Constituents from the Stems of *Aristolochia manshuriensis*

Pei-Lin Wu,* Gwo-Ching Su, and Tian-Shung Wu*

Department of Chemistry, National Cheng Kung University, Tainan, 701, Taiwan, Republic of China

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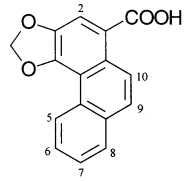
In a continuing search for bioactive compounds from *Aristolochia* species, 28 compounds, including three new constituents, demethylaristofolin E (**1**), aristomanoside (**2**), and dehydrooxoperezinone (**3**), were isolated from an extract of the stems of *Aristolochia manshuriensis*. The structures of these compounds were established by extensive 1D and 2D NMR spectral studies. Among these compounds, dehydrooxoperezinone (**3**) was found to inhibit the replication of HIV, with an EC_{50} value of 17.5 μg/mL and a therapeutic index of 1.43.

Aristolochia (Aristolochiaceae) is a large tropical and subtropical genus estimated to contain 400 species that is used extensively in traditional Chinese folk medicine.[1−3] The aristolochic acids have been proved to be the most potent constituents of *Aristolochia* species[4] and are known to be nephrotoxins and carcinogens.[5] However, additional research is needed to understand the toxicity of these compounds. As a part of our continuing search for bioactive compounds from *Aristolochia* species,[6] the stems of *A. manshuriensis* Komarovis were investigated. It is widely distributed in the middle part of mainland China and in Korea. Stems of this plant, "Kwan-Mu-Tong" (in Chinese), are used as a cardiotonic drug in Korean and Chinese traditional medicines.[7] Previous reports on this plant haved described the presence of aristolochic acids, aristolactams, and sesquiterpenes.[7−11] The present study has resulted in the isolation of 28 compounds including demethylaristofolin E (**1**), aristomanoside (**2**), and dehydrooxoperezinone (**3**) from the stems of *A. manshuriensis*. This paper deals with the isolation and structural elucidation of **1**−**3** and the biological evaluation in anti-HIV and cytotoxicity assays by selected *A. manshuriensis* constituents.



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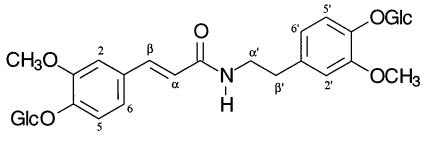

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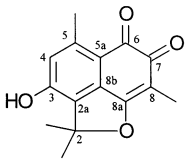

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The methanol extract of the stems of *A. manshuriensis* was suspended in water and partitioned with chloroform. Each layer was repeatedly separated by conventional chromatographic techniques to give three new compounds, demethylaristofolin E (**1**), aristomanoside (**2**), and dehydrooxoperezinone (**3**), together with 25 known compounds, which included one denitroaristolochic acid, aristofolin E;[12] seven aristolochic acids, aristolochic acid-I,[13] aristolochic acid-II,[14] aristolochic acid-I methyl ester,[13] aristolochic acid-II methyl ester,[13] aristoloside,[7] sodium aristolochate-I,[13] and aristolochic acid-IVa methyl ester;[15] four aristolactams, aristolactam-I,[13] aristolactam-II,[16] 9-methoxyaristolactam-I,[17] and aristolactam AII;[13] one aristolactone, aristolide-B;[18] one aporphine, demethylsonodione;[19] three amides, *N-trans*-feruloylmethoxytyramine,[20] *N-p*-coumaroyltyramine,[21] and *N-trans*-feruloytyramine;[21] five benzenoids, vanillic acid,[22] *p*-hydroxybenzoic acid,[23] *p*-coumaric acid,[13] methyl vanillate,[23] and ferulic acid;[13] one lipid, glyceryl-α-linocerate;[24] and three steroids, β-sitosteryl-β-D-glucoside and a mixture of β-sitosterol and stigmasterol. The known compounds were identified by comparing their physical and spectral data with those listed in the literature.

