Two New Aristolochic Acid Derivatives from the Roots of *Aristolochia fangchi* and Their Cytotoxicities

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Phytochemical investigation of 90% ethanol extracts of the roots of *Aristolochia fangchi* yielded two new aristolochic acid derivatives named Aristolochic Acid F and Aristolochic Acid G, together with three known compounds. Their structures were elucidated by spectral analysis. The cytotoxicity of the isolated compounds was also determined.

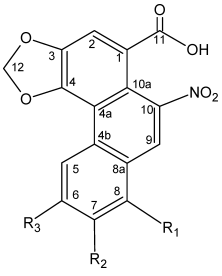
Key words *Aristolochia fangchi*; Aristolochic Acid F; Aristolochic Acid G; cytotoxicity

Guang Fang Ji, the root of *Aristolochia fangchi* Y. C. WU *ex* L. D. CHOU *et* S. M. HWANG (Aristolochiaceae), is distributed in many places of southeastern of China. For thousands of years it has been used in traditional Chinese medicine to treat arthritis, rheumatism, and edema of the lower extremities.[1)] However, in the last 20 years it has been found that Aristolochia plants (which contain aristolochic acids compounds) can cause serious kidney damage, and the products of aristolochia species have also been linked to certain types of cancer, most often occurring in the urinary tract.[2—6)] Therefore many countries including China have banned the use of plants that contain aristolochic acid.[7)] Much attention has been paid to the chemical constituents, the nephrotoxic and carcinogenic effects of aristolochic acid derivatives, and the corresponding plants for years. Several aristolochic acids and aristolactams have been isolated from this plant.[8—10)] In the course of our systemic investigation of the chemical constitutes and their toxicities, two new and three known compounds from the roots of *Aristolochia fangchi* were isolated. This paper deals with the structure determination of the new compounds and cytotoxicities of the compounds obtained.

Results and Discussion

The air-dried and powdered roots of Guang Fang Ji were extracted with 90% ethanol, and the extracts were separated as described in the experimental section to yield compounds **1**—**5**. The three known compounds were identified as aristolochic acid A (**1**), aristolochic acid B (**2**), and aristolochic acid C (**3**) by comparison of their spectral data with data reported in the literature[11—15)] (Fig. 1).

Compound **4** was obtained as a yellow amorphous powder. The high resolution MS analysis of quasimolecular ion peaks in the positive-ion FAB-MS of **4** showed a molecular ion peak at *m*/*z* 328.2459, indicating a molecular formula of $C_{16}H_9NO_7$. The UV absorption of **4** at 218, 256, 295, 351, and 371 nm showed it was a phenanthro type derivative.[11,15)] Its IR spectrum showed absorption bands due to hydroxyl, nitro, and carboxyl functions at 3438, 1337, 1520, and 1715 cm^{-1}, indicating that **4** was an aristolochic acid derivative. This was also confirmed by fragment ion peaks at *m*/*z* 328 $[M+H]^+$, *m*/*z* 282 $[M+H-NO_2]^+$, *m*/*z* 265 $[M+H-NO_2-OH]^+$, and *m*/*z* 237 $[M+H-NO_2-COOH]^+$, separately. Comparison of the ^{1}H-NMR spectral data (Table 1) and ^{13}C-NMR spectral data (Table 2) of **4** with those of **3** revealed they had many similarities. Both **3** and **4** had 9 proton signals and 16 carbon signals, and their chemical shifts are very similar. The only difference was the substitution



No.	Compound name	R_1	R_2	R_3
1	Aristolochic acid A	OCH_3	H	H
2	Aristolochic acid B	H	H	H
3	Aristolochic acid C	H	H	OH
4	Aristolochic acid F	H	OH	H
5	Aristolochic acid G	OH	H	OH

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

Table 1. ^{1}H-NMR Data for Compounds **1**—**5** (δ ppm, *J* in Hz) (500 MHz in DMSO-d_6, and TMS as Internal Standard)

