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A PAIR OF NEW NON-GLYCOSIDIC IRIDOID EPIMERS FROM *SCROPHULARIA NINGPOENSIS*

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Abstract – A pair of new non-glycosidic iridoid epimers, ningpogenins A and B (**1a/1b**), together with eighteen known ones (**2-19**) were isolated from the roots of *Scrophularia ningpoensis*. Their structures were elucidated by spectroscopic analyses, including 1D-, 2D-NMR, and HR-ESI-MS. Compound **1** is an unusual example of natural ningpogenin—L-pyroglutamic adducts. In addition, the cytotoxic effects of the new compound against HepG2, U251 and SH-SY5Y cell lines were evaluated.

Scrophularia ningpoensis Hemsl., belonging to the family Scrophulariaceae is widely distributed in China.¹ The dried roots of *S. ningpoensis* (termed “xuanshen” in Chinese Pharmacopeia) had been used as a traditional and popular Chinese medicine to treat pharyngitis, laryngitis, tonsillitis, fever, erythema, furunculosis, and cancer.^{2,3} Previously chemical and pharmacological investigation of *S. ningpoensis* had led to the discovery of a number of iridoids and phenylpropanoid glycosides,^{4,5} some of which exhibited antimicrobial, anti-inflammatory, antitumor, and neuroprotective activities.⁶ As a part of our ongoing efforts to search for structurally diverse and bioactive compounds from traditional Chinese medicine, the chemical constituents of the dried roots of *S. ningpoensis* were investigated. Here we reported the isolation and structure elucidation of compounds **1-19**. Among them, compounds **1a** and **1b** were a pair of new non-glycosidic iridoid epimers. Compound **1** was a natural adduct that is constructed through ningpogenin and L-pyroglutamic acid derivative. In addition, the cytotoxic effects of the new compound against HepG2 (human hepatocellular carcinoma), U251 (human glioma) and SH-SY5Y (human neuroblastoma) cell lines were evaluated.

