Two New Puriniums and Three New Pyrimidines from *Heterostemma brownii*

Yun-Lian Lin* and Ray-Ling Huang

National Research Institute of Chinese Medicine, Taipei, Taiwan

Chung-Ming Chang

Graduate Institute of Microbiology and Immunology, National Yang-Ming University, Taipei, Taiwan

Yueh-Hsiung Kuo*

Department of Chemistry, National Taiwan University, Taipei, Taiwan, Republic of China

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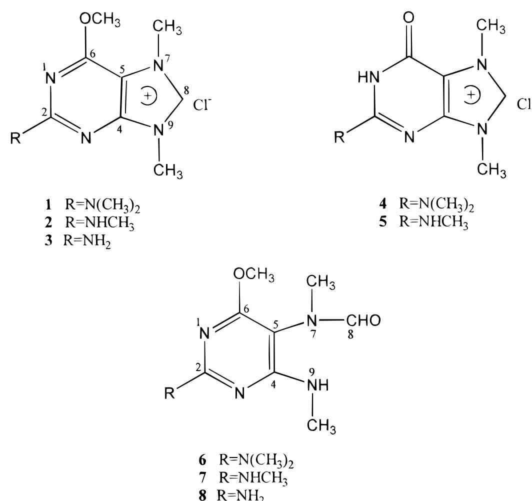
Two new puriniums, heteromines D (**4**) and E (**5**), and three new pyrimidines, heteromines F (**6**), G (**7**), and H (**8**), were isolated from the aerial parts of *Heterostemma brownii* Hay. Their structures were determined as 7,9-dimethyl-2-(*N*,*N*-dimethylamino)guaninium chloride, 7,9-dimethyl-2-(*N*-methylamino)guaninium chloride, 6-methoxy-4-(*N*-methylamino)-2-(*N*,*N*-dimethylamino)-5-(*N*-methylformamido)pyrimidine, 6-methoxy-2,4-bis(*N*-methylamino)-5-(*N*-methylformamido)pyrimidine, and 2-amino-6-methoxy-4-(*N*-methylamino)-5-(*N*-methylformamido)-pyrimidine, respectively.

The aerial parts of *Heterostemma brownii* Hay. (Asclepiadaceae) are used as a folk medicine for treatment of tumors.[1] In previous investigations on this plant, we found flavonoids, flavonoid glycosides, adenine, uridine,[2] and three new purinium derivatives, heteromines A (**1**), B (**2**), and C (**3**).[3] Further detailed reinvestigation of the plant has yielded five new compounds by reversed-phase chromatography (Diaion HP-20 and Sephadex LH-20). Two new puriniums, heteromines D (**4**) and E (**5**), and three new pyrimidines, heteromines F (**6**),G (**7**), and H (**8**), were elucidated as 7,9-dimethyl-2-(*N*,*N*-dimethylamino)guaninium chloride, 7,9-dimethyl-2-(*N*-methylamino)guaninium chloride, 6-methoxy-4-(*N*-methylamino)-2-(*N*,*N*-dimethylamino)-5-(*N*-methylformamido)pyrimidine, 6-methoxy-2,4-bis(*N*-methylamino)-5-(*N*-methylformamido)pyrimidine, and 2-amino-6-methoxy-4-(*N*-methylamino)-5-(*N*-methylformamido)pyrimidine, respectively.



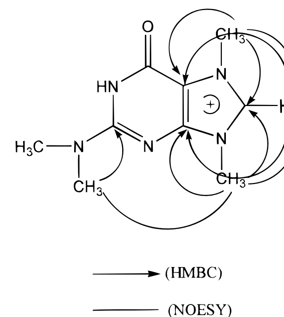


Figure 1. Correlation of **4** by HMBC and NOESY.

