Daphnane-Type Diterpenoid Glucosides and Further Constituents of *Euphorbia pilosa*

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Phytochemical investigation of whole plants of *Euphorbia pilosa* led to the isolation and identification of two new daphnane-diterpenoid glucosides, euphopilosides A and B (**1** and **2**, resp.), and a new *ent*-abietane, euphopilolide (**3**), together with eight known compounds. Compounds **1** and **2** are the first daphnane-type diterpenoid glycosides. Their structures were elucidated by a combination of 1D- and 2D-NMR, and MS analyses, and acid hydrolysis. Compounds **1**–**9** were evaluated for their *in vitro* cytotoxicities against five human tumor cell lines, HL-60, SMMC-7721, A-549, MCF-7, and SW-480. Compound **7** showed moderate inhibitory activity against all five cell lines.

Introduction. – The genus *Euphorbia* is the largest in the spurge family, comprising more than 2000 species [1]. Some species of *Euphorbia* have been used as medicinal plants for the treatment of skin diseases, gonorrhea, migraine, and intestinal parasites, and as wart cures [2]. More than 550 compounds including sesquiterpenoids, diterpenoids, triterpenoids, steroids, cerebrosides and glycerols, phenolics, flavonoids, and miscellaneous compounds have been reported from the *Euphorbia* species [3]. Diterpenoids (more than 446 compounds) are the characteristic compounds of the genus with many different core frameworks such as jatrophanes, lathyranes, viglianes, daphnanes, ingenanes, and myrsinols, and they possess many biological features such as antiproliferative activity, cytotoxicity, effects on the cell division, and DNA-damaging, PEP-inhibitory, and anti-HIV-1 activities [3].

Euphorbia pilosa L. is a perennial, erect herb and grows in subalpine meadow, grassland, or forest edge [4]. Its chemical composition has not been reported. As part of our ongoing efforts to discover new bioactive constituents from *Euphorbia* species [5], the AcOEt-soluble extract of *E. pilosa* was investigated, resulting in the isolation of two new daphnane-type diterpenoid glucosides, named euphopilosides A and B (**1** and **2**, resp.), and a new *ent*-abietane, named euphopilolide (**3**), together with eight known compounds, langduin A (**4**) [6], phorbol (**5**) [7], jokiolide A (**6**) [6], jokiolide B (**7**) [6], *ent*-atis-16-ene-3,14-dione (**8**) [8], 13β-hydroxy-*ent*-abiet-8(14)-en-7-one (**9**) [9], annphenone (**10**) [10], and obtusifoliol (**11**) [11]. Componds **1** and **2** are the first daphnane-type diterpenoid glycosides. Herein, we report the isolation and structure elucidation of the new compounds. In addition, compounds **1**–**9** were evaluated for their cytotoxicities against five human tumor cell lines.

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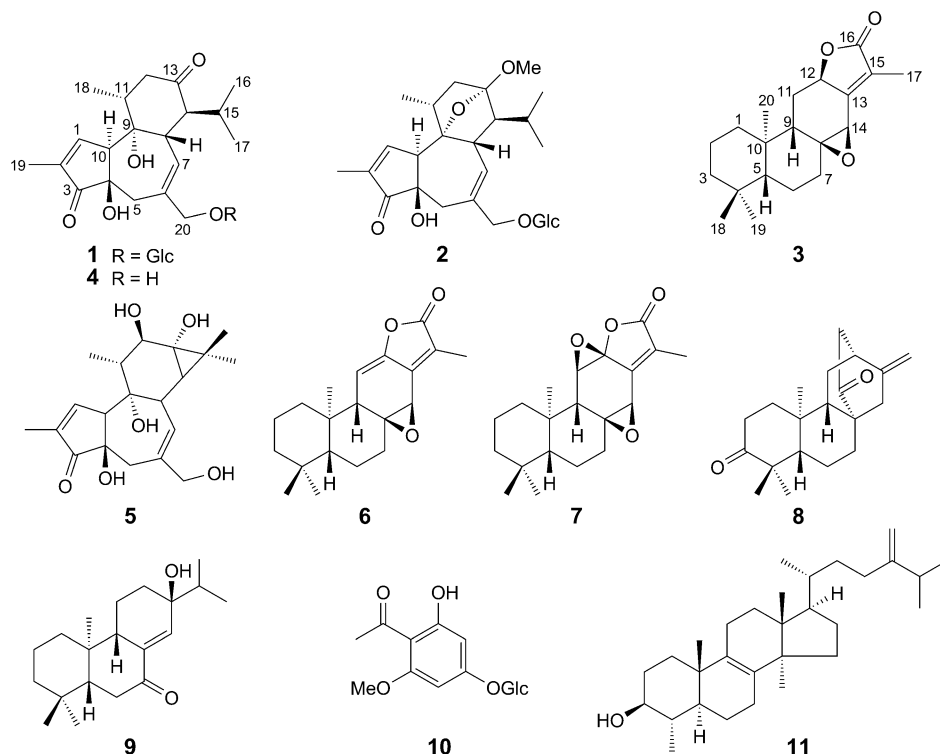

