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Quinone Derivatives from the Rhizomes of *Acorus gramineus* and Their Biological Activities

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A further phytochemical investigation of the rhizomes of *Acorus gramineus* afforded three new quinone derivatives (1–3), together with two known compounds (4 and 5). The identification and structural elucidation of these new compounds were based on 1D and 2D NMR (COSY, HMQC, HMBC and NOESY) and MS data. The absolute configurations were established on the basis of their circular dichroism (CD) data. To investigate the anti-neuroinflammatory effects of the isolated compounds (1–5), the nitric oxide (NO) production was evaluated in the lipopolysaccharide-activated microglia cell line, BV-2. Compounds (1–5) were also tested for their cytotoxicity against four human tumor cell lines (A549, SK-OV-3, SK-MEL-2, and HCT-15) *in vitro* by using the SRB assay.

Key words: *Acorus gramineus*; Araceae; quinone derivative; neuroinflammation; cytotoxicity

Acorus gramineus belongs to the Araceae family and is a perennial aromatic herb that is widely distributed in Korea, Japan, and China.[1)] The rhizomes of this plant have long been used in traditional Chinese medicine as a remedy for cognitive problems, for sedation, and for analgesia.[2)] The herb has been used in traditional Korean medicine for treating stomach ache and edema, and for improving learning and memory.[3,4)] The rhizomes of *A. gramineus* contain essential oils, such as β-asarone, α-asarone, caryophyllene, isoasarone, methylis-oeugenol, safrole, and asaylaldehyde as its main chemical constituents.[5)] The methanol extract of this plant has shown neuroprotective, cytotoxic, antibacterial, antifungal and antioxidative activities.[6–9)] In addition, the water extract has produced sedation, decreased locomotor activity, increased pentobarbital-induced sleeping time, and attenuated apomorphine-induced stereotypic behavior in mice.[2,10)]

We have recently reported the isolation of lignan derivatives[11,12)] and phenylpropanoides[13)] from the methanol extract of *A. gramineus*, their inhibitory effects on nitric oxide (NO) production in lipopolysaccharide (LPS)-activated macrophages, and their cytotoxic activity.[11)] Continuing research on this material led us to, perform a phytochemical investigation of the petroleum ether extract from the rhizomes of *A. gramineus*. Dried and chopped rhizomes of *A. gramineus* were extracted three times with petroleum ether at room temperature. Repeated column chromatographic separation of the extract resulted in the isolation of three new quinone derivatives (**1**–**3**), together with two known compounds (**4** and **5**) (Fig. 1). The structures were determined by spectroscopic methods including 1D and 2D NMR. We report here the isolation and structural elucidation of the new compounds, and the biological effects of these isolated compounds (**1**–**5**).

Materials and Methods

General experimental procedures. Optical rotation values were measured with a P-1020 polarimeter (Jasco, Easton, MD, USA). IR spectra were recorded with an IFS-66/S FT-IR spectrometer (Bruker, Karlsruhe, Germany), and CD spectra were measured with a Jasco J-810 spectropolarimeter. UV spectra were recorded with a UV-1601 UV-visible spectrophotometer (Shimadzu, Tokyo, Japan). HRESI mass spectra were obtained with a VG Biotech platform LC-mass spectrometer and HRFAB mass spectra were obtained with a JMS700 mass spectrometer (Jeol, Peabody, MA, USA). NMR spectra, including ^{1}H-^{1}H COSY, HMQC, HMBC, and NOESY experiments, were recorded by a Unity Inova 500 NMR spectrometer (Varian, Palo Alto, CA, USA) operated at 500 MHz (^{1}H) and 125 MHz (^{13}C), with chemical shifts given in ppm (δ). The preparative high-performance liquid chromatography (HPLC) used a type 306 pump (Gilson, Middleton, WI, USA) fitted to a Shodex refractive index detector (Shodex, New York, NY, USA). Silica gel 60 (Merck, 70–230 mesh and 230–400 mesh) and reverse phase (RP)-C_{18} silica gel (Merck, 230–400 mesh) were used for column chromatography. The packing material for molecular sieve column chromatography was Sephadex LH-20 (Pharmacia, Uppsala, Sweden). Precoated silica gel F_{254} plates and RP-18 F_{254s} plates (Merck, Darmstadt, Germany) were used for thin-layer chromatography (TLC), spots being detected on TLC traces under UV light or by heating after spraying with 10% H_2SO_4 in C_2H_5OH (v/v).