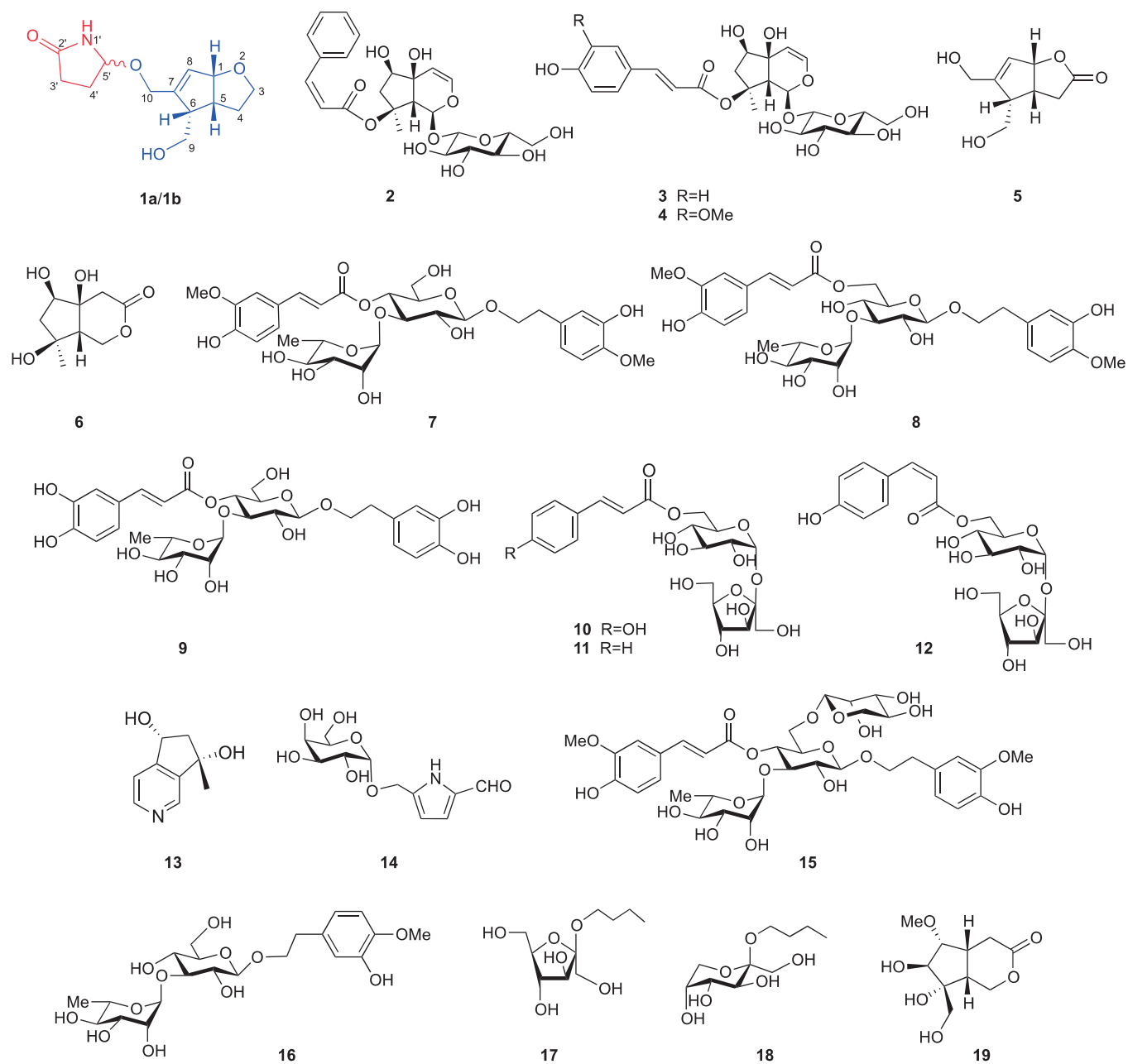


Figure 1. Chemical structures of compounds 1-19

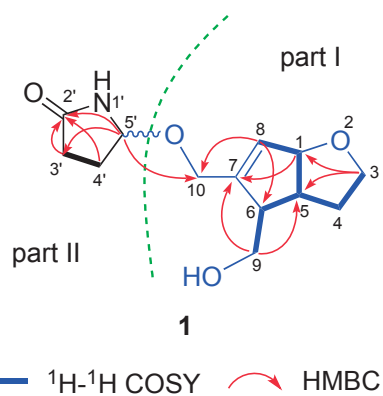


Figure 2. Key $^1\text{H}-^1\text{H}$ COSY and HMBC correlations of 1

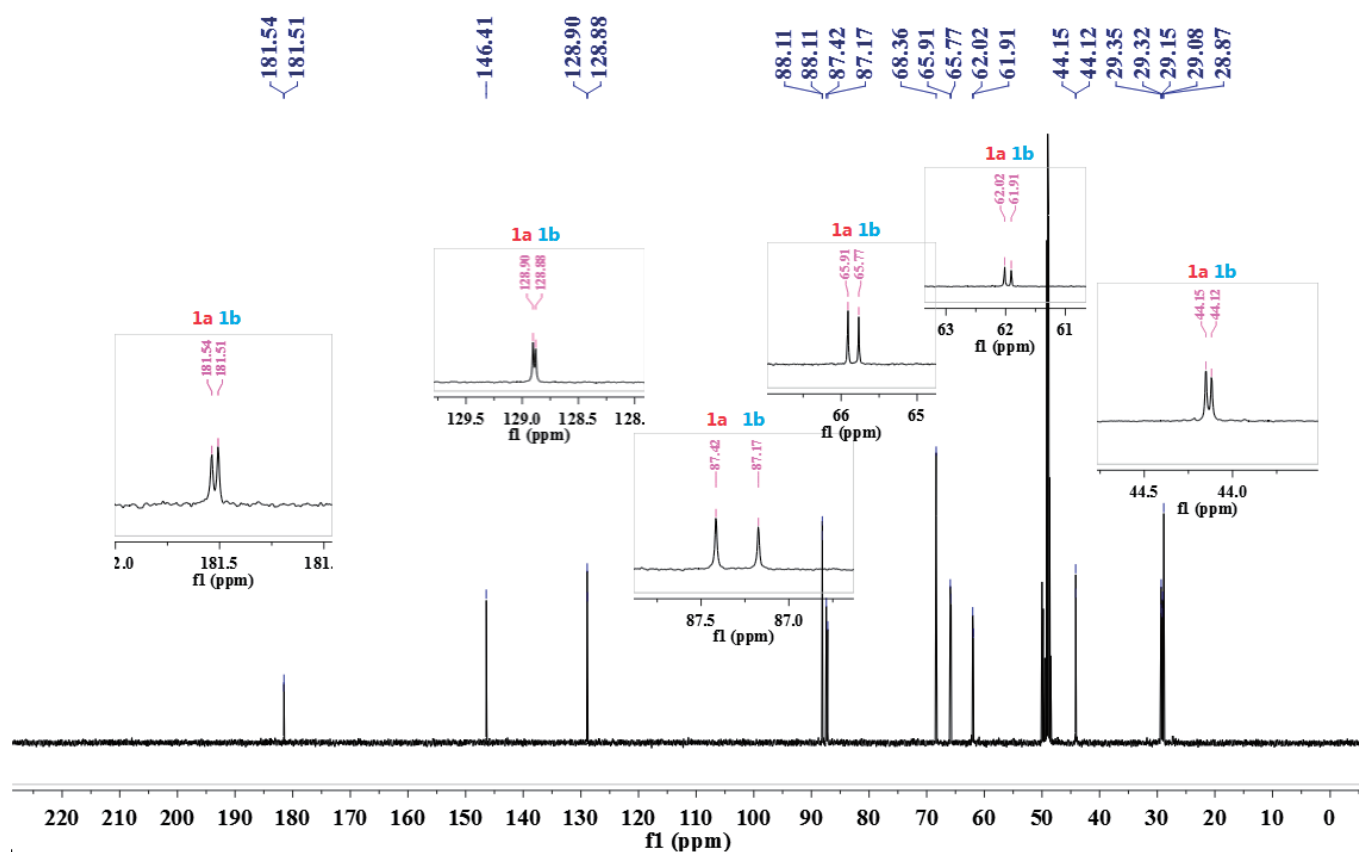


Figure 3. ^{13}C NMR spectrum for the mixture of **1a** (red) and **1b** (blue)

Ninggenins A and B (**1a/1b**) was obtained as white amorphous powder. The molecular formula of **1** was determined as $\text{C}_{13}\text{H}_{19}\text{NO}_4$ by its HRESIMS at m/z 254.1390 $[\text{M}+\text{H}]^+$ (calcd for $\text{C}_{13}\text{H}_{20}\text{NO}_4$, 254.1392). The UV spectrum of **1** showed absorption maximum at 212 nm. The IR spectrum exhibited the characteristic absorptions for lactam (3425 , 1683 cm^{-1}) and double bonds (1667 cm^{-1}) groups. Compound **1** exists as a pair of inseparable epimers (Figure 1). The ^1H and ^{13}C NMR spectra of **1** displayed two sets of signals in a ratio of approximately 1:1 (**1a/1b**, Figure 3). The ^1H NMR spectrum for one isomer **1a** showed signals for two