Results and Discussion. – Euphopiloside A (**1**), isolated as a white amorphous powder, displayed a $[M+Na]^+$ ion peak at *m/z* 533.2370 (calc. 533.2362) in the HR-ESI-MS, which, combined with ^{13}C-NMR data (*Table 1*), provided the molecular formula $C_{26}H_{38}O_{10}$, requiring eight degrees of unsaturation. The IR spectrum indicated the presence of OH (3426 cm^{-1}) and (1698 cm^{-1}) C=O groups, and C=C bond (1629 cm^{-1}). The ^{1}H-NMR spectrum of **1** (*Table 1*) exhibited signals for four Me group (δ(H) 1.02 (*d*, *J* = 6.9), 1.23 (*d*, *J* = 6.9), 1.27 (*d*, *J* = 6.8), and 1.67 (*s*)), two trisubstituted olefinic H-atom (δ(H) 5.92 (*d*, *J* = 5.4) and 7.75 (br. *s*)), and an anomeric H-atom (δ(H) 4.90 (*d*, *J* = 7.8)). The ^{13}C-NMR and DEPT spectra (*Table 1*) revealed the presence of two C=O groups (δ(C) 213.0 and 208.9), two trisubstituted C=C bonds (δ(C) 131.0, 135.2, 139.2, and 157.9), four Me, three CH_2 (one oxygenated one), and five CH groups, and two oxygenated quaternary C-atoms (δ(C) 74.6 and 76.7), as well as of a glucopyranosyl moiety (δ(C) 104.4, 75.1, 78.7, 71.9, 78.6, and 63.0). Acid hydrolysis of **1** yielded langduin A (**4**) and D-glucose as sugar residue, which was identified by GC analysis of its trimethylsilated L-cysteine adduct. The β-configuration of glucopranosyl was determined by the coupling constant ($^3J(1,2) > 7$) of the anomeric H-atom. Comparison of the NMR data of **1** (*Table 1*) with those of langduin A (**4**) [6] confirmed that compound **1** was a glucoside derivative of **4**. This conclusion was verified by ^{1}H,^{1}H-COSY, HMBC, HSQC, and ROESY experiments (*Fig.*). The HMBC between the signals at δ(H) 4.90 (H–C(1′)) and δ(C) 75.6 (C(20)) implied that the glucopyranosyl

Table 1. *^{1}H- and ^{13}C-NMR Data for Compounds* **1** *and* **2** (δ in ppm, J in Hz). Atom numbering as indicated in the *Formulae.*

