New Sesquiterpenoids from *Ligularia duciformis*

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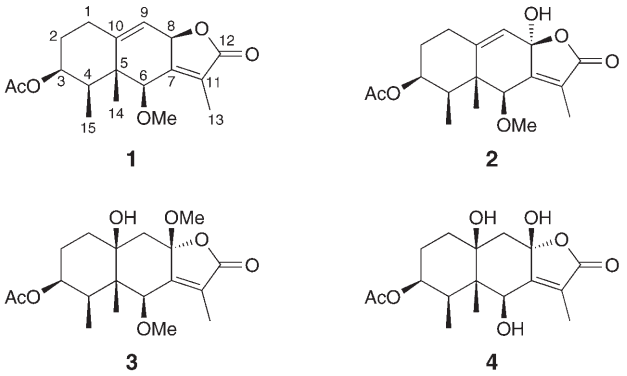
From the whole plants of *Ligularia duciformis*, four new sesquiterpenoids, 3β-acetoxy-6β-methoxyeremophila-7(11),9(10)-dien-12,8β-olide (**1**), 3β-acetoxy-8α-hydroxy-6β-methoxyeremophila-7(11),9(10)-dien-12,8β-olide (**2**), 3β-acetoxy-10β-hydroxy-6β,8β-dimethoxyeremophil-7(11)-en-12,8α-olide (**3**), and 3β-acetoxy-6β,8β,10β-trihydroxyeremophil-7(11)-en-12,8α-olide (**4**) were isolated. Their structures were established by high-field NMR techniques (^{1}H,^{1}H-COSY, ^{13}C-APT, HMQC, HMBC, and NOESY) and HR-ESI-MS analysis, together with comparison of the spectroscopic data with those of structurally related compounds. In addition, the cytotoxicity of the new compounds against human hepatic cancer cells Bel-7402, human pneumonic cancer cells A-549, and human colonic cancer cells HCT-8 were evaluated, the new compounds showed no cytotoxicity against the three tumor cells (all IC_{50} values >200 μM).

Introduction. – *Ligularia duciformis* (Compositae) is a perennial grass, which is native in the southwest area of mainland China. Its roots are used as a Chinese folk medicine for the treatment of inflammation and apoplexy [1], showing effect on nourishing lung and relieving a cough. Sesquiterpenoids, especially eremophil-7(11)-en-12,8-olides have been isolated from other plants in *Ligularia* as the characteristic components of the genus [2–4]. However, phenolic compounds were previously reported from the dried root of *L. duciformis* collected in Hubei Province [5]. Herein, we studied the constituents of the MeOH extract of the whole plant of *L. duciformis*, collected in the southwest area of Sichuan Province, where the average height is more than 3000 meters. As a result, four new eremophil-7(11)-en-12,8-olides **1**–**4** were isolated. In this work, we describe the isolation and structural elucidation of these compounds. Furthermore, all new compounds were evaluated for their cytotoxicity against human hepatic cancer cells Bel-7402, human pneumonic cancer cells A-549, and human colonic cancer cells HCT-8.

Results and Discussion. – Compound **1** was obtained as colorless needles. Its molecular formula was deduced as $C_{18}H_{24}O_5$ from HR-ESI-MS ($[M+H]^+$ at m/z 321.1691), and showed seven degrees of unsaturation. The IR spectrum showed absorptions of an α,β-unsaturated γ-lactone (1741 cm^{-1}) and an AcO group (1710 cm^{-1}), as well as of a C=C bond (1610 cm^{-1}). The ^{13}C-NMR spectrum displayed 18 C-atoms including five Me, two CH_2, and five CH groups, as well as six quaternary C-atoms, assigned by an APT experiment. Additionally to two C=O groups with signals at δ(C) 173.80 and δ(C) 170.77, and two C=C bonds with signals at δ(C) 157.82, δ(C) 120.70, δ(C) 148.57, and δ(C) 118.00 in the ^{13}C-NMR spectrum, the compound should