oxymethine [δ_{H} 5.01 (1H, m), 5.00 (1H, br d, $J = 7.0$ Hz)], three oxymethylene protons [δ_{H} 3.72 (1H, m), 3.64 (1H, m), 3.80 (1H, m), 3.62 (1H, m), 4.12 (1H, br d, $J = 13.0$ Hz), 3.99 (1H, br d, $J = 13.0$ Hz)], and an olefin proton [δ_{H} 5.63 (1H, br s)]. The ^{13}C NMR and DEPT spectra of **1a** exhibited thirteen carbon signals, including two oxygenated methines [δ_{C} 88.1 (C-1), 87.4 (C-5')], three oxygenated methylenes [δ_{C} 68.4 (C-3), 62.0 (C-9), 65.9 (C-10)], two olefin carbons [δ_{C} 146.4 (C-7), 128.9 (C-8)], two methines [δ_{C} 44.1 (C-5), 50.0 (C-6)], three methylenes [δ_{C} 28.9 (C-4), 29.3 (C-3'), 29.1 (C-4')], and a carbonyl [δ_{C} 181.5 (C-2')]. With the aid of ^1H - ^1H COSY, HSQC, and HMBC experiments, all the ^1H and ^{13}C NMR signals of **1** were assigned as shown in Table 1. The ^1H - ^1H COSY spectrum of **1a** revealed the presence of the spin systems as shown in Figure 2. In the HMBC spectrum, correlations between H-1 and C-7, between H-3 and C-1/C-5, between H-8 and C-6/C-10, as well as between H-9 and

