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New aristolochic acid, aristololactam and renal cytotoxic constituents from the stem and leaves of *Aristolochia contorta*

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Two novel phenanthrene derivatives, aristololactam IVa (**1**) and 9-hydroxy aristolochic acid I (**2**) were isolated from the stem and leaves of *Aristolochia contorta* Bunge, together with 17 known compounds (**3**–**19**). The structures of these compounds were determined by spectroscopic analysis. The phenanthrenes obtained were tested for cytotoxicity against renal proximal tubular epithelial cell line (HK-2). Aristololactam IVa and 7-methoxy aristololactam IV were found to have strong cytotoxic activity against HK-2 cells with a potency similar to or even stronger than those of aristolochic acid I and aristololactam I.

1. Introduction

Much attention has been paid to the nephrotoxic and carcinogenic effects of aristolochic acid I (AA-I) (Mengs 1988; Mengs and Stotzem 1993) and the corresponding plants for years. Recently, our research group found and proved that aristololactam I (AL-I) was also a renal cytotoxic compound (Li et al. 2004). However, the cytotocities of other aristolochic acids (AAs) and aristololactams (ALs) have not been reported. Up to now, AAs have been found in the family Aristolochiaceae, while ALs have been isolated or detected in Aristolochiaceae, Annonaceae, Menispermaceae, Piperaceae, Monimiaceae and Saururceae (Carl et al. 1993; Choudhury et al. 2001; Mix et al. 1982; Michinori et al. 1974; Rao and Reddy 1990). As some plants of these families are used as herbal medicines, vegetables or spices, it is important to investigate whether other AAs and ALs have renal toxicity.

Aristolochia contorta Bunge (Aristolochiaceae) is a perennial herbaceous vine, and is distributed in China, Korea, Japan and so on. The stems and leaves of *A. contorta* are commonly called Tianxianteng in Chinese (The Pharmacopoeia Commission of PRC 2000) and have been used in Traditional Chinese Medicine for the treatment of stomachache and arthritis for about one thousand years (Tang 1976). Although ristolochic acid IVa, magnoflorine and β-sitosterol were detected in the stem and leaves of *A. contorta* (Fang et al. 1990), there are no systematical chemical studies of the stems and leaves of *A. contorta*.

According to the results of our preliminary study performed by means of HPLC-DAD, it was found that in Tianxianteng there were more than ten constituents with UV spectra similar to those of AAs and ALs. So we decided to isolate and elucidate constituents from the drug Tianxianteng and to test the renal cytotoxicity of AAs and ALs obtained.

The present paper deals with the isolation and structural elucidation of two new compounds, aristololactam IVa (**1**) and 9-hydroxy aristolochic acid I (**2**) from the ethanol extract of Tianxianteng. In addition, ten AAs and ALs obtained from this plant were tested for the cytotoxicity against a renal proximal tubular epithelial cell line (HK-2).

2. Investigations, results and discussion

The ethanol extract of Tianxianteng was subjected to partition and column chromatography to afford compounds **1**–**16** from the ethyl acetate-soluble part, and compounds **17**–**19** from the *n*-butanol-soluble part.

Aristololactam IVa (**1**), yellow powder, exhibited an $[M + H]^+$ ion peak at m/z 310 in the positive-TOF-MS, indicating the molecular weight to be 309. The molecular formula of $C_{17}H_{11}NO_5$ was established by the negative HR-FAB-MS $[M-H]^-$ at m/z 308.0559. The UV absorption at 221.5, 240.0, 261.5, 339.0 and 411.5 nm was characteristic of a phenanthrene chromophore. The IR absorption bands at 3438 cm^{-1} and 1670 cm^{-1} indicated the presence of hydroxy and lactam carboxyl groups (Leu et al. 1998). In the ^1H NMR of **1**, a set of meta coupled aromatic protons appeared at δ 7.59 (1 H, d, J = 2.1 Hz), and 6.69 (1 H, d, J = 2.1 Hz), which were assigned to H-5 and H-7, respectively. A singlet signal at δ 7.61 (1 H, s) could be assigned to H-2 as it showed HMBC correlation with the carbonyl signal at δ 167.8. Similary, a methylenedioxy group at δ 6.46 (2 H, s) could be positioned to C-3 and C-4 according to HMBC analysis. Another singlet signal at δ 7.23 (1 H, s) could be assigned to H-9, which showed the typical higher chemical shift of aristololactam (Li et al. 1994). Signals of a hydroxy and a methoxy groups appeared at δ 9.91 (1 H, br) and δ 3.93 (3 H, s) respectively. In its NOESY spectrum, NOE were observed between H-7 (δ 6.69) or H-9 and the methoxy signal, but not between H-5 and the methoxy signal. Therefore, the methoxy group was located at C-8 and the hydroxy group was at C-6. Consequently, the structure of compound **1**