Demethylaristofolin E (**1**) was isolated as a crystalline yellow solid. The molecular ion peak at *m*/*z* 266.0579 in the HREIMS was consistent with the molecular formula $C_{16}H_{10}O_4$. The latter was corroborated by the ^{13}C NMR and DEPT spectra, which showed 16 carbon resonances consisting of one methylene, seven aromatic methines, and eight quaternary carbons. The UV absorptions at 221, 240, 260, 284, 323, 353, and 370 nm together with the IR absorption bands at 3000 (COOH) and 1670 (C=O) cm^{-1} as well as the lack of a typical NO_2 band around 1550 and 1350 cm^{-1} suggested that compound **1** is a denitroaristolochic acid derivative.[25] In the ^1H NMR spectrum, one set of four mutually coupled proton signals at δ 7.68 (2H, m), 7.97 (1H, m), and 9.05 (1H, m) corresponded to H-6 and -7, H-8, and H-5, respectively, indicating that the C ring was unsubstituted. An AB set of doublets at δ 7.79 and 8.80 with a coupling constant of 9.6 Hz was assigned to H-9 and H-10, respectively, which was supported by the presence of a NOE interaction between H-8 (δ 7.97) and H-9 (δ 7.79). A typical aromatic singlet at δ 7.88 which showed no NOE interactions with H-5 and H-10 was attributed to H-2. Hence, a singlet at δ 6.44 assignable to a methylenedioxy group fused to the C-3 and C-4 positions of ring A could be proposed, as this proton showed long-range $^1H-^{13}C$ correlations with C-3 (δ 144.6) and C-4 (δ 146.7) in the HMBC spectrum. A broad and D_2O-exchangeable singlet at δ 13.13 and a carbonyl carbon signal at δ 168.4 further suggested the presence of a carboxylic group. The attachment of the carboxylic acid group to C-1 was inferred by the HMBC correlations between H-2 (δ 7.88) and the carbonyl carbon. Therefore, the structure of demethylaristofolin E (**1**) was deduced as 3,4-methylenedioxyphenanthrene-1-carboxylic acid.

* To whom correspondence should be addressed. Fax: 886-6-2740552. E-mail: wupl@mail.ncku.edu.tw; tswu@mail.ncku.edu.tw.

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Aristomanoside (**2**), obtained as a yellow amorphous powder, was determined to have the molecular formula $C_{31}H_{41}NO_5$ from its protonated molecular ion at *m/z* 668.2552 $[M + H]^+$ by HRFABMS. It showed UV absorption maxima at 219, 228, 284, and 313 nm, consistent with a cinnamamide chromophore. In turn, the IR spectrum displayed absorption bands for an amido NH at 3200 cm^{-1} and a carbonyl group at 1651 cm^{-1}. In the ^1H NMR spectrum, two ethylene triplets at δ 2.69 and 3.41 (each 2H, $J = 7.6$ Hz), two downfield-shifted olefinic doublets at δ 6.50 and 7.33 with large coupling constants ($J = 15.2$ Hz), and a broad NH singlet at δ 8.02 indicated the presence of a *trans*-CH═CH−CONH−CH_2−CH_2− partial structure.[26] Two sets of ABX aromatic proton signals at δ 7.04 (dd, $J = 8.4$, 1.9 Hz, H-6), 7.07 (d, $J = 8.4$ Hz, H-5), and 7.15 (d, $J = 1.9$ Hz, H-2) and δ 6.68 (dd, $J = 8.4$, 2.0 Hz, H-6′), 6.82 (d, $J = 2.0$ Hz, H-2′), and 6.97 (d, $J = 8.4$ Hz, H-5′) were indicative of two 1,3,4-trisubstituted benzene moieties in **2**. The connectivity of these units was established on the basis of HMBC correlations such as H-β (δ 7.33) with C-2 (δ 110.9) and C-6 (δ 121.2), H-α (δ 6.50) with C-1 (δ 129.0) and C═O (δ 165.3), and H-2′ (δ 6.82) and H-6′ (δ 6.68) with C-β' (δ 34.9). Two methoxyls at δ 3.73 and 3.78 were placed at C-3′ and C-3, respectively, on the basis of their NOE interactions with H-2′ (δ 6.82) and H-2 (δ 7.15). The remaining ^1H and ^{13}C NMR signals, especially the anomeric proton signals at δ 4.82 (d, $J = 7.2$ Hz, H-1‴) and 4.93 (d, $J = 7.6$ Hz, H-1″) and the carbon signals at δ 60.8 (C-6″ and -6‴), 69.8 (C-4″ and -4‴), 73.3 (C-2″ and -2‴), 77.0−77.3 (C-3″, -3‴, -5″, and -5‴), 99.9 (C-1‴), and 100.4 (C-1″), revealed the existence of two glucose units in **2**. NOE interactions of the anomeric protons with H-5′ (δ 6.97) and H-5 (δ 7.07) confirmed the placement of glucosyl units on C-4 and C-4′. On the basis of the above analysis, aristomanoside (**2**) could be formulated as the 4,4′-diglucopyranoside of *N*-feruloyl-3-methoxytyramine.