Results and Discussion

Heteromine D (**4**), $C_9H_{14}N_5OCl$, was isolated as colorless needles and was soluble in H_2O. It was presumed to be a quaternary ammonium chloride because it formed a precipitate with $AgNO_3$ as did heteromines A−C (**1**−**3**). A compound having a purinium ring with an amino group was suggested by UV and IR. The ^{1}H-NMR spectrum of **4** (Table 1) exhibited signals for a dimethylamino group [δ 3.00 (6H, s)], two methyl groups attached on two quaternary amines (δ 3.63 and 3.98) each, and a typical purinium base H-8 (δ 9.27).[4] The spectrum was similar to that of heteromine A (**1**) [3] except for the presence of a carbonyl group in **4** in place of the methoxy group. The ^{13}C-NMR data of **4** (Table 1) indicated that it was a guanine or an isoguanine derivative. HMBC and NOESY spectra (Figure 1) confirmed heteromine D (**4**) was 7,9-dimethyl-2-(*N*,*N*-dimethylamino)guaninium chloride.

Heteromine E (**5**), colorless needles, H_2O soluble, $C_8H_{12}N_5OCl$, was a quaternary ammonium chloride due to giving AgCl precipitation as reaction with $AgNO_3$. Based on the ^{1}H-NMR signals [δ 2.80 (3H, d, J = 6.9 Hz), 3.68, 3.98 (each 3H, s), 7.15 (1H, br s, −N*H*CH3), and 8.99 (1H, s)], the ^{13}C-NMR data (Table 1), HMBC, and NOESY techniques, the structure of heteromine E (**5**) was assigned as 7,9-dimethyl-2-(*N*-methylamino)-guaninium chloride.

* To whom correspondence should be addressed.
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Table 1. ^{1}H- and ^{13}C-NMR (δ values) Data for **4–8** (300 MHz and 75 MHz)

	4[a]		**5**[a]		**6**[b]		**7**[b]		**8**[b]	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
2		163.2		158.6		160.1		161.0		160.1
4		150.3		157.4		161.0		161.3		160.3
5		107.6		109.9		94.0		95.0		94.0
6		162.7		163.9		165.3		165.6		164.0
8	9.27 s	134.2	8.99 s	141.3	7.87 s	166.0	7.83 s	166.0	7.88 s	164.3
7-CH_3	3.98 s	34.8	3.98 s	38.1	2.91 d (4.8)	31.7	2.94 s	28.3	2.96 s	31.0
9-CH_3	3.63 s	30.3	3.68 s	33.6	2.93 d (4.8)	27.7	2.92 d (4.8)	27.7	2.91 d (4.8)	27.2
N-CH_3	3.00 s	37.1	2.80 d (6.9)	28.8	3.13 s	36.7	2.78 s	31.7		
O-CH_3					3.80 s	53.0	3.74 s	53.2	3.80 s	51.9
N-H			7.15 br s		4.63 br s		4.58 br s		4.65 br s	
							4.80 br s		4.77 br s	

[a] In DMSO-d_6. [b] In $CDCl_3$.

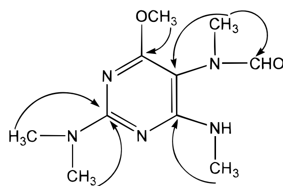


Figure 2. Correlation of **6** by HMBC.

Heteromine F (**6**) was isolated as colorless needles, was soluble in an organic solvent, and had molecular the formula $C_{10}H_{17}N_5O_2$. The UV absorption at 247 and 268 nm, and IR bands at 3385, 1675 cm^{-1} indicated that **6** was a pyrimidine derivative with amino and amide groups. The ^{1}H-NMR spectrum (Table 1) of **6** showed the presence of NCH_3, $NHCH_3$, $N(CH_3)_2$, OCH_3, NH, and HCON at δ 2.91, 2.93, 3.13, 3.80, 4.63, and 7.87, respectively. The ^{13}C-NMR data of **6** (Table 1) indicated a pyrimidine derivative with methoxy, *N,N*-dimethylamino, *N*-methylamino, and *N*-methylformamido groups. Using the HMBC technique (see Figure 2), the four functional groups were located at C-6, C-2, C-4, and C-5, respectively. This compound was also obtained from **1** by treatment in 10% aqueous NH_4OH, confirming that the *N*-methylformamido group of **6** was located at C-5.[5] Therefore, the structure of heteromine F was elucidated as 6-methoxy-4-(*N*-methylamino)-2-(*N,N*-dimethylamino)-5-(*N*-methylformamido)pyrimidine.