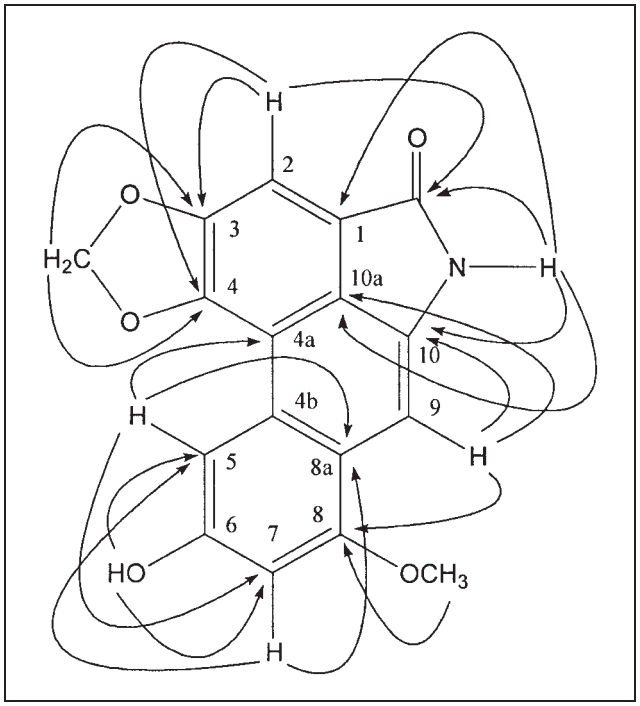


Fig. 1: Important HMBC correlations of **1**

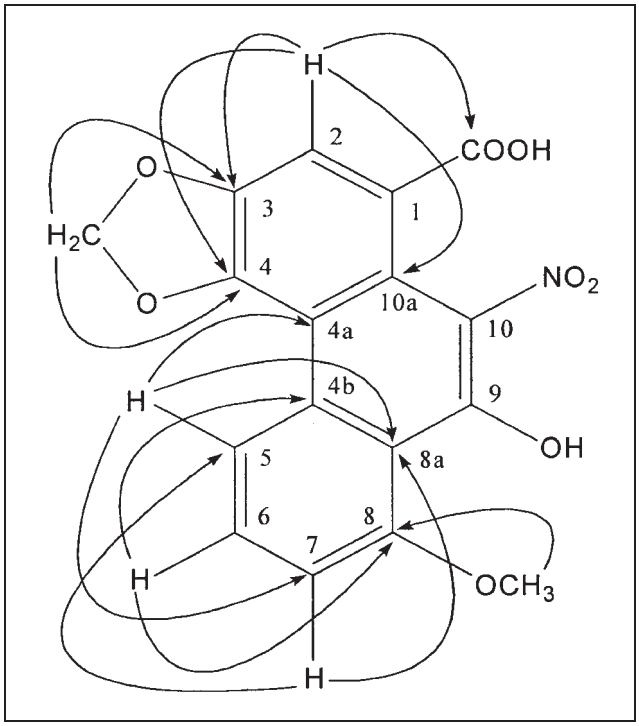


Fig. 2: Important HMBC correlations of **2**

was determined as shown in Fig. 1. According to the spectroscopic evidence from the HMQC and HMBC of compound **1**, all the carbon and proton signals were assigned as shown in the Experimental section.