C-5/C-7 allowed the establishment of the planar structure of ningpogenin moiety (**part I**).^{7,8} The spin system from H-3' to H-4' in the ¹H-¹H COSY spectrum, as well as the HMBC correlations between H-5' and C-2'/C-3', between H-3' and C-2'/C-5', between H-4' and C-2'/C-5' established the planar structure of 5-hydroxypyrrolidin-2-one residue (**part II**). Furthermore, the two units **part I** and **part II** could be connected through an oxygen atom by HMBC cross-peaks from δ_{H} 5.01 (H-5') to δ_{C} 65.9 (C-10) and from δ_{H} 4.12, 3.99 (H-10) to δ_{C} 87.4 (C-5'). Analysis of the 1D and 2D NMR spectroscopic data of another isomer **1b** reveals that the two isomers have the same 2D structure. The main differences between **1a** and **1b** involved resonances for CH₂-10 (δ_{H} 4.12/3.99 for **1a**, and δ_{H} 4.08/4.03 for **1b**), which implies that **1b** is a C-5' isomer of **1a**. Compound **1** being as a pair of inseparable stereoisomers (**1a/1b**) was isolated as a mixture. Unfortunately, no more samples were available to do more research to demonstrate the absolute configuration at C-5'. Therefore, it was determined only that **1a/1b** possessed opposite configurations at C-5'.

Table 1. NMR data of **1** (CD₃OD, *J* in Hz)^c

No.	1a		1b	
	δ_{H}^a	δ_{C}^b	δ_{H}^a	δ_{C}^b
1	5.00 (br d, 7.0)	88.1	5.00 (br d, 7.0)	88.1
3	3.72 (m), 3.64 (m)	68.4	3.72 (m), 3.64 (m)	68.4
4	1.93 (m), 1.84 (m)	28.9	1.93 (m), 1.84 (m)	28.9
5	3.05 (m)	44.1	3.05 (m)	44.1
6	2.92 (m)	50.0	2.92 (m)	49.8
7		146.4		146.4
8	5.63 (br s)	128.9	5.63 (br s)	128.9
9	3.80 (m), 3.62 (m)	62.0	3.80 (m), 3.62 (m)	61.9
10	4.12, 3.99 (each br d, 13.0)	65.9	4.08, 4.03 (each br d, 13.0)	65.8
2'		181.5		181.5
3'	2.46, 2.19 (m)	29.3	2.46, 2.19 (m)	29.3
4'	2.31, 2.04 (m)	29.1	2.31, 2.04 (m)	29.1
5'	5.01 (m)	87.4	5.01 (m)	87.2

^aMeasured at 500 MHz. ^bMeasured at 125 MHz.

^cOverlapped signals are reported without designating multiplicity.

In the NOESY spectrum, correlations between H-1 and H-5/H-6, and between H-5 and H-6 suggested that the relative configurations of C-1, C-5 and C-6 as shown in Figure 4. Based on the above evidences, the structure of **1** was elucidated.