Heteromine G (**7**), a second pyrimidine derivative, was obtained as colorless needles ($C_9H_{15}N_5O_2$). The UV absorption at 242 and 266 nm, and the IR bands at 3440, 3350, 1685 cm^{-1}, and the ^{1}H- and ^{13}C-NMR data (Table 1) of **7** were all similar to those of heteromine F (**6**). The assignment of NMR signals was confirmed by ^{1}H–^{13}C COSY and HMBC experiments. The only difference between these two compounds was the *N*-methylamino group for **7** instead of the *N,N*-dimethylamino group for **6**. Compound **7** was obtained from heteromine B (**2**) in 10% aqueous NH_4OH by opening the ring.[5] This result confirmed the structure of heteromine G (**7**) as 6-methoxy-2,4-bis(*N*-methylamino)-5-(*N*-methylformamido)pyrimidine.

The third pyrimidine derivative was heteromine H (**8**), obtained as colorless needles. The UV absorption, IR spectrum, and ^{1}H- and ^{13}C-NMR data (Table 1) were similar to those of heteromines F (**6**) and G (**7**), except there was one nonsubstituted amino group instead of a substituted one. Assignment of NMR signals utilized ^{1}H–^{13}C COSY and HMBC experiments. Therefore, the structure of **8** was elucidated as 2-amino-6-methoxy-4-(*N*-methylamino)-5-(*N*-methylformamido)pyrimidine. Heteromine C (**3**) afforded heteromine G (**8**) upon reaction in 10% aqueous NH_4OH.

Hecht *et al.*[5] found that 7,9-disubstituted purinium ions were converted to 7,9-disubstituted 7,8-dihydropurines by reduction with $NaBH_4$ in water. The reoxidation of 7,8-dihydropurines to the corresponding purinium ion was attributed to the O_2 dissolved in solvent. In the aqueous NH_4OH, the ring of 7,9-disubstituted purinium was opened and converted to a N^5-formylpyrimidine derivative. When treated with nucleophiles other than hydroxide, it demethylated to give a 9-substituted derivative. In our experiment, heteromine A was reduced with aqueous $NaBH_4$, and then the product dihydropurine (**11**)[3] was dissolved in MeOH under stirring for 3 days. After purification on Si gel, three products, **6**, **9**, **10**, and recovered **1** were isolated. Compounds **6** and **10** were derived from **1** by $NaBH_4$ reduction, and dihydropurine (**11**) yielded **9** and **1** via air oxidation. The EIMS molecular peak at *m/z* 237 (100%) indicated the molecular formula of **9** to be $C_{10}H_{15}N_5O_2$. ^{1}H and ^{13}C spectral data of **9** were in good agreement with the assigned structure. Compound **10** was a demethylation product. On the basis of its MS, ^{1}H-NMR, and ^{13}C-NMR spectra, compound **10** was assigned the structure shown.[5] Formation of the products **6** and **11**, by the reaction with hydride reduction, and then reoxidization to **1** may be rationalized in terms of the mechanism shown in Scheme 1.[5] Formation of **10** was presumably from **1** by the attacking hydride. The ring opening is postulated to involve addition of a hydroxide ion at C-8 of **1**, followed by a base-catalyzed reaction to yield N^7-formyl structure **6**. The addition of hydride to C-8 in **1** to afford 7,8-dihydropurines **11** was followed by oxidation with O_2 in solution. The initial intermediate **12** combined with O_2 to form a peroxide radical and then coupled with **11** to produce hydroperoxide **13**. This was not stable and converted to **1** (path a) by elimination of hydroperoxide ion, or was converted to **9** by dehydration (path b).

Heteromines A and B showed cytotoxicity in five cancer cell lines: esophageal carcinoma (HCE-6), hepatoma (HuH-7), lymphoma (Molt-4), and leukemia (HL-60 and K 562). Their cytotoxic activities (IC_{50}) given in Table 2.