Position	**1**[a])		**2**[b])	
	δ(H)	δ(C)	δ(H)	δ(C)
1	7.75 (br. *s*)	157.9	7.55 (*s*)	160.0
2		135.2		135.2
3		208.9		209.2
4		74.6		72.6
5	3.16 (br. *s*, 2 H)	38.8	2.63 (*d*, J=19.0), 2.34 (*d*, J=19.0)	38.4
6		139.2		133.6
7	5.92 (*d*, J=5.4)	131.0	5.48 (br. *s*)	133.8
8	4.33–4.35 (*m*)	46.0	2.77 (br. *s*)	53.3
9		76.7		84.7
10	3.66 (br. *s*)	58.7	3.43 (br. *s*)	51.2
11	3.25–3.29 (*m*)	37.8	2.30–2.32 (*m*)	42.6
12	2.71 (*dd*, J=7.9, 14.6), 2.54 (*dd*, J=7.5, 14.6)	48.0	2.20 (*dd*, J=9.0, 11.9), 1.26 (*dd*, J=9.0, 13.9)	36.7
13		213.0		110.9
14	2.89 (*dd*, J=2.7, 11.9)	55.9	1.59–1.61 (*m*)	57.3
15	2.11–2.15 (m)	29.2	1.57–1.59 (*m*)	32.0
16	1.23 (*d*, J=6.9)	22.1	1.00 (*d*, J=5.5)	21.8
17	1.02 (*d*, J=6.9)	17.9	0.98 (*d*, J=5.5)	19.6
18	1.27 (*d*, J=6.8)	18.9	0.92 (*d*, J=6.7)	20.7
19	1.67 (*s*)	10.5	1.75 (*s*)	8.81
20	4.58 (*d*, J=11.7), 4.29 (*d*, J=11.7)	75.6	4.17 (*d*, J=11.2), 4.08 (*d*, J=11.2)	76.0
MeO			3.44 (*s*)	50.5
1′	4.90 (*d*, J=7.8)	104.4	4.27 (*d*, J=7.8)	101.2
2′	3.97–4.00 (*m*)	75.1	3.15–3.17 (*m*)	73.6
3′	3.90–3.93 (*m*)	78.7	3.19–3.21 (*m*)	70.0
4′	4.16–4.18 (*m*)	71.9	3.26–3.29 (*m*)	73.6
5′	4.18–4.20 (*m*)	78.6	3.30–3.35 (*m*)	76.5
6′	4.55–4.58 (*m*), 4.33 (*dd*, J=6.0, 12.0)	63.0	3.83 (*d*, J=11.9), 3.65 (*dd*, J=5.3, 11.9)	61.0

[a]) Recorded in C_5D_5N, at 500 and 125 MHz for ^{1}H and ^{13}C, respectively. [b]) Recorded in CD_3OD, at 400 and 100 MHz for ^{1}H and ^{13}C, respectively.

was attached at C(20) of langduin A (**4**). The downfield shift exhibited by C(20) (δ(C) 75.6) compared to that of langduin A (**4**) (+6.4) corroborated this deduction. Based on these results, the structure of compound **1** was elucidated as 4β,9α,20-trihydroxy-13,15-secotiglia-1,6-diene-3,13-dione 20-*O*-β-D-glucopyranoside. This is the first daphnane-type diterpenoid glycoside.

Euphopiloside B (**2**) had a molecular formula $C_{27}H_{40}O_{10}$ deduced from HR-ESI-MS (*m/z* 547.2522 ([*M*+Na]$^+$; calc. 547.2519)) and ^{13}C-NMR data (*Table 1*). Comparison of the ^{1}H- and ^{13}C-NMR data indicated that **2** differed from **1** by the presence of a ketal (δ(C) 110.9) and a MeO group (δ(H) 3.44; δ(C) 50.5) instead of a C=O group (δ(C) 213.0) moiety at C(13) in **1**. The HMBC of the C(13) signal (δ(C) 110.9) with those of

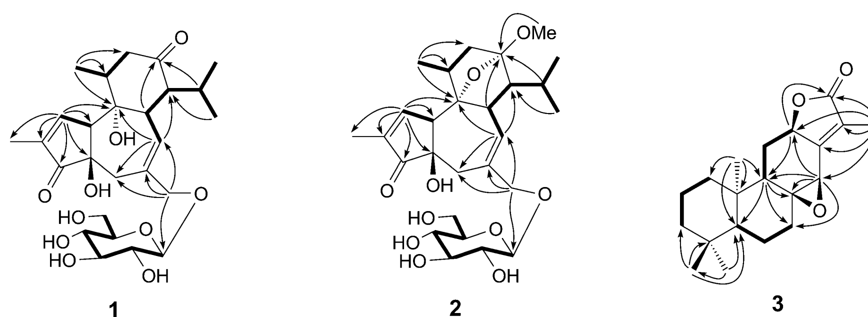

Figure. *Key* ^{1}H,^{1}H-COSY (—) *and HMB* (H → C) *correlations of* **1**–**3**