consist of three rings to satisfy the degrees of unsaturation. In the ^{1}H-NMR spectrum, an AcO signal at δ(H) 2.06 (*s*) and a MeO signal at δ(H) 3.46(*s*) could be observed. Based on the above data and comparison of the spectral data with those of reported eremophilenolides, the structure was proposed to be an eremophil-7(11)-en-12,8-olide [6]. Assignments of the ^{1}H- and ^{13}C-NMR data (*Tables 1* and *2*) were based on an HMQC experiment. In the ^{1}H,^{1}H-COSY spectrum, Me(15) (δ(H) 1.15) showed a correlation with H_α–C(4) (δ(H) 1.92–1.99), and H_α–C(4) showed a correlation with H_α–C(3) (δ(H) 4.94), while H–C(9) (δ(H) 5.61) showed a correlation with H_α–C(1) (δ(H) 2.46–2.53) and H–C(8) (δ(H) 5.28), respectively. In the HMBC spectrum, H_α–C(3) showed a correlation with the C-atom at δ(C) 170.77 of the AcO group, H–C(9) showed correlations with C(7) (δ(C) 157.82) and C(5) (δ(C) 50.12). Thus, the AcO group should be attached to C(3), and the second C=C bond should be between C(9) and C(10) (*Fig. 1*). The relative configuration at C(3) was established as β-oriented (orientation of the AcO group) by the small coupling constants between H_α–C(3) (equatorial bond) and CH_2(2) and H_α–C(4) ($J(3\alpha,2\beta) = 2.5$, $J(3\alpha,2\alpha) = J(3\alpha,4\alpha) = 3.0$). The cross-peaks of H_α–C(6) and H_α–C(4), and H_α–C(6) and H_α–C(8) in the NOESY spectrum indicated that the MeO group at C(6) and the α,β-unsaturated lactone at C(8) were both β-oriented (*Fig. 2*). Therefore, compound **1** was elucidated as 3β-acetoxy-6β-methoxyeremophila-7(11),9(10)-dien-12,8β-olide.

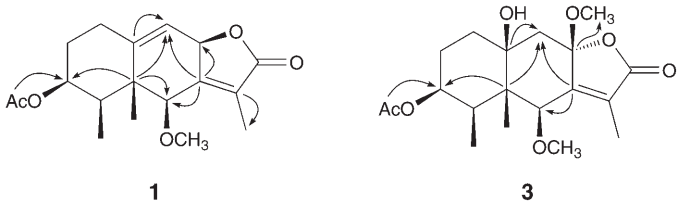


Fig. 1. *Key HMBC correlations of compounds* **1** *and* **3**

Compound **2** was obtained as a colorless oil. Its molecular formula was assigned as $C_{18}H_{24}O_6$ on the basis of the HR-ESI-MS ($[M+Na]^+$ at *m*/*z* 359.1463). The ^{1}H- and ^{13}C-NMR spectra (*Tables 1* and *2*) were close to those of **1**. However, no H–C(8) H-

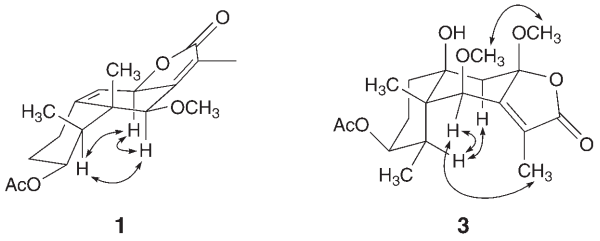
Table 1. *^{1}H-NMR Data of Compounds* **1**–**4** (500 MHz, (D_6)acetone, δ in ppm)

	1	**2**	**3**	**4**
H_α–C(1)	2.46–2.53 (*m*)	2.46–2.50 (*m*)	1.85–1.93 (*m*)	2.13–2.18 (*m*)
H_β–C(1)	2.05–2.09 (*m*)	2.01–2.03 (*m*)	1.22–1.27 (*m*)	1.43–1.49 (*m*)
H_α–C(2)	1.55–1.64 (*m*)	1.59–1.66 (*m*)	1.72–1.79 (*m*)	1.78–1.87 (*m*)
H_β–C(2)	1.95–2.03 (*m*)	1.94–2.03 (*m*)	1.66–1.70 (*m*)	1.59–1.66 (*m*)
H–C(3)	4.94 (*dt*, *J* = 3.0, 2.5)	4.94 (*dt*, *J* = 3.0, 2.5)	4.87 (*dt*, *J* = 2.5, 1.5)	4.90 (*dt*, *J* = 3.0, 1.5)
H–C(4)	1.92–1.99 (*m*)	1.98–2.02 (*m*)	1.67–1.72 (*m*)	1.31–1.36 (*m*)
H–C(6)	4.23 (*q*, *J* = 1.0)	4.24 (*q*, *J* = 1.0)	4.39 (*s*)	4.70 (*s*)
H–C(8)	5.28 (*d*, *J* = 1.5)	–	–	–
H_α–C(9)	5.61 (*t*, *J* = 1.5)	5.74 (*d*, *J* = 1.0)	2.40 (*d*, *J* = 15)	2.37 (*d*, *J* = 15)
H_β–C(9)			2.22 (*d*, *J* = 15)	2.22 (*d*, *J* = 15)
Me(13)	1.93 (*d*, *J* = 1.0)	1.94 (*d*, *J* = 1.0)	1.96 (*s*)	1.88 (*s*)
Me(14)	1.14 (*s*)	1.14 (*s*)	1.36 (*s*)	1.43 (*s*)
Me(15)	1.15 (*d*, *J* = 7.0)	1.14 (*d*, *J* = 7.0)	0.96 (*d*, *J* = 7.0)	0.91 (*d*, *J* = 8.0)
6-MeO	3.46 (*s*)	3.43 (*s*)	3.40 (*s*)	–
8-MeO	–	–	3.24 (*s*)	–
AcO	2.06 (*s*)	2.06 (*s*)	2.05 (*s*)	2.11 (*s*)