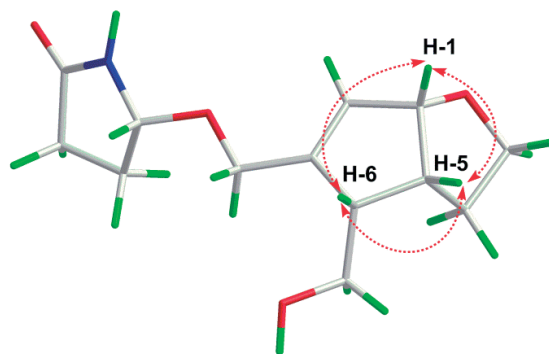


Figure 4. Key NOESY correlations of **1**

The eighteen known compounds were identified as 8-*O*-*cis*-cinnamoylharpagide (**2**),⁹ 8-*O*-*p*-coumaroylharpagide (**3**),¹⁰ 8-*O*-feruloylharpagide (**4**),¹¹ pedicularis-lactone (**5**),¹² buergerinin B (**6**),¹³ martynoside (**7**),¹⁴ isomartynoside (**8**),¹⁵ acteoside (**9**),¹⁶ 6-*trans*-*p*-coumaroylsucrose (**10**),¹⁷ sibirioside A (**11**),² 6-*cis*-*p*-coumaroylsucrose (**12**),¹⁷ (-)-oxerine (**13**),¹⁸ 5-[(α -D-galactopyranosyloxy)methyl]-1*H*-pyrrole-2-carbaldehyde (**14**),¹⁹ angoroside C (**15**),^{20,22} darendoside B (**16**),²¹ *n*-butyl β -D-fructofuranoside (**17**),²² *n*-butyl β -D-fructopyranoside (**18**)²² and buddlin (**19**)²³ by comparing their spectral data with those reported in the literature (Figure 1). Compounds **13** and **14** were firstly isolated from Scrophulariaceae species. Compounds **10** and **12** were firstly isolated from Scrophularia genus. Compounds **6** and **19** were firstly isolated from *S. ningpoensis*.

The cytotoxicities of the new compound against three cancer cell lines (HepG2, U251 and SH-SY5Y) were evaluated by MTT assay. However, the mentioned compound was found to be devoid of significant cytotoxic activity at the concentration of 50 μ M.

EXPERIMENTAL

General

UV spectra were recorded on a PERSEE TU-1810 spectrometer (PERSEE, Beijing, China). IR spectra were determined on a Nicolet Impact 410 FTIR spectrophotometer (Thermo Electron Corporation, Massachusetts, USA) using KBr pellets. Optical rotations were determined using a Hanon P800 digital polarimeter (HANON, Jinan, China) at 25 °C. NMR spectra were recorded on Bruker Avance DRX-500 spectrometer (Bruker, Bremerhaven, Germany). HR-ESI-MS and ESI-MS were carried out on a Micromass ZabSpec mass spectrometer and a VG-20-250 mass spectrometer, respectively. Column chromatographies (CC) were performed on Sephadex LH-20 (GE Healthcare Bio-Sciences, Sweden), silica gel (200-300 mesh, Qingdao Marine Chemical Inc, Qingdao, P. R. China), Diaion HP2 MGL Macroporous adsorptive resins (Mitsubishi, Korea), and ODS (50 μ m; Merck; Germany). TLC was pre-coated with silica gel GF-254 (Qingdao Marine Chemical Inc, Qingdao, China). All the reagents were

of analytical grade.

Plant Material

The dried roots of *Scrophularia ningpoensis* were collected from Bozhou Medical Material Market in November 2013. A voucher specimen (No. 081218) identified by Prof. Chun-Sheng Liu (Beijing University of Chinese Medicine) was deposited with School of Chinese Materia Medica, Beijing University of Chinese Medicine, Beijing, P. R. of China.