No.	1	2	3	4	5
2	7.78 (1H, s)	7.75 (1H, s)	7.73 (1H, s)	7.72 (1H, s)	7.76 (1H, s)
12-H	6.46 (2H, s)	6.41 (2H, s)	6.345 (2H, s)	6.43 (2H, s)	6.50 (2H, s)
5-H	8.61 (1H, d, *J*=8)	8.52 (1H, m)	8.42 (1H, s)	8.69 (1H, d, *J*=8.2)	8.13 (1H, d, *J*=2.0)
6-H	7.80 (1H, m)	7.70 (1H, m.)		7.31 (1H, dd, *J*=8.2, 2.2)	
7-H	7.33 (1H, d, *J*=8.2)	7.74 (1H, m)	7.23 (1H, d. *J*=8)		6.72 (1H, d, *J*=2.0)
8-H		7.90 (1H, m)	8.02 (1H, d, *J*=8)	7.42 (1H, d, *J*=2.2)	
9-H	8.54 (1H, s)	8.39 (1H, s)	8.42 (1H, s)	8.21 (1H, s)	8.70 (1H, s)
8-OCH_3	4.03 (3H, s)				
COOH	10.90 (1H, s)	10.96 (1H, s)	10.88 (1H, s)	10.90 (1H, s)	10.82 (1H, s)
6-OH			11.36 (1H, s)		11.50 (1H, s)
7-OH				11.80 (1H, s)	
8-OH					11.36 (1H, s)

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Table 2. ^{13}C-NMR Data for Compounds **1**—**5** (δ ppm) (125 MHz in DMSO-d_6, and TMS as Internal Standard)

No.	1	2	3	4	5
1	125.4	124.6	124.3	123.6	124.3
2	113.1	113.8	112.6	112.1	113.9
3	146.9	147.3	146.3	146.3	146.4
4	146.3	146.8	146.8	147.5	146.8
4a	117.6	117.3	117.6	118.3	117.4
4b	130.7	130.2	133.2	136.9	135.0
5	119.7	127.8	112.0	129.8	115.6
6	132.5	126.1	160.7	110.3	150.8
7	109.8	128.0	119.6	159.4	106.9
8	157.2	129.8	131.8	116.4	152.4
8a	119.3	135.5	122.4	126.3	120.3
9	120.3	121.6	127.2	124.8	121.8
10	145.9	146.0	144.3	145.6	146.3
10a	118.2	118.3	118.3	118.4	118.6
11	168.0	169.6	168.8	168.9	169.8
12	103.8	103.1	103.7	103.2	103.8
OCH_3	57.2				

position of the hydroxyl group. The ^{1}H-NMR spectrum of **4** showed two downfield exchangeable proton signals at δ 11.80 (1H, s) and 10.90 (1H, s) which were attributed to hydroxyl and carboxyl groups, respectively. In the aromatic region, three mutual coupled ABX pattern signals at δ 7.42 (1H, d, J=2.2 Hz), 8.69 (1H, d, J=8.2 Hz), and 7.31 (1H, dd, J=8.2, 2.2 Hz) were attributed to H-8, H-5, and H-6, respectively,[14] and a hydroxyl group was believed to be connected at C-7. The remaining 2 one-proton signals at δ 7.72 (1H, s) and 8.21 (1H, s) could be ascribed to H-2 and H-9, respectively, due to the anisotropic effect of the carbonyl group at C-1.[14,15] One singlet signal at δ 6.48 (2H, s) was attributed to methylenedioxyl protons.[11,13] All substituted groups and positions were confirmed by heteronuclear multiple bond connectivity (HMBC) and nuclear Overhauser effect spectroscopy (NOESY) experiments (Fig. 2). In the HMBC spectrum, H-8 (δ 7.42) showed ^2J or ^3J correlations with signals for C-7 (δ 159.4), C-6 (δ 110.3), C-8a (δ 126.3), and C-9 (δ 124.8). C-7 (δ 159.4) showed ^2J correlations with H-6 (δ 7.31) and H-8 (δ 7.42). In the NOESY spectrum, H-8 (δ 7.42) showed correlation with a signal for H-9 (δ 8.21), and H-6 (δ 7.31) showed correlation with signal for H-5 (δ 8.69). These spectral findings confirmed that the hydroxyl group was substituted at C-7. Based on the above evidence, compound **4** was determined as a new constituent, and named Aristolochic Acid F.