CH_2(12) (δ(H) 1.26 (*dd*, *J* = 9.0, 13.9) and 2.20 (*dd*, *J* = 9.0, 11.9)), H–C(14) (δ(H) 1.59–1.61 (*m*)), H–C(15) (δ(H) 1.57–1.59 (*m*)), and MeO (δ(H) 3.44) indicated the location of the ketal group at C(13) (*Fig.*). ROESY Correlation of the MeO signal (δ(H) 3.44) with those of H–C(8) (δ(H) 2.77 (br. *s*)), H–C(11) (δ(H) 2.30–2.32 (*m*)), H–C(15) (δ(H) 1.57–1.59 (*m*)), Me(16) (δ(H) 1.00 (*d*, *J* = 5.5)), and Me(17) (δ(H) 0.98 (*d*, *J* = 5.5)) evidenced that the MeO group at C(12) was β-oriented. Therefore, the structure of **2** was established as 9α,13α-epoxy-4β,20-dihydroxy-13β-methoxy-13,15-secotiglia-1,6-diene-3-one 20-*O*-β-D-glucopyranoside.

Euphopilolide (**3**) gave rise to $[M + Na]^+$ ion peak in the HR-ESI-MS at *m/z* 339.1932, corresponding to the molecular formula $C_{20}H_{28}O_3$. The UV and IR spectra displayed absorptions due to an α,β-unsaturated-γ-lactone (moiety 222 nm, and 1754 and 1683 cm^{-1}, resp.). The ^{1}H-NMR spectrum (*Table 2*) exhibited signals for three tertiary Me groups (δ(H) 0.88, 0.92, and 1.05), one olefinic Me group (δ(H) 1.95), two CH–O groups (δ(H) 3.75 and 4.97–5.01). The ^{13}C-NMR spectrum of **3** (*Table 2*) displayed signals of a C=O group (δ(C) 173.9), one tetrasubstituted C=C bond (δ(C)

Table 2. *^{1}H- and ^{13}C-NMR* (in $CDCl_3$, at 400 and 125 MHz, resp.) *Data for Compound* **3** (δ in ppm, *J* in Hz)

Position	δ(H)	δ(C)	Position	δ(H)	δ(C)
1	1.45–1.47 (*m*), 1.18 (*dd*, *J* = 6.1, 12.5)	41.3	11	2.27 (*dd*, *J* = 5.5, 13.3), 1.35–1.37 (*m*)	23.7
2	1.52–1.56 (*m*, 2 H)	18.5	12	4.97–5.01 (*m*)	75.6
3	1.92–1.94 (*m*), 1.06–1.09 (*m*)	40.8	13		155.8
4		33.2	14	3.75 (*s*)	56.0
5	1.01–1.03 (*m*)	54.0	15		128.5
6	1.74 (*dd*, *J* = 2.9, 11.3), 1.41–1.43 (*m*)	20.9	16		173.9
7	1.96–1.99 (*m*), 1.63 (*d*, *J* = 13.8)	34.7	17	1.95 (*s*)	8.7
8		61.0	18	0.92 (*s*)	34.0
9	1.94–1.96 (*m*)	49.1	19	0.88 (*s*)	22.0
10		39.2	20	1.05 (*s*)	19.2

128.5 and 155.8), four Me, six CH_2, and four CH groups (two oxygenated ones at δ(C) 56.0 and 75.6), and three quaternary C-atoms (one oxygenated one at δ(C) 61.0). These data are very similar to those of jolkinolide A (**6**) except for the presence of one more CH–O (δ(C) 75.6) and CH_2 group (δ(C) 23.7) instead of a trisubstituted C=C bond between C(11) and C(12). This was supported by the correlations from the signal of CH_2(11) (δ(H) 1.35–1.37 (*m*) and 2.27 (*dd*, J=5.5, 13.3)) to that of H–C(12) (δ(H) 4.97–5.01 (*m*)) in the $^1H,^1H$-COSY spectrum (*Fig.*) and the long-range correlations of the signal of C(12) with H–C(9) (δ(H) 1.94–1.96 (*m*)), CH_2(11) (δ(H) 1.35–1.37 (*m*) and 2.27 (*dd*, J=5.5, 13.3)), those of H–C(14) (δ(H) 3.75 (*s*)), and Me(17) (δ(H) 1.95 (*s*)) in the HMBC spectrum (*Fig.*). The H–C(12) was α-oriented on the basis of ROESY correlation of H–C(12) (δ(H) 4.97–5.01 (*m*)) with Me(20) (δ(H) 1.05 (*s*)). Thus, the structure of compound **3** was elucidated as 8β,14β-epoxy-*ent*-abiet-13(15)-en-16,12β-olide.