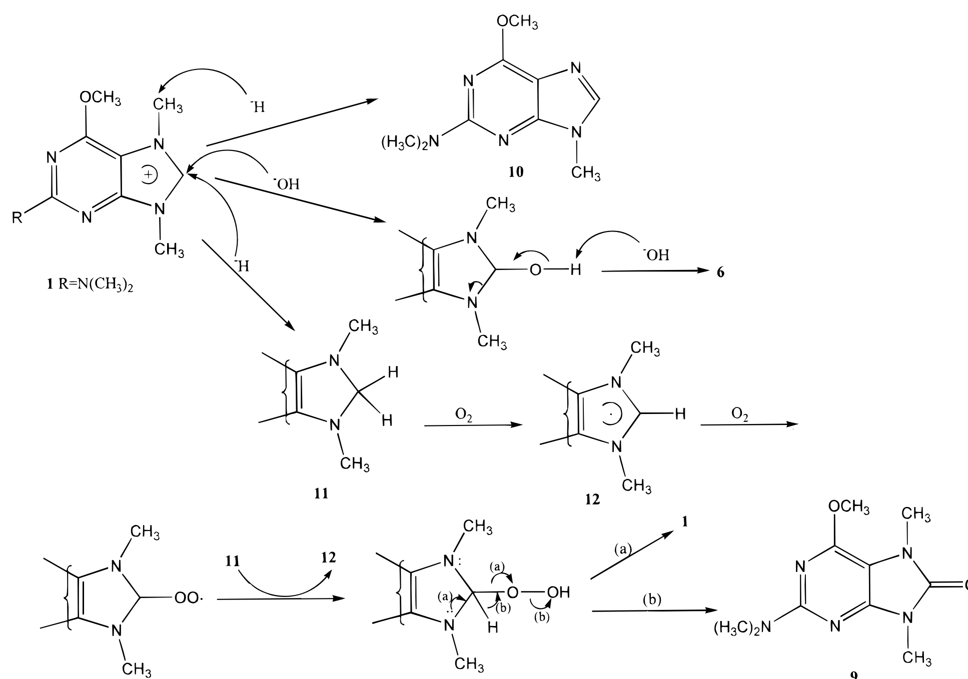
Experimental Section

General Experimental Procedures. Melting points were determined on a Yanagimoto micromelting point apparatus and were uncorrected. IR spectra were recorded on a Perkin-Elmer 781 spectrophotomer. ^{1}H and ^{13} C-NMR were run on a Bruker AC-300 MHz

Table 2. MTT Assay of Heteromines A and B (% of treated per control)

cancer cell line	10^{-3}M	10^{-4}M	10^{-5}M	10^{-6}M	10^{-7}M	IC_{50} M
esophageal carcinoma (HCE-6)						
heteromine A	1.7	8.2	26.2	87.4	92.4	4.08×10^{-6}
heteromine B	10.6	22.1	85.0	82.2	82.2	3.60×10^{-5}
hepatoma (Huh-7)						
heteromine A	11.1	43.1	91.1	101.9	97.3	7.18×10^{-5}
heteromine B	28.8	92.5	94.3	102.8	104.5	4.64×10^{-4}
lymphoma (Molt-4)						
heteromine A	5.6	24.8	54.8	90.7	94.7	1.44×10^{-5}
heteromine B	17.4	35.9	86.0	91.0	106.9	5.23×10^{-5}
leukemia (HL-60)						
heteromine A	5.7	8.2	42.7	99.3	94.5	7.43×10^{-6}
heteromine B	14.9	20.3	72.6	99.1	113.9	2.70×10^{-5}
leukemia (K562)						
heteromine A	5.9	33.5	49.7	107.2	94.3	9.88×10^{-6}
heteromine B	27.6	40.4	88.2	95.5	96.8	6.29×10^{-5}

Scheme 1



spectrometer in DMSO-d_6 or $CDCl_3$ solution. Chemical shift data are in parts per million downfield from TMS as internal reference. ^{1}H−^{1}H COSY and ^{1}H−^{13}C COSY, HMBC, and NOESY were measured based on standard pulse sequences. UV spectra were taken on a Hitachi U-3200 spectrophotometer. EIMS were measured on a Finnigan TSQ-46C MS spectrometer. FABMS were run on a JEOL JMS-HX 110 MS spectrometer. Elementary analysis was performed on a Perkin-Elmer 2400 elemental analyzer.

Plant Material. The aerial parts of *Heterostemma brownii* were collected in April 1991, in Wen-Sun mountains, Taipei Hsien, Taiwan. Plant material was identified by comparison with a voucher specimen, which was deposited at the Herbarium of the Department of Botany of National Taiwan University.