Plant materials. The rhizomes of *A. gramineus* were collected on Jeju Island, Korea, in March 2009, and the plant was identified by one of the authors (K.R. Lee). A voucher specimen (SKKU-NPL-0910) has been deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

Extraction and isolation. The rhizomes of *A. gramineus* (6.0 kg) were dried and chopped, extracted at room temperature with petroleum ether and then filtered. The filtrate was evaporated under vacuum to obtain a petroleum ether extract (180 g). This petroleum ether extract (180 g) was separated by silica gel (230–400 mesh, 3.6 kg) column chromatography using a solvent system of *n*-hexane-EtOAc (11:1–1:1,

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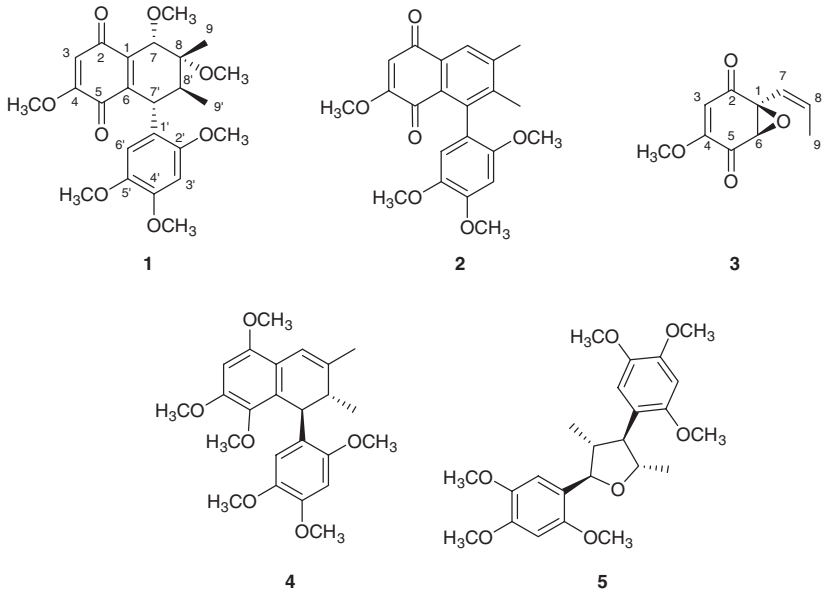


Fig. 1. Chemical Structures of Compounds **1**–**5**.

v/v) as the eluent to yield five fractions (P1–P5). Fraction P5 (50 g) was separated in a silica gel column with *n*-hexane-EtOAc (50:1, v/v) to yield seven fractions (P51–P57). Fraction P51 (15 g) was separated in an RP-C_{18} silica gel column with a solvent system of MeOH-H_2O (70:30) to afford six subfractions (P511–P516). Subfraction P511 (2 g) was separated in a Sephadex LH-20 column with a solvent system of 100% MeOH and then purified by preparative normal-phase high-performance liquid chromatography (HPLC) using a solvent system of *n*-hexane-EtOAc (5:1, v/v) as the eluent to yield **3** (20 mg, $t_R = 15.0$ min). Subfraction P514 (5 g) was separated in an RP-C_{18} silica gel column with a solvent system of MeOH-H_2O (70:30, v/v) and then purified by preparative normal-phase HPLC with a solvent system of *n*-hexane-EtOAc (3.5:1, v/v) as the eluent to yield **5** (10 mg, $t_R = 15.0$ min). Subfraction P515 (500 mg) was separated in a Sephadex LH-20 column with a solvent system of 100% MeOH and then purified by preparative reversed-phase HPLC using a solvent system of MeOH-H_2O (80:20, v/v) as the eluent to afford **4** (6 mg, $t_R = 16.5$ min). Subfraction P516 (1 g) was applied to a 100% MeOH Sephadex LH-20 column and purified by preparative normal-phase HPLC with a solvent system of *n*-hexane-EtOAc (1:1, v/v) as the eluent to yield **1** (8 mg, $t_R = 12.5$ min) and **2** (10 mg, $t_R = 14.0$ min).