Compound **5** was obtained as a yellowish-brown amorphous powder. The high resolution MS analysis of quasimolecular ion peaks in the positive-ion FAB-MS of **5** showed a molecular ion peak at m/z 344.2462, indicating a molecular formula of $C_{16}H_9NO_8$. The UV absorption of **5** at 216, 258, 286, 364, and 373 nm showed it was a phenanthro type derivative.[11,15] Its IR spectrum showed absorption bands due to hydroxyl, nitro, and carboxyl functions at 3480, 1312, 1510, and 1718 cm^{-1}, indicating that **5** was also an aristolochic acid derivative. This was also confirmed by fragment ion peaks at m/z 344 $[M+H]^+$, m/z 298 $[M+H-NO_2]^+$, m/z 281 $[M+H-NO_2-OH]^+$, and m/z 253 $[M+H-NO_2-COOH]^+$, separately. Comparison of the ^{1}H-NMR spectral data (Table 1) and ^{13}C-NMR spectral data (Table 2) of **5** with those of **4** revealed they had many similarities. Both **5** and **4** had 9 proton signals and 16 carbon signals, and most of their chemical shifts were very similar. The main differences were focused on the numbers and substitution positions of hydroxyl groups in the aromatic ring. The ^{1}H-NMR spectrum of **5** showed three downfield exchangeable proton signals at δ 11.50 (1H, s), 11.36 (1H, s), and 10.82 (1H, s), which were attributed to hydroxyl and carboxyl groups, respectively. In the aromatic region, two mutual coupled signals at δ 8.13 (1H, d, J=2.0 Hz) and 6.72 (1H, d, J=2.0 Hz) were attributed to H-5 and H-7, respectively,[15] and two hydroxyl groups were believed to be connected at C-6 and C-8. The remaining 2 one-proton signals at δ 7.76 (1H, s) and 8.70 (1H, s) could be ascribed to H-2 and H-9, respectively, due to the anisotropic effect of the carbonyl group at C-1. One singlet signal at δ 6.50 (2H, s) was attributed to methylenedioxyl protons. In the HMBC spectrum (Fig. 3), H-7 (δ 6.72) showed ^2J or ^3J correlations with signals for C-8 (δ 152.4), C-6 (δ 150.8), C-8a (δ 120.3), and C-5 (δ 115.6). These spectral findings confirmed that two hydroxyl groups were substituted at C-6 and C-8. Based on the above evidence, compound **5** was determined as a new constituent, and named Aristolochic Acid G.

From the results of cytotoxic activity testing (Table 3), compound **1**, as predicted from previous data,[16] showed intensive cytotoxic activity against LLC-PK$_1$ cells with an

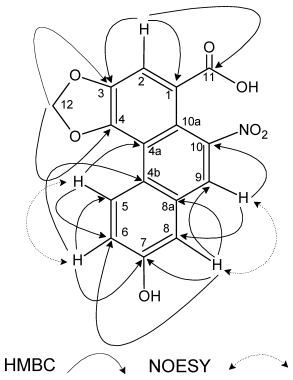

Fig. 2. Structure, Key HMBC and NOESY Correlations of Compound **4**

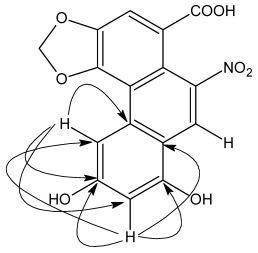

Fig. 3. Structure, Key HMBC Correlations of Compound **5**

Table 3. Cytotoxic Potential of Compounds **1**—**5** against LLC-PK$_1$ Cells

Compounds	IC_{50} (μmol/l)[a]
1 (Aristolochic acid A)	11
2 (Aristolochic acid B)	78
3 (Aristolochic acid C)	85
4 (Aristolochic Acid F)	82
5 (Aristolochic Acid G)	93
Doxorubicin (positive control)	1.8

a) IC_{50} (μmol/l) was the content of compound that inhibited cell growth to 50%.

IC_{50} value of 11 μmol/l. Compound **2**—**5** exhibited moderate cytotoxic activity against LLC-PK_1 cells with IC_{50} values of 78, 85, 82 and 93 μmol/l, respectively. Aristolochic acid A is more toxic because of the presence of a methoxy ($-OCH_3$) group at the R_1 position. The addition of one hydroxyl group will decrease the toxic nature greatly, and the results are consistent with the published data.[16)]