Extraction and Isolation. The aerial parts of *H. brownii* (5 kg) were extracted with 60% MeOH (80 L) at 50 °C (overnight for three times). The extract (954 g) was subjected to Diaion HP-20 column chromatography, and eluted with H_2O−MeOH gradient solvent system. The fraction eluted with 50−80% aqueous MeOH was rechromatographed on Diaion HP-20 (40% aqueous MeOH) and Sephadex LH-20 (MeOH). Heteromines A (**1**) (146 g), B (**2**) (86 g), C (**3**) (16 g), D (**4**) (0.86 g), and E (**5**) (0.015 g) were eluted in this order. The MeOH eluted fraction (45 g) was rechromatographed on Sephadex LH-20 (MeOH) and Si gel (5−20% MeOH in $CHCl_3$) to afford heteromines F (**6**) (1.6 g), G (**7**) (0.65 g), and H (**8**) (0.15 g).

Heteromine D (4): colorless needles (MeOH); mp 196−198 °C; UV (MeOH) λ_{max} (log ϵ) 240 (4.00), 258 sh (3.85), 300 (3.69) nm; IR (KBr) υ_{max} 3320, 1705, 1620, 1595, 1365 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; EIMS *m/z* 207 [M$^+$ − Cl − 1] (100), 192 (82), 178 (55), 163 (58), 136 (58); *anal.* C 44.45%, H 5.73%, N 28.69%, calcd for $C_9H_{14}N_5OCl$, C 44.36%, H 5.79%, N 28.74%.

Heteromine E (5): colorless needles (MeOH); mp 295 °C (decomp); UV (MeOH) λ_{max} (log ϵ) 236 (3.90), 253 sh (3.98), 291 (3.79) nm; IR (KBr) υ_{max} 3345, 1700, 1645, 1615, 1575, 1515, 1385 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; FABMS *m/z* 194 [M$^+$ − Cl] (100), 180 (5), 163 (5), 138 (8); *anal.* C 41.94%, H 5.30%, N 30.58%, calcd for $C_8H_{12}N_5OCl$, C 41.84%, H 5.27%, N 30.49%.

Heteromine F (6): colorless needles (MeOH); mp 176−177 °C; UV (MeOH) λ_{max} (log ϵ) 247 (4.14), 268 (3.91) nm; IR (KBr) υ_{max} 3385, 1675, 1610, 1580, 1545, 1515, 1165, 1130, 815, 790 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; EIMS *m/z* 239 [M$^+$] (100), 210 (50), 196 (41),

181 (11); *anal.* C 50.28%, H 7.15%, N 29.34%, calcd for $C_{10}H_{17}N_5O_2$, C 50.19%, H 7.16%, N 29.27%.

Heteromine G (7): colorless needles (MeOH): mp 105−106 °C; UV (MeOH) λ_{max} (log ϵ) 242 (3.61), 266 (3.62) nm; IR (KBr) υ_{max} 3440, 3350, 1685, 1585, 1525, 1050, 815, 790 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; EIMS *m/z* 225 [M$^+$] (100), 208 (33), 196 (51), 168 (53), 167 (43), 106 (62); *anal.* C 48.20%, H 6.69%, N 31.15%, calcd for $C_9H_{15}N_5O_2$, C 47.99%, H 6.71%, N 31.01%.

Heteromine H (8): colorless needles (MeOH); mp 220−222 °C; UV (MeOH) λ_{max} (log ϵ) 236 (3.44), 258 (3.46), 290 (3.30) nm; IR (KBr) υ_{max} 3325, 3220, 1685, 1655, 1580, 1520, 1380, 820, 790 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; EIMS *m/z* 211 [M$^+$] (100), 194 (54), 182 (42), 168 (53), 167 (43), 166 (62); *anal.* C 45.60%, H 6.15%, N 33.24%, calcd for $C_8H_{13}N_5O_2$, C 45.49%, H 6.20%, N 33.16%.

Reaction of 1, 2, or 3 in 10% Aqueous Ammonium Hydroxide. Each of the heteromines A (**1**), B (**2**), and C (**3**) (35 mg) was dissolved in 3 mL of 10% NH_4OH, maintained at room temperature for 3 h, and extracted with EtOAc (15 mL) three times to give heteromines F (**6**), G (**7**), or H (**8**) (each of 25 mg), respectively.