Compound 1. A brown-red gum, $[\alpha]_D^{25}$ +9.0 (*c* 0.25, $CHCl_3$). IR (KBr) ν_{max} cm^{-1}: 3385, 2948, 2835, 1652, 1451, 1114, 1024, 695. UV (MeOH) λ_{max} (log ε) nm: 280 (3.4). CD (MeOH) λ_{max} ($\Delta\varepsilon$) nm: 244 (5.5), 310 (−2.9). ^{1}H- and ^{13}C-NMR spectra, see Table 1. HRESIMS (positive-ion mode) *m/z* 469.1838 $[M + Na]^+$ (calcd. for $C_{24}H_{30}O_8Na$, 469.1836).

Compound 2. A brown-red gum; $[\alpha]_D^{25}$ +30.5 (*c* 0.15, $CHCl_3$). IR (KBr) ν_{max} cm^{-1}: 3375, 2947, 2833, 2522, 1650, 1451, 1213, 1114, 1032, 673. UV (MeOH) λ_{max} (log ε) nm: 253 (3.4), 293 (3.5). ^{1}H- and ^{13}C-NMR spectra, see Table 1. HRESIMS (positive-ion mode) *m/z* 405.1307 $[M + Na]^+$ (calcd. for $C_{22}H_{22}O_6Na$, 405.1314).

Compound 3. A brown-red gum; $[\alpha]_D^{25}$ +7.0 (*c* 0.15, $CHCl_3$). IR (KBr) ν_{max} cm^{-1}: 3384, 2947, 2834, 1660, 1452, 1246, 1118, 1031, 701. UV (MeOH) λ_{max} (log ε) nm: 295 (3.7). CD (MeOH) λ_{max} ($\Delta\varepsilon$) nm: 239 (−3.1), 251 (2.5), 263 (−4.0). ^{1}H- and ^{13}C-NMR spectra, see Table 1. HRFABMS (positive-ion mode) *m/z* 195.0655 $[M + H]^+$ (calcd for $C_{10}H_{11}O_4$, 195.0657).

Measurement of the nitric oxide (NO) production and cell viability. BV-2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 1% penicillin-streptomycin. The BV-2 cells were plated onto a 96-well plate (3×10^4 cells/well) to measure NO production. After 24 h, the cells were pretreated for 30 min with compounds and then stimulated for 24 h with 100 ng/mL of LPS. Nitrite, a soluble oxidation product of NO, was measured in the culture medium by using the Griess reaction. The resulting supernatant was harvested and mixed with an equal volume of the Griess reagent (1% sulfanilamide, 0.1% *N*-1-naphthylethylenediamine dihydrochloride in 5% phosphoric acid). The absorbance at 540 nm was measured after 10 min by using an Emax microplate reader (Molecular Devices, Sunnyvale, CA, USA). Sodium nitrite was used as the standard to calculate the nitrite concentration. Cell viability was measured by using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. N^G-Monomethyl-L-arginine (L-NMMA; Sigma, St. Louis, MO, USA), a well-known NOS inhibitor, was tested as a positive control.[14]

Cytotoxicity assay. A sulforhodamine B (SRB) bioassay was used to determine the cytotoxicity of each isolated compound against four cultured human tumor cell lines.[15] The assays were performed at the Korea Research Institute of Chemical Technology, using the cell lines A549 (non-small cell lung carcinoma), SK-OV-3 (ovary malignant ascites), SK-MEL-2 (skin melanoma), and HCT-15 (colon adenocarcinoma). Doxorubicin was used as the positive control. The respective cytotoxicity of doxorubicin against the A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines was IC_{50} 0.001, 0.008, 0.001, and 0.021 μM.[15]