Experimental

General IR studies were conducted with KBr disks in a Shimadzu FTIR-8100 Spectrometer. FAB-MS and high resolution mass spectrometry (HR-MS) analysis employed a JOEL JMS-SX 102A mass spectrometer. NMR studies employed a Varian INOVA-500 Spectrometer operating at 500 MHz for ^{1}H- and 125 MHz for ^{13}C-NMR including heteronuclear multiple-bond correlation (HMBC), and homonuclear Overhauser enhancement spectroscopy (NOESY). The chemical shifts were given in δ relative to tetramethylsilane (TMS) as internal standard. Silica gel (300—400 mesh, Qingdao Marine Chemical Factory, China) and Sephadex LH-20 (Pharmacia) were was used for column chromatography, and silica gel GF_{254} plates (Yantai Marine Chemical Co., Ltd. China) were used for thin-layer chromatography. For preparative HPLC (pump, Beckman 126P; detector, 125P; Beckman Separation Products, U.S.A.) separation, an ODS column [Phenomenex LUNA 10 μ, C_{18} (250×21.2 mm I.D.), U.S.A.] column was used. For analytical HPLC (Waters Alliance 2690, 996 detector at wavelength 254 nm; Waters Separation Products, U.S.A.) separation, an ODS column [YWG C_{18} 150 mm L×4.6 mm I.D. 10 μ, U.S.A.] column was used. LLC-PK_1 cells were obtained from Beijing NC Inc. (Beijing, P. R. China), and dimethyl sulfoxide (DMSO) was obtained from Beijing Chemical Co. (Beijing, P. R. China).

Plant Material The roots of *Aristolochia fangchi* were collected at Zhaoqing, Guangdong Province, P. R. China, in October 2006, and were identified by Professor Zhang Ji. The voucher specimen (No. S2587-22) was deposited at the Herbal Museum of the National Institute for the Control of Pharmaceutical and Biological Products, State Food and Drug Administration (Beijing, P. R. China).

Extraction and Isolation The powdered air-dried roots (5 kg) were extracted three times with 95% EtOH under reflux for 2 h. After removal of the solvent *in vacuo*, the extract (0.6 kg) was further partitioned between H_2O and $CHCl_3$ to give H_2O-soluble and $CHCl_3$-soluble fractions. The $CHCl_3$-soluble (0.2 kg) fraction was subjected to column chromatography on silica gel and eluted with $CHCl_3$/MeOH (30 : 0, 28 : 1, 15 : 1, 10 : 1, 8 : 1, 4 : 1) to yield 6 subfractions. The $CHCl_3$/MeOH (15 : 1) subfraction was purified on a Sephadex LH-20 column with $CHCl_3$/MeOH (1 : 2) as eluent, then followed by preparative HPLC (70% MeOH) to yield compound **1** (67 mg). The $CHCl_3$/MeOH (10 : 1) subfraction was purified by preparative HPLC (68% MeOH) to yield compound **3** (19 mg). The $CHCl_3$/MeOH (8 : 1) subfraction was purified by preparative HPLC [MeOH–0.5% HOAc aq. (65 : 35, v/v), flow rate, 25 ml/min] to yield **2** (36 mg), **4** (13 mg), and **5** (15 mg). The purity of the compounds was analyzed by HPLC [MeCN–0.5% HOAc aq. (65 : 35, v/v); flow rate, 1.0 ml/min; column temperature, 25 °C].

Assay of Cytotoxic Activities against LLC-PK_1 Cells The isolated compounds **1**—**5** were evaluated for their cytotoxic activity against LLC-PK_1 cells using a method described in the literature.[16)] Doxorubicin was used as the positive control and hederasaponin C was used as the negative control (Table 3). An IC_{50} value over 300 μmol/l was considered to indicate no cytotoxic activity against LLC-PK_1 cells.

Aristolochic Acid A (**1**): Yellow amorphous powder, FAB-MS *m/z*: 341, 296; HR-FAB-MS (positive mode) *m/z*: 342.2740 $[M+H]^+$ (Calcd for $C_{17}H_{12}NO_7$: 342.2723); UV λ_{max} (MeOH) nm: 221, 253, 317, 390; IR (KBr) ν_{max} cm^{-1}: 1695, 1515, 1340, 1266, 1036. ^{1}H-NMR (DMSO-d_6, 500 MHz) and ^{13}C-NMR (DMSO-d_6, 125 MHz): see Tables 1 and 2.

Aristolochic Acid B (**2**): Yellow amorphous powder, FAB-MS *m/z*: 311, 294; HR-FAB-MS (positive mode) *m/z*: 312.2473 $[M+H]^+$ (Calcd for $C_{16}H_{10}NO_6$: 312.2465); UV λ_{max} (MeOH) nm: 220, 251, 297, 353; IR (KBr) ν_{max} cm^{-1}: 1718, 1515, 1548. ^{1}H-NMR (DMSO-d_6, 500 MHz) and ^{13}C-NMR (DMSO-d_6, 125 MHz): see Tables 1 and 2.