9-Hydroxy aristolochic acid I (**2**), pale yellow powder, exhibited an $[M-H]^-$ ion peak at m/z 356 and an $[M-H-NO_2]^-$ ion peak at m/z 310 in the negative-TOF-MS, indicating the molecular weight to be 357 and nitro to exist. The molecular formula of $C_{17}H_{11}NO_8$ was established by the negative HR-FAB-MS $[M-H]^-$ at m/z 356.0414. The UV absorption at 220.0, 256.5, 274.0 (sh), 309.5, 387.5 nm and the IR absorption bands at 1701 cm^{-1} (C=O) and 1513, 1350 cm^{-1} (NO_2) suggested the existence of a typical phenanthrene skeleton (Chen and Zhu 1987). In the ^1H NMR spectrum of **2**, a broad singlet signal at δ 13.01 indicated the presence of COOH. In the aromatic region of the ^1H NMR spectrum, an ABC-type system at δ 8.76 (1 H, d, J = 8.4 Hz), 7.74 (1 H, t, J = 8.4 Hz) and 7.42 (1 H, d, J = 8.4 Hz) was attributed to H-5, H-6 and H-7, respectively. One proton signal at δ 7.51 (1 H, s) was assigned to H-2 because a HMBC correlation was observed between this proton signal and the carbonyl signal at δ 171.4. Clear correlations were also observed between H-2 and two carbon signals at δ 145.8 (C-4) and δ 144.3 (C-3). A methylenedioxy group, observed as one singlet at δ 6.42 (2 H, s) could be positioned at C-3 and C-4, as HMBC showed the corresponding correlations. In a NOESY experiment, NOE were observed between the methoxy proton at δ 4.16 (3 H, s) and H-7 (δ 7.42), between H-5 (δ 8.76) and H-6 (δ 7.74) as well as between H-6 and H-7. According to the above evidences and considering the molecular formula of compound **2**, it is proved that a hydroxy group should be located at C-9. Consequently, the structure of **2** was determined as 9-hydroxy aristolochic acid I. According to the spectroscopic data from the HMQC and HMBC of compound **2**, all the carbon and proton signals were assigned as shown in experimental. Fig. 2 showed the HMBC correlation of **2**.

The 17 known compounds, aristololide (**3**), aristolochic acid Va (**4**), aristololactam II (**5**), 7-methoxy aristololactam IV (**6**), 9-hydroxy aristololactam I (**7**), syringic acid (**8**), vanillic acid (**9**), esculetin (**10**), p-coumaric acid (**11**), daucosterol (**12**), aristolochic acid I (**13**), aristolochic acid II (**14**), aristolochic acid IVa (**15**), aristololactam I (**16**), adenosine (**17**), adenine (**18**) and glucose (**19**) (Basudeb et al. 1983; Feng et al. 2001; Hans et al. 1992; Mix et al. 1982; Sholi et al. 1990; Yue et al. 1994; Zhang and Bao 2000; Zhang et al. 2002; Zhao et al. 1991) were characterized by comparing their spectroscopic data with literature values. All these compounds were isolated from the stem and leaves of *A. contorta* for the first time.

As summarized in Fig. 3, all aristolochic acids and aristololactams were evaluated for their cytotoxicity against HK-2 cells. Compound **6** exhibited a strong cytotoxic effect, whereas compounds **1**, **13** and **16** showed moderate cytotoxicity. Consequently, it is deduced from their struc-

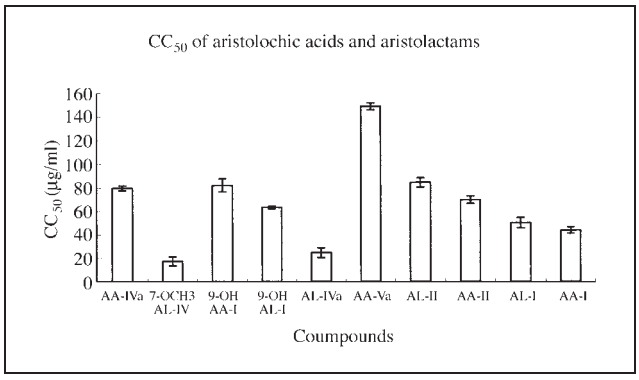
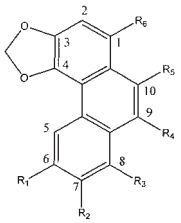


Fig. 3: Cytotoxic activity against renal cells (HK-2) of compounds

Results are expressed as CC_{50} values (μg/ml); CC_{50} is the 50 percent cytotoxic concentration. AA-I: aristolochic acid I; AA-II: aristolochic acid II; AA-IVa: aristolochic acid IVa; AA-Va: aristolochic acid Va; 9-OH AA-I: 9-hydroxy aristolochic acid I; AL-I: aristololactam I: AL-II: aristololactam II; AL-IVa: aristololactam IVa; 7-OCH_3 AL-IV: 7-methoxy aristololactam IV; 9-OH AL-I: 9-hydroxy aristololactam I