Results and Discussion

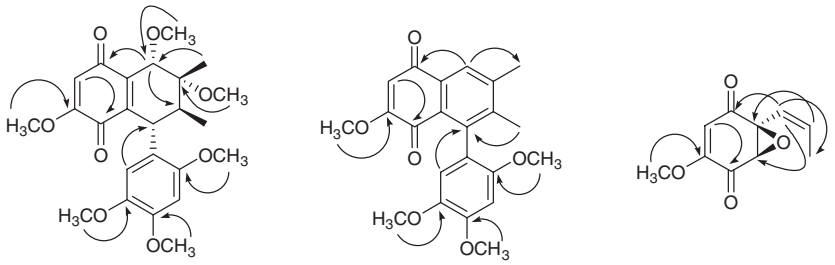
*Structural elucidation of **1**–**3***

Compound **1** was obtained as a brown-red gum. The molecular formula was determined to be $C_{24}H_{30}O_8$ from the molecular ion peak $[M + Na]^+$ at *m/z* 469.1838 (calcd. for $C_{24}H_{30}O_8Na$, 469.1836) in the positive-ion HRESIMS data. The IR spectrum of **1** indicated the presence of hydroxyl (3385 cm^{-1}) and carbonyl groups (1652 cm^{-1}). The ^{1}H NMR spectrum of **1** showed signals for a tertiary methyl group at δ_H 0.98 (3H, s), a secondary methyl group at δ_H 0.97 (3H, d, $J = 7.0$ Hz), an oxygenated methine at δ_H 4.62 (1H, s), two methine protons at δ_H 2.39 (1H, m) and 4.08 (1H, m), an olefinic proton at δ_H 5.93 (1H, s), two aromatic protons at δ_H 6.54 (1H, s) and 6.66 (1H, s), and six methoxy groups at δ_H 3.39, 3.57, 3.73, 3.76, 3.87, and 3.96 (each 3H, s). The ^{13}C-NMR spectrum showed the appearance of 24 carbon signals, including two methyl carbons at δ_C 11.5 and 14.7, an oxygenated methine

Table 1. ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) Spectral Data for **1**–**3** in $CDCl_3$ (δ in ppm)

Position	**1**		**2**		**3**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		139.3		130.8		60.8
2		187.0		185.5		190.5
3	5.93 (s)	106.7	6.08 (s)	108.0	5.81 (s)	108.0
4		158.6		160.8		158.9
5		181.4		179.8		187.6
6		144.9		127.0	3.76 (s)	59.4
7	4.62 (s)	70.7	7.98 (s)	127.2	5.64 (ddd, 11.0, 2.0, 1.5)	119.1
8		76.9		143.7	6.00 (dd, 11.0, 7.0)	134.6
9	0.98 (s)	14.7	2.47 (s)	21.5	1.68 (dd, 7.0, 2.0)	14.8
4-OMe	3.76 (s)	56.1	3.82 (s)	56.6	3.78 (s)	56.5
7-OMe	3.57 (s)	58.6				
8-OMe	3.39 (s)	49.6				
1′		123.3		120.4		
2′		151.5		150.4		
3′	6.54 (s)	98.2	6.65 (s)	97.8		
4′		148.1		148.9		
5′		143.3		143.6		
6′	6.66 (s)	112.8	6.43 (s)	112.6		
7′	4.08 (m)	40.2		139.4		
8′	2.39 (m)	40.4		143.8		
9′	0.97 (d, 7.0)	11.5	2.03 (s)	16.9		
2′-OMe	3.96 (s)	57.2	3.67 (s)	56.1		
4′-OMe	3.87 (s)	57.0	3.97 (s)	56.4		
5′-OMe	3.73 (s)	56.4	3.79 (s)	56.2		

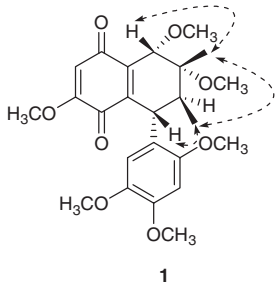
Assignments are based on 2D NMR data including COSY, HMQC and HMBC. Well-resolved couplings is expressed with the coupling patterns and Hz coupling constants in parentheses.