Aristolochic Acid C (**3**): Yellow amorphous powder, FAB-MS *m/z*: 327, 312, 281; HR-FAB-MS (positive mode) *m/z*: 328.2466 $[M+H]^+$ (Calcd for $C_{16}H_{10}NO_7$: 328.2458); UV λ_{max} (MeOH) nm: 226, 256, 317, 358; IR (KBr) ν_{max} cm^{-1}: 3400, 1681, 1508, 1328. ^{1}H-NMR (DMSO-d_6, 500 MHz) and ^{13}C-NMR (DMSO-d_6, 125 MHz): see Tables 1 and 2.

Aristolochic Acid F (**4**): Yellow amorphous powder, FAB-MS *m/z*: 328, 282, 265, 237; HR-FAB-MS (positive mode) *m/z*: 328.2459 $[M+H]^+$ (Calcd for $C_{16}H_{10}NO_7$: 328.2453); UV λ_{max} (MeOH) nm: 218, 256, 295, 351, 371; IR (KBr) ν_{max} cm^{-1}: 3438, 1715, 1520, 1337. ^{1}H-NMR (DMSO-d_6, 500 MHz) and ^{13}C-NMR (DMSO-d_6, 125 MHz): see Tables 1 and 2.

Aristolochic Acid G (**5**): Yellowish-brown amorphous powder, FAB-MS *m/z*: 344, 298, 281, 253; HR-FAB-MS (positive mode) *m/z*: 344.2462 $[M+H]^+$ (Calcd for $C_{16}H_{10}NO_8$: 344.2455); UV λ_{max} (MeOH) nm: 216, 258, 286, 364, 373; IR (KBr) ν_{max} cm^{-1}: 3480, 1718, 1510, 1312. ^{1}H-NMR (DMSO-d_6, 500 MHz) and ^{13}C-NMR (DMSO-d_6, 125 MHz): see Tables 1 and 2.

References

1) State Administration of Traditional Chinese Medicine, *Chinese Materia Medica*, **2007**, 2069—2070 (2007).
2) Liang Q., Ni C., Xie M., Zhang Q., Zhang Y. X., Yan X. Z., Yang M. J., Peng S. Q., Zhang Y. Z., *Zhong Xi Yi Jie He Xue Bao*, **7**, 746—752 (2009).
3) Nortier J. L., Martinez M. C., Schmeiser H. H., Arlt V. M., Bieler C. A., Petein M., Depierreux M. F., Pauw L., Abramowicz D., Vereerstraeten P., Vanherweghem J. L., *N. Engl. J. Med.*, **342**, 1686—1692 (2000).
4) Heinrich M., Chan J., Wanke S., Neinhuis C., Simmonds M. S., *J. Ethnopharmacol.*, **125**, 108—144 (2009).
5) Vanherweghem L. J., *J. Altern. Complement. Med.*, **4**, 9—13 (1998).
6) Debelle F. D., Vanherweghem J. L., Nortier J. L., *Kidney Int.*, **74**, 158—169 (2008).
7) Schwetz B. A., *JAMA*, **285**, 2705 (2001).
8) Xu L. Z., *Zhong Yao Tong Bao*, **9**, 14—15 (1984).
9) Chen H., *Zhongguo Zhong Yao Za Zhi*, **15**, 707—708 (1990).
10) Chen H. A., Xu G. J., Jing R. L., Xu L. S., *Zhongguo Zhong Yao Za Zhi*, **19**, 323—324 (1994).
11) Che C. T., Ahmed M. S., Kang S. S., Waller D. P., Bingel A. S., Martin A., Rajamahendran P., Bunyapraphatsara N., Lankin D. C., Cordell G. A., *J. Nat. Prod.*, **47**, 331—341 (1984).
12) Wu T. S., Chan Y. Y., Leu Y. L., *Chem. Pharm. Bull.*, **48**, 357—361 (2000).
13) Wu T. S., Chan Y. Y., Leu Y. L., *Chem. Pharm. Bull.*, **48**, 1006—1009 (2000).
14) Wu T. S., Leu Y. L., Chan Y. Y., *Biol. Pharm. Bull.*, **23**, 1216—1219 (2000).
15) Zhang C. Y., Wang X., Su T., Ma C. M., Wen Y. J., Shang M. Y., Li X. M., Liu G. X., Cai S. Q., *Pharmazie*, **60**, 785—788 (2005).
16) Balachandran P., Wei F., Lin R. C., Khan I. A., Pasco D. S., *Kidney Int.*, **67**, 1797—1805 (2005).