Table: Structures of aristolochic acids and aristololactams



Compounds	R_1	R_2	R_3	R_4	R_5	R_6
1 (AL-IVa)	OH	H	OCH_3	H	NH−CO	
2 (9-OH AA-I)	H	H	OCH_3	OH	NO_2	COOH
4 (AA-Va)	OH	OCH_3	H	H	NO_2	COOH
5 (AL-II)	H	H	H	H	NH−CO	
6 (7-OCH_3 AL-IV)	OCH_3	OCH_3	OCH_3	H	NH−CO	
7 (9-OH AL-I)	H	H	OCH_3	OH	NH−CO	
13 (AA-I)	H	H	OCH_3	H	NO_2	COOH
14 (AA-II)	H	H	H	H	NO_2	COOH
15 (AA-IVa)	OH	H	OCH_3	H	NO_2	COOH
16 (AL-I)	H	H	OCH_3	H	NH−CO	

tures that a hydroxy group at C-9 might decrease the renal cytotoxicity, while a methoxy group at C-8 might contribute to the cytotoxicity of aristolochic acids and aristololactams.

As aristolochic acid I, aristololactam I, aristololactam IVa and 7-methoxy aristololactam IV obtained from the stems and leaves of *A. contorta* showed potential renal cytotoxicity, the use of the drug Tianxianteng should be cautious.

According to the cytotoxic test, the aristololactams also exhibited potential cytotoxicity against HK-2 cells similar to or even stronger than aristolochic acid I which was only found in the plants of Aristolochiaceae in the vegetable kingdom. Whereas there are well authenticated reports of the occurrence of aristololactams in some plants of families Annonaceae, Menispermaceae, Piperaceae, Monimiaceae and Saururceae (Carl et al. 1993; Choudhury et al. 2001; Mix et al. 1982; Michinori et al. 1974; Rao and Reddy 1990), we should say that it is very important to evaluate the renal toxicity of the plants of these families to avoid health risks when they are used as medicines or food.

3. Experimental

3.1. General procedures

Melting points were determined on an XT-4_A micromelting point apparatus without correction. The IR spectra were recorded on a Thermo Nicolet Nexus 470 FT-IR spectrometer. UV spectra were measured on a Varian Cary Eclipse 300 spectrometer using MeOH as the solvent. Both ^1H and ^{13}C NMR experiments were performed on JEOL JNM-AL 300 NMR spectrometer and a Bruker DRX 500 NMR spectrometer using solvents as internal standards. The HR-FAB-MS spectra were detected with a Bruker APEX II mass spectrometer. Column chromatography was carried out with silica gel (200–300 mesh) provided by Tsingtao Marine Chemistry Co. Ltd., Sephadex LH-20 (18–110 μm) manufactured by Pharmacia Co. Ltd., ODS (100–200 mesh) made by Fuji Silysia Chemical Co. Ltd., and R-A macroporous adsorptive resin (0.23–0.9 mm) produced by Peking Chemical No. 7 factory. HK-2 cells were purchased from the American Type Culture Collection (Rockville, MD). DMEM were obtained from GIBICO Co. Ltd.

3.2. Plant material

The plant material of Tianxianteng was purchased in March, 2002, from Zhengzhou Chinese Crude Drugs Co. Ltd., in Henan province of China and authenticated as the stems and leaves of *Aristolochia contorta* Bunge by Prof. Shao-Qing Cai. The voucher sample. (No. 4001) was deposited in the Herbarium of Pharmacognosy, School of Pharmaceutical Sciences, Peking University.

3.3. Extraction and isolation

The dried stems and leaves of *A. contorta* (17 kg) were powdered and then extracted with 95% and 50% ethanol under infiltration in room temperature to give a dark brown syrup (1.2 kg), which was suspended in water and partitioned successively with cyclohexane, petroleum ether, ethyl acetate (EtOAc) and *n*-butanol. The EtOAc-soluble part (245 g) was subjected to R-A macroporous adsorptive resin column chromatography using 70% EtOH as the eluant. The ethanol eluate was concentrated *in vacuo* to obtain an extract (146 g), and then the extract was subjected to silica gel column chromatography using $CHCl_3$−MeOH (100 : 0–9 : 1) as the eluant to afford six fractions (Fr. A–Fr. F). Fr. C (15 g) was further chromatographed on silica gel column eluted with gradient $CHCl_3$−MeOH (100 : 0–4 : 1) to give four sub-fractions. Sub-fraction 2 (1.1 g) of Fr. C was chromatographed using $CHCl_3$−MeOH (50 : 1–20 : 1) as eluant on silica gel column repeatedly. The obtained fraction was purified with ODS column chromatography using 50% MeOH as eluant to yield **1** (20 mg). Fr. E (17 g) was divided into several sub-fractions using gradient $CHCl_3$−MeOH (50 : 1–9 : 1) as eluant to get the crude **2**. The crude **2** was purified by Sephadex LH-20 (with 50% MeOH as eluant), then by ODS (with 40% MeOH as eluant) to yield **2** (9 mg).