1 **2** **3**

Fig. 2. Key HMBC (⟶) Correlations of **1**–**3**.

carbon at δ_C 70.7, two methine carbons at δ_C 40.2 and 40.4, four quaternary carbons at δ_C 76.9, 139.3, 144.9, and 158.6, an olefinic carbon at δ_C 106.7, six aromatic carbons at δ_C 98.2, 112.8, 123.3, 143.3, 148.1, and 151.5, two quinone carbonyl carbons at δ_C 181.4 and 187.0, and six methoxy groups at δ_C 49.6, 56.1, 56.4, 56.9, 57.2, and 58.6, which were classified by HMQC experiments. The ^{1}H- and ^{13}C-NMR spectra (Table 1) of **1** were almost identical to those of garcillisquinone A isolated from *Spallerocarpus gracillis*,[16] except for the presence of signals for two additional methoxy groups (δ_H 3.39, 3.57; δ_C 49.6, 58.6) in **1** and the absence of a methine signal in garcillisquinone A. The positions of two methoxy groups were determined at C-7 and C-8 on the basis of the HMBC correlations between a methoxy proton (δ_H 3.57) and C-7 (δ_C 70.7), and the other methoxy proton (δ_H 3.39) and C-8 (δ_C 76.9) (Fig. 2). The relative configuration of **1** was deduced by a NOESY experiment (Fig. 3). The results of the NOESY experiments showed correlations between H-7′ (δ_H 4.08) and H_3-9′ (δ_H 0.97), H-8′ (δ_H 2.39) and H-6′ (δ_H 6.66), H_3-9′ (δ_H 0.97) and H_3-9 (δ_H 0.98), and H_3-9 (δ_H 0.98)



1

Fig. 3. Key NOESY (⟷) Correlations of **1**.

and H-7 (δ_H 4.62). The absolute configuration of **1** was established as 7′*R*,8*R*,8′*S* by comparing its CD spectrum showing a negative Cotton effect at 310 nm and a positive Cotton effect at 244 nm with that of (−)-8′-*epi*-8-hydroxy-aristoligone isolated from *Holostylis reniformis*.[17] However, the absolute stereochemistry at C-7 could not be determined. The α-orientation of the methoxy group at C-7 was established by the NOESY experiment. The structure of **1** was therefore determined

to be (7′*R*,8*R*,8′*S*)-7′-(2′,4′,5′-trimethoxyphenyl)-4,7α,8-trimethoxy-8,8′-dimethyl-2,5-quinone.

Compound **2** was obtained as a brown-red gum. The molecular formula was determined to be $C_{22}H_{22}O_6$ from the molecular ion peak $[M+Na]^+$ at m/z 405.1307 (calcd. for $C_{22}H_{22}O_6Na$, 405.1314) in the positive-ion HRESIMS. The ^{1}H- and ^{13}C-NMR spectroscopic data (Table 1) for **2** were similar to those for **1**. The main differences were the appearance of aromatic signals [δ_H 7.98 (H-7); δ_C 127.2 (C-7), 139.4 (C-7′), 143.7 (C-8) and 143.8 (C-8′)] in **2**, as well as the disappearance of two methoxy groups and a methine signal in **1**, impling that **2** was aromatized by demethoxylation at C-7 and C-8 in **1**. This was confirmed by the HMBC experiment, which showed correlations between the following protons and carbons: H-7 and C-1, C-6, C-9, and C-8′; H-9 and C-7′, C-8′, and C-8; and H-9′ and C-7, C-8, and C-8′ (Fig. 2). Hence, the structure of **2** was elucidated as 7′-(2′,4′,5′-trimethoxyphenyl)-4-methoxy-8,8′-dimethyl-2,5-quinone.