Euphopilosides A and B (**1** and **2**, resp.) are the first daphnane-type diterpenoid glycosides. In 2011, we reported the first tigliane-type glycosides, fischerosides A–C, isolated from *Euphorbia fischeriana* [5]. Interestingly, they had the same glucosylation position, *i.e.*, C(20). Compounds **1**–**9** were evaluated for their cytotoxic activities against five human cancer cell lines (HL-60, SMMC-7721, A-549, MCF-7, and SW-480) using the MTT (=3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay, and cisplatin was used as the positive control. Only jokiolide B (**7**) showed cytotoxic activities against the five cell lines with IC_{50} values of 4.18±0.18, 6.06±0.27, 4.12±0.34, 17.64±0.98, and 11.40±0.08 μM, respectively.

Experiment Part

General. TLC: SiO_2 plates; detection by UV_{254} lamp and 10% H_2SO_4 in EtOH, followed by heating. Column chromatography (CC): silica gel (SiO_2, 200–300 mesh; *Qingdao Marine Chemical Inc.*, P. R. China), *RP-18* (40–63 μm, *Merck*), *MCI GEL CHP20P* (75–150 μm, *Mitsubishi Chemical Co.*), and *Sephadex LH-20* (25–100 μm, *GE Healthcare*). GC: *HP5890* Gas chromatograph; H_2 flame ionization detector. Optical rotations: *JASCO P-1020* digital polarimeter. UV Spectra: *Shimadzu UV-2401PC* spectrophotometer, MeOH solns.; λ_{max} (log ε) in nm. IR Spectra: *Bruker Tensor-27* infrared spectrophotometer, KBr pellets; $\tilde{\nu}$ in cm^{-1}. NMR Spectra: *Bruker AM-400* and *DRX-500* instruments; δ in ppm rel. to Me_4Si as internal standard, J in Hz. ESI- and HR-ESI-MS: *API QSTAR* time-of-flight (TOF) spectrometer.

Plant Material. Whole plants of *E. pilosa* were collected in June 2010 in Panshi, Jilin Province, P. R. China, and identified by Dr. *En-De Liu* of Kunming Institute of Botany. A voucher specimen (No. HY00011) was deposited with the State Key Laboratory of Phytochemistry and Plant Resources in West China.

Extraction and Isolation. The dried and powered material (10 kg) was extracted with 95% EtOH (25 l × 3) under reflux for a total of 6 h to give a crude extract. After removal of the solvent *in vacuo*, the residue was suspended in H_2O (10 l) and successively extracted with AcOEt (4 × 10 l). The solvent was removed, and the AcOEt extract (1.17 kg) was subjected to CC (SiO_2; petroleum ether (PE)/AcOEt 1:0 → 0:1) to give four fractions, *Fr. A–D*. *Fr. B* (68 g) was subjected to MPLC (SiO_2; PE/acetone (500:1 → 50:1) to give *Subfr.* B_1 and B_2. And then *Fr.* B_2 was subjeced to MPLC (*RP-18* gel MeOH/H_2O 40% → 70%) to give two combined fractions, B_{21}–B_{22}. *Fr.* B_{21} was purified by CC (SiO_2 PE/aectone 25:1) to afford **3** (5.1 mg), **6** (5.1 mg), **7** (3.1 mg), and **8** (2.7 mg). Compounds **4** (1.6 mg), **5** (3.6 mg), **9** (2.0 mg), and **11** (500 mg) were obtained after crystallization from PE/aectone from the *Fr.* B_{22}. *Fr. D* (86 g) was separated by CC (*MCI GEL CHP20P*; MeOH/H_2O 40% → 70%) to give *Fr.* D_1 and D_2. *Fr.* D_1 was submitted to CC (SiO_2; $CHCl_3$/MeOH 40:1 → 25:1) and purified by prep. TLC ($CHCl_3$/MeOH

20:1) to give **1** (53 mg) and **2** (6.1 mg). *Fr. D_2* was subjected to CC (SiO_2; $CHCl_3$/MeOH 25:1 → 10:1) to afford **10** (3.9 mg).