Reduction of Heteromine A (1) with $NaBH_4$ and Then Reoxidation. Excess of $NaBH_4$ (200 mg) was added in small portions into a solution of **1** (280 mg) in H_2O (3 mL), and the reaction mixture was allowed to stand for 30 min. The reaction mixture was then extracted with EtOAc (35 mL) three times. After evaporation of EtOAc *in vacuo*, the extract was dissolved in MeOH (10 mL). The MeOH solution was stirred at room temperature for 3 days after 7,8-dihydroheteromine A (**11**)[3] disappeared.

The product was subjected to SiO_2 column chromatography with 2% MeOH−$CHCl_3$ to afford three products **6** (10 mg), **9** (135 mg), **10** (15 mg), and recovered **1** (17 mg).

Compound 9: mp 178−180 °C; IR (KBr) υ_{max} 1707, 1632, 1602, 1545, 1310, 1030, 767 cm^{-1}; UV (MeOH) λ_{max} (log ϵ) 210 (4.51), 254 (3.63), 300 (3.56) nm;^{1}H-NMR ($CDCl_3$, 300 MHz) δ 3.11 (6H, s), 3.31, 3.43, 3.94 (each 3H, s); ^{13}C-NMR ($CDCl_3$, 300 MHz) δ 26.1 (N-CH$_3$), 29.2 (N-CH$_3$), [N(CH$_3$)$_2$], 52.9 (OCH$_3$), 98.7 (C-5), 151.0 (C-4), 153.2 (C-6), 153.6 (C-2), 157.9 (C-8); EIMS (70 eV) *m/z* 237 [M$^+$] (100), 222 (28), 208 (26), 194 (24); *anal.* C 50.71%, H 6.35%, N 29.60%, calcd for $C_{10}H_{15}N_5O_2$, C 50.62%, H 6.37%, N 29.52%.

Compound 10: mp 154−156 °C; IR (KBr) υ_{max} 1610, 1573, 1543, 1385, 1261 cm^{-1}; UV (MeOH) λ_{max} (log ϵ) 217 (4.20), 254 (3.64), 296 (3.48) nm;^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.18 (6H, s), 3.66, 4.06 (each 3H, s), 7.48 (1H, s); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 29.2 (NCH$_3$), 37.3 [N(CH$_3$)$_2$], 53.3 (OCH$_3$), 113.7 (C-5), 139.1(C-8), 154.6 (C-4), 159.5 (C-6), 160.6 (C-2); EIMS (70 eV) *m/z* 207 [M$^+$] (100), 192 (48), 178 (53), 163 (37), 149 (35); *anal.* C 52.23%, H 6.29%, N 33.90%, calcd for $C_9H_{13}N_5O$, C 52.16%, H 6.32%, N 33.80%.

Cytotoxicity Assay. Cell lines were provided by the Cell Bank of Veterans General Hospital, Taipei, Taiwan. Cells were grown in RPMI 1640 supplemented with 10% (v/v) fetal calf serum, 100 IU/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine, 1% nonessential amino acid. Cytotoxicity *in vitro* was done by the method of Carmichael *et al.*[6] In all, 2×10^4 cells/well were incubated in the presence or absence of test compound for 72 h. Then, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma M2128] was added, and plates were incubated at 37 °C for 4 h. Dimethylsulfoxide (DMSO, E. Merck) 100 μL was added to all wells and mixed throughly to dissolve the dark blue crystals. After a few minutes at room temperature to ensure that all crystals were dissolved, the plates were read on a Dynatech MR5000 Microelisa reader, using 570 nm as a test wavelength, a reference wavelength of 630 nm, and a calibration setting at 1.99 (or 1.00 if the samples were strongly colored). Plates were normally read within 1 h after adding DMSO. Each experiment was carried out in triplicate, and the percent inhibition was calculated as follows: % inhibition = [1 − OD (570 nm) of sample well/OD (570 nm) of control well] $\times$ 100. IC_{50} was given as the concentration (in μM) required for 50% inhibition of cell growth.

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