Table 2. *^{13}C-NMR Data of Compounds* **1**–**4** (125 MHz, (D_6)acetone, δ in ppm)

	1	**2**	**3**	**4**
CH_2(1)	26.90 (*t*)	26.79 (*t*)	29.68 (*t*)	27.47 (*t*)
CH_2(2)	31.05 (*t*)	30.95 (*t*)	26.76 (*t*)	31.00 (*t*)
H–C(3)	74.29 (*d*)	74.29 (*d*)	72.77 (*d*)	72.40 (*d*)
H–C(4)	45.67 (*d*)	45.61 (*d*)	35.54 (*d*)	36.47 (*d*)
C(5)	50.12 (*s*)	50.29 (*s*)	47.81 (*s*)	46.97 (*s*)
H–C(6)	86.76 (*d*)	85.95 (*d*)	80.21 (*d*)	70.76 (*d*)
C(7)	157.82 (*s*)	156.75 (*s*)	151.36 (*s*)	154.39 (*s*)
H–C(8) or C(8)	77.54 (*d*)	100.86 (*s*)	106.41 (*s*)	103.23 (*s*)
H–C(9) or CH_2(9)	118.00 (*d*)	119.86 (*d*)	41.80 (*t*)	43.55 (*t*)
C(10)	148.57 (*s*)	148.87 (*s*)	73.68 (*s*)	76.77 (*s*)
C(11)	120.70 (*s*)	122.11 (*s*)	131.48 (*s*)	126.40 (*s*)
C(12)	173.80 (*s*)	170.97 (*s*)	170.70 (*s*)	170.92 (*s*)
Me(13)	7.91 (*q*)	7.47 (*q*)	8.05 (*q*)	8.74 (*q*)
Me(14)	13.90 (*q*)	13.82 (*q*)	12.28 (*q*)	12.57 (*q*)
Me(15)	14.64 (*q*)	14.00 (*q*)	12.03 (*q*)	12.25 (*q*)
6-MeO	56.99 (*q*)	56.84 (*q*)	58.70 (*q*)	–
8-MeO	–	–	50.27 (*q*)	–
AcO	20.20, 170.77	20.21, 169.97	20.29, 170.60	21.28, 170.92

atom was observed in the ^{1}H-NMR spectrum, and a signal for a dioxygenated quaternary C-atom at δ(C) 100.86 in the ^{13}C-NMR spectrum was observed instead of the signal at δ(C) 77.54 in the spectrum of **1**. The IR spectrum showed an absorption for a OH group at 3454 cm^{-1}, in addition to absorptions for an α,β unsaturated γ-lactone at 1733 cm^{-1}, an AcO group at 1715 cm^{-1}, and a C=C bond at 1669 cm^{-1}. Based on the above data, compound **2** could be deduced as 3-acetoxy-8-hydroxy-6-methoxyeremophila-7(11),9(10)-dien-12,8-olide. The coupling pattern of the ^{1}H-NMR signal of H–C(3) (δ(H) 4.94, *dt*, *J* = 3.0, 2.5) also showed that the AcO group has β-orientation.