Compound **3** was obtained as a brown-red gum. The molecular formula was determined to be $C_{10}H_{10}O_4$ from the molecular ion peak $[M+H]^+$ at m/z 195.0655 (calcd. for $C_{10}H_{11}O_4$, 195.0657) in the positive-ion HRFABMS data. The IR spectrum of **3** indicated the presence of hydroxyl (3384 cm^{-1}) and carbonyl groups (1660 cm^{-1}). The ^{1}H-NMR data (Table 1) showed the presence of a methyl group at δ_H 1.68 (3H, dd, $J = 7.0$, 2.0 Hz), an oxygenated methine proton at δ_H 3.76 (1H, s), three olefinic protons at δ_H 5.64 (1H, ddd, $J = 11.0$, 7.0, 2.0 Hz), 5.81 (1H, s), and 6.00 (1H, dd, $J = 11.0$, 7.0 Hz), and one methoxy group at δ_H 3.78 (3H, s). The ^{13}C-NMR spectrum showed nine carbon resonances, which were classified by HMQC experiments as a methyl carbon at δ_C 14.8, an oxygenated methine carbon at δ_C 59.4, two quaternary carbons at δ_C 60.8 and 158.9, three olefinic carbons at δ_C 108.0, 119.1, and 134.6, two carbonyl carbons at δ_C 187.6 and 190.5, and one methoxy group at δ_C 56.5. In the ^{1}H-NMR spectrum, the coupling constant (11.0 Hz) of H-7 (δ_H 5.64) indicated that **3** possessed *cis*-olefinic functionality.[5] Based on the above consideration and analysis of 2D-NMR experiments (COSY, HMQC, and HMBC) the planar structure of **3** was established to be 1-propenyl-1,6-epoxy-4-methoxy-2,5-quinone. The absolute configurations at C-1 and C-6 were respectively assigned to be 1*S* and 6*R*, based on the CD spectrum, which showed a negative Cotton effect at 239 nm and 263 nm, and a positive Cotton effect at 251 nm. The corresponding quinone epoxide[18] showed a negative Cotton effect at 231 nm and 257 nm, and a positive Cotton effect at 243 nm. Therefore, the structure of **3** was assigned as 1-*cis*-propenyl-1*S*,6*R*-epoxy-4-methoxy-2,5-quinone. This is the first report of compound **3** as a natural product, although it has been synthesized without details of its absolute configuration.[19]

The known compounds were identified as magnoshinin (**4**),[20] and (2*S*,3*S*,4*R*,5*R*)-2,4-dimethyl-1,3-bis(2′,4′,5′-trimethoxyphenyl) tetrahydrofuran (**5**),[21] by comparing their spectroscopic and physical data with reported values.

Anti-neuroinflammatory effect

We tested the anti-neuroinflammatory effects of compounds (**1**–**5**) isolated from *A. gramineus* by measuring the NO level, using the bacterial endotoxin LPS in murine microglia BV-2 cells, the inhibitory activity of each toward NO production being expressed as the 50% inhibitory concentration (IC_{50}). All compounds significantly inhibited the effects of NO production in LPS-activated BV-2 cells ($IC_{50} = 10.24$–$17.46\,\mu M$). The positive control, L-NMMA, showed a low IC_{50} value of 12.88 μM. Among the active compounds, **1** also reduced the cell viability of BV-2 cells, indicating that the inhibitory effect of **1** toward NO production may have been caused by its high cytotoxic activity.

Table 2. Inhibitory Effect on Nitric Oxide (NO) Production of Compounds **1**–**5** in Lipopolysaccharide (LPS)-Activated BV-2 Cells

Sample	IC_{50} (μM)[a]	Cell viability (%)[b]
1	10.24	49.8 ± 1.4
2	11.54	113.8 ± 6.1
3	10.30	100.9 ± 6.0
4	17.46	113.8 ± 7.1
5	14.94	110.0 ± 6.0
NMMA[c]	12.88	105.8 ± 6.1

[a]IC_{50} value of each compound is expressed as the concentration (μM) that caused 50% inhibition of NO production in LPS-activated BV-2 cells.
[b]The cell viability after treating with 20 μM of each extract is expressed as a percentage of the LPS-only treatment group. each result is the average of three independent experiments, and the data are expressed as mean ± standard deviation.
[c]NMMA is shown as a positive control.

Cytotoxic effects

The cytotoxic activities of the isolated compounds (**1**–**5**) against the A549, SK-OV-3, SK-MEL-2, and HCT-15 human cancer cell lines were evaluated by using the SRB assay *in vitro*. Compounds **1** and **3** showed significant cytotoxicity in this assay against the A549, SK-OV-3, SK-MEL-2, and HCT-15 human tumor cell lines (IC_{50} values 12.28, 13.17, 5.19, and 7.11 μM in **1**; 17.59, 13.70, 6.91, and 7.45 μM in **3**, respectively). Compounds **2**, **4** and **5** showed little cytotoxicity against the four tested cell lines ($IC_{50} > 30\,\mu M$).

Acknowledgments

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