Compounds **3** (3 mg), **4** (20 mg), **5** (7 mg), **6** (9 mg), **7** (20 mg), **8** (30 mg), **9** (3 mg), **10** (7 mg), **11** (25 mg), **12** (50 mg), **13** (6 mg), **14** (10 mg), **15** (500 mg), **16** (10 mg) were obtained from the other fractions of the EtOAc-soluble part, and compounds **17** (20 mg), **18** (15 mg), **19** (15 mg) were obtained from the *n*-butanol-soluble part (160 g).

3.3.1. Aristololactam IVa (**1**)

Yellow powder, m.p. 277–279 °C, UV λ_{max} (MeOH) nm: 221.5, 240.0, 261.5, 288.8, 305.4 (sh), 339.0, 411.5; IR ν_{max} (KBr) cm^{-1}: 3438, 1670 (C=O), 1362, 1272, 1189, 1048, 966, 673; TOF-MS (pos.) m/z: 310 $[M+H]^+$; HR-FAB-MS (neg.) m/z: 308.0572 $[M-H]^-$ (calcd. for $C_{17}H_{11}NO_5$: 308.0559). ^1H NMR (300 MHz, DMSO-d_6) δ: 10.61 (s, NH), 9.90 (br, OH), 7.61 (1 H, s, H-2), 7.59 (1 H, d, J = 2.1 Hz, H-5), 7.23 (1 H, s, H-9), 6.69 (1 H, d, J = 2.1 Hz, H-7), 6.46 (2 H, s, $-OCH_2O-$), 3.93 (3 H, s, $-OCH_3$); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 167.8 (C=O), 156.5 (C-8), 156.0 (C-6), 148.4 (C-3), 147.2 (C-4), 134.7 (C-10), 126.0 (C-4b), 125.4 (C-10a), 119.3 (C-1), 117.0 (C-8a), 110.8 (C-4a), 105.6 (C-2), 103.5 (C-5), 103.1 ($-OCH_2O-$), 99.1 (C-7), 98.6 (C-9), 55.8 ($-OCH_3$).

3.3.2. 9-Hydroxy aristolochic acid I (**2**)

Yellow powder, m.p. >300 °C, UV λ_{max} (MeOH) nm: 220.0, 256.5, 274.0 (sh), 309.5, 387.5; IR ν_{max} (KBr) cm^{-1}: 3446, 1701 (C=O), 1636, 1569, 1513 (NO_2), 1473, 1415, 1395, 1350, 956; TOF-MS (neg.) m/z: 356 $[M-H]^-$, 310 $[M-H-NO_2]^-$; HR-FAB-MS (neg.) m/z: 356.0414 $[M-H]^-$ (calcd. for $C_{17}H_{11}NO_8$: 356.0406). ^1H NMR (300 MHz, DMSO-d_6) δ: 13.01 (br, s, −COOH), 8.76 (1 H, d, J = 8.4 Hz, H-5), 7.74 (1 H, t, J = 8.4, 8.4 Hz, H-6), 7.51 (1 H, s, H-2), 7.42 (1 H, d, J = 8.4 Hz, H-7), 6.42 (2 H, s, $-OCH_2O-$), 4.16 (3 H, s, $-OCH_3$), ^{13}C NMR (75 MHz, DMSO-d_6) δ: 171.4 (C=O), 161.4 (C-9), 153.9 (C-8), 145.8 (C-4), 144.3 (C-3), 128.6 (C-6), 128.2 (C-10), 128.0 (C-4b), 126.8 (C-1), 119.6 (C-5), 118.7 (C-10a), 117.1 (C-8a), 115.3 (C-4a), 109.2 (C-7), 108.6 (C-2), 102.3 ($-OCH_2O-$), 56.3 ($-OCH_3$).

3.4. Assay for cytotoxic activity

HK-2 cells used in this study were cultured human renal proximal tubular epithelial cell line (HK-2), which was an immortalized cell line from normal human kidney proximal tubules. The colorimetric method (Tang et al. 2002) was employed in cytotoxic assay.

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