1 **3**

Fig. 2. *Important NOESY correlations of compounds* **1** *and* **3**

The cross-peaks between H_α–C(4) and H–C(6) in the NOESY spectrum indicated a β-orientated MeO group. Due to the presence of a homoallylic coupling between H_α–C(6) (δ(H) 4.24, *q*, $J = 1.0$) and Me(13) (δ(H) 1.94, *d*, $J = 1.0$), the relative configuration of the OH group at C(8) was established to be α, from which the angle between H_α–C(6) and Me(13)–C(11) could be determined to be *ca.* 90° [7][8]. Therefore, compound **2** was elucidated as 3β-acetoxy-8α-hydroxy-6β-methoxyeremophila-7(11),9(10)-dien-12,8β-olide.

Compound **3** was obtained as colorless needles. Its molecular formula was determined as $C_{19}H_{28}O_7$ by HR-ESI-MS ($[M + Na]^+$ at *m*/*z* 391.1728). Its ^{1}H- and ^{13}C-NMR spectra were similar to the data of compounds **1** and **2** (*Tables 1* and *2*). Differences were found for an oxygenated quaternary C-atom at δ(C) 73.68 and a secondary C-atom δ(C) 41.80 in the ^{13}C-NMR, instead of the $\Delta^{9(10)}$ C=C signals of **1** and **2**. Furthermore, an additional MeO signal was observed at δ(H) 3.24 (*s*) in the ^{1}H-NMR spectrum, corresponding to the second MeO C-atom at δ(C) 50.27 in the ^{13}C-NMR spectrum. In accordance with the IR absorptions for an OH group at 3536 cm^{-1}, for an α,β-unsaturated γ-lactone at 1772 cm^{-1}, and for an AcO group at 1733 cm^{-1}, compound **3** should be deduced as 3-acetoxy-6,8-dimethoxyeremophil-7(11)-en-12,8-olide. Its ^{1}H- and ^{13}C-NMR signals were assigned by an HMQC experiment, which was similar with the reported 3β-acetoxy-8β,10β-dihydroxy-6β-methoxyeremophilenolide [9]. The MeO–C(8) bond was confirmed by the cross-peak between the signal of the MeO group at δ(H) 3.24 and C(8) δ(C) 106.41 in the HMBC experiment (*Fig. 1*). Rules about the relative configuration at C(8) reported by *Naya et al.* indicate that in 12,8α-eremophilenolides, the *singlet* of Me(14) appeares in a lower field in the ^{1}H-NMR specturm than the *doublet* of Me(15), while in 12,8β-eremophilenolide, the signals are found *vice versa* [10]. Thus, the ^{1}H-NMR data of compound **3** indicated an 12,8α-eremophilenolide. Furthermore, the absence of a homoallylic coupling between Me(13)–C(11) and H_α–C(6) in the 12,8α-olide showed that the MeO group at C(6) was β-oriented, as the angle between H_α–C(6) and Me(13)–C(11) was around 0° [8]. The upfield signal for Me(15) also indicates a *cis*-eremophilane skeleton, supporting a β-orientation of the OH group at C(10) [11][12], confirmed by the key cross peaks in the NOESY spectrum (*Fig. 2*). As a result, compound **3** was elucidated as 3β-acetoxy-10β-hydroxy-6β,8β-dimethoxyeremophil-7(11)-en-12,8α-olide.

Compound **4** was obtained as colorless needles. Its molecular formula was determined as $C_{17}H_{24}O_7$ by the $[M + NH_4]^+$ peak in HR-ESI-MS ($[M + NH_4]^+$ at *m*/*z* 358.1862). The IR spectrum showed absorptions for two OH groups at 3493 and

3325 cm^{-1}, an α,β-unsaturated γ-lactone at 1750 cm^{-1}, and an AcO group at 1711 cm^{-1}. The ^1H- and ^{13}C-NMR spectra were very similar to those of compound **3** (*Tables 1* and *2*), except for the absence of two MeO groups. The ^1H- and ^{13}C-NMR data were also assigned by an HMQC experiment. The AcO group was attached to C(3) as deduced by the cross peak between the AcO C-atom (δ(C) 170.92) and H−C(3) (δ(H) 4.90, *dt*, $J = 3.0, 1.5$). Furthermore, compared with compound **3**, the three OH groups in **4** were attached to C(6), C(8), and C(10) respectively. The small coupling constants of $J(3\alpha,2\alpha)$, $J(3\alpha,2\beta)$, and $J(3\alpha,4\alpha)$ indicated that H−C(3) was in equatorial position, which indicated that the AcO-group at C(3) had β-orientation. The relative configuration at C(8) was deduced as 12,8α-olide by the difference between chemical shifts of Me(14) and Me(15) (*Table 1*). The absence of a homoallylic coupling between H_α−C(6) and Me(13)−C(11) showed that the 6-OH group was in β-orientation. The upfield Me(15) also implied a *cis*-eremophilane, showing the 10-OH group in β-orientation [11][12]. Thus, compound **4** was elucidated as 3β-acetoxy-6β,8β,10β-trihydroxyeremophil-7(11)-en-12,8α-olide.