Euphopiloside A (= *[(3a*R*,6a*S*,7*S*,10*R*,10a*R*,10b*S*)-3a,10a-3,3a,4,6a,7,8,9,10,10a,10b-decahydrodihydroxy-2,10-dimethyl-3,8-dioxo-7-(propan-2-yl)benzo[*e*]azulen-5-yl]methyl β-*D*-Glucopyranoside*; **1**). White amorphous powder. $[\alpha]_D^{14} = +38.5$ ($c = 0.14$, MeOH). UV (MeOH): 202 (4.02). IR (KBr): 3426, 2959, 2926, 1698, 1629, 1460, 1077, 1044. ^{1}H- and ^{13}C-NMR: *Table 1*. ESI-MS (pos.): 533 $[M+Na]^+$. HR-ESI-MS: 533.2370 ($[M+Na]^+$, $C_{26}H_{38}NaO_{10}^+$; calc. 533.2362).

Euphopiloside B (= *[(3a*R*,6a*S*,7*S*,8*S*,10*R*,10a*R*,10b*R*)-3a,4,6a,7,8,9,10,10b-octahydro-3a-hydroxy-8-methoxy-2,10-dimethyl-3-oxo-7-(propan-2-yl)-3*H*-8,10a-epoxybenzo[*e*]azulen-5-yl]methyl β-*D*-Glucopyranoside*; **2**). White amorphous powder. $[\alpha]_D^{13} = +12.3$ ($c = 0.13$, MeOH). UV (MeOH): 235 (3.71), 201 (3.96). IR (KBr): 3426, 2957, 2925, 1703, 1629, 1461, 1077. ^{1}H- and ^{13}C-NMR: *Table 1*. ESI-MS (pos.): 547 $[M+Na]^+$. HR-ESI-MS: 547.2522 ($[M+Na]^+$, $C_{27}H_{40}NaO_{10}^+$; calc. 547.2519).

Euphopilolide (= *(1a*S*,3a*S*,7a*R*,7b*S*,8a*R*,11b*R*)-3,3a,4,5,6,7,7a,7b,8,8a-Decahydro-4,4,7a,11-tetramethyl-2*H*-oxireno[1,10a]phenanthro[3,2-*b*]furan-10(11b*H*)-one*; **3**). White needle. $[\alpha]_D^{13} = +51.4$ ($c = 0.09$, $CHCl_3$). UV (MeOH): 222 (4.12). IR (KBr): 3486, 2951, 2928, 1754, 1683, 1456, 1091, 1022. ^{1}H- and ^{13}C-NMR: *Table 2*. ESI-MS (pos.): 339 $[M+Na]^+$. HR-ESI-MS: 339.1932 ($[M+Na]^+$, $C_{20}H_{28}NaO_3^+$; calc. 339.1936).

Acid Hydrolysis, and GC and HPLC Analyses. Compounds **1** and **2** (2 mg) were separately hydrolyzed with 4M CF_3COOH/dioxane 1:1 (2 ml) for 4 h at 90°. After extraction with AcOEt (3 × 5 ml), the aq. layer was evaporated to dryness. The dried residue was dissolved in 0.5 ml of anh. pyridine and treated with L-cysteine methyl ester hydrochloride (1.5 mg), and the mixture was stirred at 60° for 1 h. 1-(Trimethylsilyl)-1*H*-imidazole (0.5 ml) was added to the mixtures, which was stirred at 60° for another 30 min. The mixture (4 μl) was analyzed by GC. D-Glucose (t_R 19.01 min) was detected in the hydrolysate of **1** and **2**, and its identity was confirmed by co-injection with a derivatized authentic sample. The AcOEt layer of compound **1** was evaporated to dryness. The residue showed the same peak at 15.23 min as that of langduin A (**4**) by HPLC analysis (conditions: *Zorbax SB-C18*, 5 μm, 46 × 150 mm; solvent, MeCN/H_2O 10 → 50% (0 → 40 min); flow rate, 1.0 ml/min; detection, UV absorbance at 203 nm) under the same conditions.

Cytotoxicity Assay. The cytotoxicity assay was performed according to the MTT method [12] by using of the following five human cancer cell lines: human myeloid leukemia HL-60, hepatocellular carcinoma SMMC-7721, lung cancer A-549, breast cancer MCF-7, and colon cancer SW-480. Each experiment was repeated three times. Cisplatin was used as the positive control antitumor drug and exhibited IC_{50} values for the cell lines of 3.29 ± 0.18, 9.26 ± 0.27, 9.98 ± 0.34, 15.92 ± 0.98, and 14.43 ± 0.08 μM, resp.

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