 100:1; 100:2; 100:4; 100:8; 0:1, each 1.5 L) to afford six fractions (Fr-1: 2.1 g, Fr-1: 1.8 g, Fr-2: 1.2 g, Fr-3: 3.8 g, Fr-4: 2.5 g, Fr-5: 1.1 g, Fr-6: 9.3 g). Bioactive fractions Fr-1 and 2 were combined and further purified by reverse phase C-18 column (60 × 5 cm) eluted with H_2O–MeOH mixtures (1:0, 85:15, 70:30, 50:50, 0:1) to give four subfractions Fr-1a: 0.2 g, Fr-1b: 0.5 g, Fr-1c: 0.3 g, Fr-1d: 0.2 g and Fr-1e: 1.5 g). Fr-1a was further purified with Sephadex LH-20 to give **1** (19 mg). Fr-1b was re-separated by a silica gel column with petroleum–acetone (2:1) to give **2** (32 mg) and physcion (8 mg) (Yang et al., 1998), lumichrome (40 mg) (Yu and Guo, 2002). Fr-1 c was also purified with Sephadex LH-20 to give **3** (21 mg) and emodin-1, 6-dimethyl ether (5 mg) (Okabe et al., 1973).

3.4. Spectroscopic data of alkaloids

3.4.1. Penicidone A (*1*)

White amorphous powder; ($[\alpha]_D^{20} = -98.3$) ($c = 0.11$, $CHCl_3$); UV (MeOH) λ_{max} ($\log\varepsilon$): 206 (4.6), 237 (3.1) nm; IR (KBr) ν_{max} 3110, 3048, 2944, 2842, 1765, 1627, 1609, 1528, 1504, 1413, 1336, 1283, 1241, 1118 cm^{-1}; ESIMS *m/z* 328 $[M+H]^+$, 350 $[M+Na]^+$; HRESIMS *m/z* $[M+H]^+$ 328.1175 (calcd. for $C_{18}H_{18}NO_5$: 328.1179, Δ1.2 ppm); for ^1H and ^{13}C NMR spectroscopic data, see Table 1.

3.4.2. Penicidone B (*2*)

White amorphous powder; ($[\alpha]_D^{20} = -87.4$) ($c = 0.20$, $CHCl_3$); UV (MeOH) λ_{max} ($\log\varepsilon$): 209 (4.4), 239 (4.0) nm; IR (KBr) ν_{max} 3281, 3119, 3057, 2941, 1736, 1666, 1627, 1530, 1466, 1392, 1349, 1289, 1230, 1183 1125 cm^{-1}; ESIMS *m/z* 314 $[M+H]^+$, 337 $[M+Na]^+$; HRESIMS *m/z* $[M+H]^+$, 314.1023 (calcd. for $C_{17}H_{16}NO_5$: 314.1013, Δ 3.1 ppm); for ^1H and ^{13}C NMR spectroscopic data, see Table 1.

3.4.3. Penicidone C (*3*)

White amorphous powder; UV (MeOH) λ_{max} ($\log\varepsilon$): 215 (4.1), 253 (4.3) nm; IR (KBr) ν_{max} 3214, 3008, 2945, 2842, 1723, 1665, 1624, 1602, 1534, 1499, 1455, 1329, 1291, 1246, 1217, 1151 cm^{-1}; ESIMS *m/z* 358 $[M+H]^+$, 380 $[M+Na]^+$; HRESIMS [M+H] *m/z* 358.1285 ($C_{19}H_{20}NO_6$: 358.1291, Δ 1.6 ppm); for ^1H and ^{13}C NMR spectroscopic data, see Table 1.

3.5. Cytotoxicity assay

The cytotoxicity of **1–3** was tested on a series of cancer cell lines, namely, human colon cancer (SW1116), leukemia (K562), human nasopharynyeal epidermoid tumor (KB) and human cervical carcinoma (Hela) cell lines. The effects of **1–3** on the viability of these cells were assayed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method (Menicagli et al., 2004). Briefly, cells at the exponential phase were collected and transferred into 96- well plates. After 24 h incubation, compound dilutions were dispensed to the established culture plates for 48 h. An MTT solution was then added to each well (0.1 mg/well). After further incubation for 4 h, the supernatant was removed, the crystals were dissolved in DMSO (150 μL), and the absorbencies of each well were read at 570 nm. The value for IC_{50} was determined at the concentration that inhibited cell growth by 50% using the MTT assay. The data represent the mean of three experiments performed in triplicate and are expressed as means ± SD.

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