Eremophilenolides **1**–**4** were tested for their cytotoxicity against human hepatic cancer cells Bel-7402, human pneumonic cancer cells A-549, and human colonic cancer cells HCT-8, using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method [13]. The results showed that compounds **1**–**4** do not have the ability to inhibit the tumor cells (all IC_{50} values > 200 μM).

Experimental Part

General. TLC: precoated SiO_2 GF_{254} plates (*Qingdao Marine Chemical Factory* P. R. China). Column chromatography (CC): SiO_2 (200–300 mesh; *Qingdao Marine Chemical Factory*, P. R. China); *Sephadex LH-20* (*Pharmacia*). M.p.: *X-6 micro-melting-point* apparatus; uncorrected. Optical rotation: *Perkin-Elmer 341* polarimeter. UV Spectra: *HITACHI-U-2800 UV/VIS* spectrophotometer; λ_{max} (log ε) in nm. IR Spectra (KBr): *Bruker-VERTEX 70 FT-IR* spectrometer; in cm^{-1}. 1D- and 2D-NMR Spectra: *Bruker-AV-500* spectrometer; δ in ppm rel. to Me_4Si, *J* in Hz. MS: *Agilent-1100-LC/MSD-Trap SL* (ESI-MS) and *Bruker APEX II* (HR-ESI-MS) mass spectrometer; in *m*/*z*.

Plant Material. The whole plants of *L. duciformis* were collected in Meigu County, Sichuan Province, P. R. China, in August 2006. The plant material was identified by Prof. *Yi-Lin Chen*, Institute of Botany, Chinese Academy of Sciences, P. R. China. A voucher specimen (No. 20060901) was deposited in the herbarium of the College of Life and Environment, Central University of Nationalities, Beijing, P. R. China.

Extraction and Isolation. The air-dried whole plants of *L. duciformis* (1.5 kg) were pulverized and extracted three times with MeOH (each for 7 d) at r.t. The extract was concentrated to give a residue (110 g), which was further separated by CC (SiO_2, petroleum ether (PE)/AcOEt 30 : 1, 20 : 1, 15 : 1, 10 : 1, 8 : 1, 5 : 1, 3;1, 2 : 1, 1 : 1, 1 : 1.5 (*v*/*v*)): *Fr. 1–10*. Each fraction was examined by TLC and combined to afford many subfractions. *Fr. 6a* (0.9 g) was subjected to CC (SiO_2, PE/AcOEt 10 : 1, 5 : 1 (*v*/*v*)) to yield **1** (20 mg). *Fr. 7a* (1.0 g) was purified by CC (SiO_2, PE/AcOEt 8 : 1, 5 : 1 (*v*/*v*)) to yield **2** (18 mg). *Fr. 8a* (1.4 g) was subjected to CC (SiO_2, PE/AcOEt 8 : 1, 5 : 1 (*v*/*v*)) to give a crude gum of **3**, which was further purified by CC (*Sephadex LH-20*, MeOH) to yield **3** (15 mg). *Fr. 9c* (0.8 g) was separated by CC (SiO_2, PE/AcOEt 5 : 1, 3 : 1 (*v*/*v*)) to give a crude gum of **4**, which was further purified by CC (*Sephadex LH-20*, MeOH) to give **4** (30 mg).

3β-Acetoxy-6β-methoxyeremophila-7(11),9(10)-dien-12,8β-olide (= *(4*S*,4a*R*,5*R*,6*S*,9a*R*)-6-(Acetyloxy)-4a,5,6,7,8,9a-hexahydro-4-methoxy-3,4a,5-trimethylnaphtho[2,3-*b*]furan-2(4*H*)-one*; **1**). Colorless needles. M.p. 219.0–220.8° (Me_2CO). $[\alpha]_D^{25} = -139$ ($c = 0.002$, $CHCl_3$). UV (MeOH): 218 (3.84). IR (KBr): 1741, 1710, 1610, 1443,1385, 1251. ^1H- and ^{13}C-NMR: *Tables 1* and *2*. ESI-MS (pos.): 343.2 ($[M + Na]^+$). HR-ESI-MS: 321.1691 ($[M + H]^+$, $C_{18}H_{25}O_5^+$; calc. 321.1702).