Extraction and Isolation

The dried roots of *S. ningpoensis* (20.0 kg) were pulverized and extracted with 95% EtOH (3×160 L) for 3 h each time, then with 60% EtOH (3×160 L) for 1.5 h each time. The combined extracts were concentrated by a rotary evaporator at 45 °C to obtain a crude extract. The extract (2.0 kg) was suspended in H₂O and partitioned with petroleum ether, CHCl₃, EtOAc and *n*-butanol successively. The EtOAc-soluble fraction was dried to yield an extract (38.0 g), which was subjected to silica gel column chromatography using CHCl₃-MeOH (50:1 to 10:1, v/v) as an eluent to afford four fractions (Fr. A-D). Fr. B (8.1 g) was subjected to silica gel column chromatography using CH₂Cl₂-MeOH (50:1 to 30:1, v/v), and then was subjected to an ODS column using MeOH-H₂O (30:70, v/v) as an eluent to obtain compounds **8** (3.0 mg) and **9** (4.0 mg). Fr. C (4.0 g) was separated by silica gel column eluted with CHCl₃-MeOH (30:1) to afford four subfractions (Fr. C1-C4). Fr. C1 (15.3 mg) was subjected to MCI column chromatography eluted with MeOH-H₂O (70:30 to 100:0, v/v) to yield compound **1** (4.2 mg). Fr. C3 was subjected to silica gel column eluted with a gradient mixture of CHCl₃-MeOH (30:1 to 15:1, v/v), followed by ODS column eluted with MeOH-H₂O (70:30 to 100:0, v/v) to obtain compounds **2** (5.0 mg) and **10** (8.0 mg). The *n*-BuOH fraction (150.0 g) was separated into two fractions (Fr. G-H) by Diaion HP-2 MGL macroporous resin eluted with MeOH-H₂O in gradient. Fr. G was loaded onto silica gel column eluted with EtOAc-MeOH (from 50:1 to 40:1) to afford two subfractions (Fr. G1 and G2). Fr. G1 was purified by Sephadex LH-20 column (MeOH) and ODS column (MeOH-H₂O in gradient) to yield compounds **11** (3.1 mg), **12** (2.4 mg), **13** (4.9 mg), **14** (6.0 mg) and **15** (3.2 mg). Fr. G2 was subjected to an ODS column eluted with a gradient of MeOH-H₂O (20:80 to 100:0, v/v) to yield compounds **3** (4.0 mg) and **4** (5.3 mg). Fr. H was subjected to silica gel column chromatography using CHCl₃-MeOH in gradient (30:1 to 5:1, v/v) to afford three subfractions (Fr. H1-H3). Fr. H1 was subjected to an ODS column using MeOH-H₂O (80:20-100:0, v/v) to afford compounds **5** (6.2 mg), **18** (4.1 mg) and **19** (4.2 mg). Fr. H2 was separated by ODS column eluted with MeOH-H₂O (80:20) to yield compounds **6** (3.2 mg), **7** (2.2 mg) and **17** (4.7 mg). Fr. H3 was first subjected to MCI column (MeOH-H₂O in gradient) and then separated by ODS column to yield compound **16** (3.4 mg).

Ningpogenin A and B (1a/1b)

White amorphous powder; $[\alpha]_{\text{D}}^{25} + 15.8$ (c 1.50, MeOH); UV (MeOH) λ_{max} (log ϵ): 212 (3.43) nm; IR (KBr) ν_{max} : 3425, 2922, 2858, 1683, 1667, 1060 cm^{-1} ; ^1H NMR (CD_3OD , 500 MHz), ^{13}C NMR (CD_3OD , 125 MHz), Table 1; ESI-MS: m/z 254.1 $[\text{M}+\text{H}]^+$, 276.1 $[\text{M}+\text{Na}]^+$; HR-ESI-MS: m/z 254.1390 $[\text{M}+\text{H}]^+$ (calcd for $\text{C}_{13}\text{H}_{20}\text{NO}_4$, 254.1392).

Cytotoxic Assay

HepG2 (human hepatocellular carcinoma), U251 (human glioma) and SH-SY5Y (human neuroblastoma) cells were obtained from American Type Culture Collection (ATCC). The cells were seeded into 96-well plates and incubated at 37 °C in an atmosphere of 5% CO_2 for 12 h. Different concentrations of the compounds were added to the cells followed by incubation for 48 h. Then 100 μL fresh culture medium and 10 μL MTT solution (5 mg/mL) were added to the cells with incubation for 4 h, after which the culture medium was removed and 150 μL DMSO was added to dissolve the formazan crystals, incubating for 10 min. The absorbance of plates was measured using SpectraMax i3x Multi-mode detection platform (Molecular Devices, Austria) at 490 nm.

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