3β-Acetoxy-8α-hydroxy-6β-methoxyeremophila-7(11),9(10)-dien-12,8β-olide (=*(4*S*,4a*R*,5*R*,6*S*,9a*R*)-6-(Acetyloxy)-4a,5,6,7,8,9a-hexahydro-9a-hydroxy-4-methoxy-3,4a,5-trimethylnaphtho[2,3*-b*]furan-2(4*H*)-one*; **2**). Colorless oil. $[\alpha]_D^{25} = -12$ ($c = 0.001$, $CHCl_3$). UV (MeOH): 234 (3.49). IR (KBr): 3454, 1733, 1715, 1669, 1456, 1377, 1247. ^{1}H- and ^{13}C-NMR: *Tables 1* and *2*. ESI-MS (pos.): 359.1 ($[M+Na]^+$). HR-ESI-MS: 359.1463 ($[M+Na]^+$, $C_{18}H_{24}NaO_6^+$; calc. 359.1471).

3β-Acetoxy-10β-hydroxy-6β,8β-dimethoxyeremophil-7(11)-en-12,8α-olide (=*(4*S*,4a*S*,5*R*,6*S*,8a*S*,9a*S*)-6-(Acetyloxy)-4a,5,6,7,8,8a,9,9a-octahydro-8a-hydroxy-4,9a-dimethoxy-3,4a,5-trimethylnaphtho[2,3*-b*]furan-2(4*H*)-one*; **3**). Colorless, needles. M.p. 218.5–219.0° (Me_2CO). $[\alpha]_D^{25} = +98$ ($c = 0.001$, $CHCl_3$). UV (MeOH): 224 (3.68). IR (KBr): 3536, 1772, 1733, 1450, 1379, 1250. ^{1}H- and ^{13}C-NMR: *Tables 1* and *2*. ESI-MS (pos.): 391.2 ($[M+Na]^+$). HR-ESI-MS: 391.1728 ($[M+Na]^+$, $C_{19}H_{28}NaO_7^+$; calc. 391.1733).

3β-Acetoxy-6β,8β,10β-trihydroxyeremophil-7(11)-en-12,8α-olide (=*(4*S*,4a*S*,5*R*,6*S*,8a*S*,9a*S*)-6-(Acetyloxy)-4a,5,6,7,8,8a,9,9a-octahydro-4,8a,9a-trihydroxy-3,4a,5-trimethylnaphtho[2,3*-b*]furan-2(4*H*)-one*; **4**). Colorless, needles. M.p. 192.4–192.7° (Me_2CO). $[\alpha]_D^{25} = +54.4$ ($c = 0.001$, $CHCl_3$). UV (MeOH): 220 (3.54). IR (KBr): 3492, 3325, 1750, 1711, 1439, 1390, 1262. ^{1}H- and ^{13}C-NMR: *Tables 1* and *2*. ESI-MS (pos.): 363.2 ($[M+Na]^+$). HR-ESI-MS: 358.1862 ($[M+NH_4]^+$, $C_{17}H_{28}NO_7^+$; calc. 358.1866).

Test of Cytotoxicities against Human Hepatic Cancer Cells Bel-7402, Human Pneumonic Cancer Cells A-549, and Human Colonic Cancer Cells HCT-8. Cells were seeded into 96 well plates at a density of 10^4 cells per well in growth medium. The plates were incubated at 37° under the condition of humidified atmosphere containing 5% CO_2. After 24 h, the medium was discarded and test solns. were added. Five wells were used for each concentration and cell controls. After 72 h incubation at 37°, the medium was removed and 200 µl of MTT solution (0.5 mg MTT dissolved into 1 ml *Dulbecco*'s Modified *Eagle*'s Medium (DMEM)) were added to each well. After four h at 37°, the supernatant was removed and the formazan product was solubilized by the addition of 200 µl DMSO. The optical density of each well was measured using an automatic plate reader (*Multiscan MK3*) with the test wavelength of 570 nm. The absorbance was directly proportional to the number of living cells. The cytotoxicity of each compound was expressed as an IC_{50} value, *i.e.*, the concentration in µM that inhibits cell growth by 50% compared with cell controls, and was calculated by linear regression analysis.

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