Dehydrooxoperezinone (**3**) was isolated as orange needles and its molecular formula $C_{15}H_{14}O_4$ obtained from the HREIMS (*m/z* 258.0893), which indicated nine degrees of unsaturation. Its UV absorptions at 218, 265, 295, and 362 nm and IR bands at 3163, 1686, and 1599 cm^{-1} were characteristic of an *o*-naphthoquinone chromophore.[27] The ^{13}C NMR and DEPT spectra showed two carbonyl carbons, eight olefinic or aromatic carbons, four methyl carbons, and an aliphatic quaternary carbon. In the ^1H NMR spectrum, a methyl group attached to the aromatic ring deshielded by a *peri*-carbonyl of the quinone appeared at δ 2.52 (CH_3-5), an olefinic methyl on the quinone ring was seen as a singlet at δ 1.77 (CH_3-8), and two geminal methyl groups were observed at δ 1.69 (2 × CH_3-2). The ^1H NMR spectrum also displayed an aromatic proton singlet at δ 6.73 and a broad D_2O-exchangeable signal at δ 11.50 for H-4 and OH-3, respectively. In accord with the above data, the tricyclic structure, 3-hydroxy-2,2,5,8-tetramethyl-2*H*-naphtho[1,8-*bc*]furan-6,7-quinone, was proposed for **3**. The HMBC (5 Hz) correlations of the aromatic proton at δ 6.73 (H-4) with the carbons at δ 20.2 (CH_3-5), 95.9 (C-2), 116.4 (C-5), 130.5 (C-2a), and 157.0 (C-3); the dimethyl protons at δ 1.69 (CH_3-2) with the carbons at δ 95.9 (C-2) and 130.5 (C-2a); the olefinic methyl at δ 1.77 (CH_3-8) with the carbons at 180.0 (C-7), 107.4 (C-8), and 167.5 (C-8a); the aromatic methyl at δ 2.52 (CH_3-5) with the carbons at δ 116.4 (C-5), 120.1 (C-4), 146.1 (C-5a), and 177.1 (C-6); and the hydroxyl proton at δ 11.50 (OH-3) with the carbons at δ 120.1 (C-4), 130.5 (C-2a), and 157.0 (C-3) confirmed the connectivities of the complete structure of compound **3**. The highly upfield-shifted carbon signal at δ 8.0 for CH_3-8 and the signal at δ 107.4 for C-8 further supported the α-methyl in the oxygenated α,β-unsaturated carbonyl compound. Although compound **3** has been named as dehydrooxoperezinone and synthesized by Joseph-Nathan et al.,[27] this is the first time that it has been isolated from a natural source.

Compounds **1**−**3** as well as aristolochic acid-I, aristolochic acid-I methyl ester, aristoloside, sodium aristolochate-I, aristolactam-II, *N-trans*-feruloylmethoxytyramine, *N-p*-coumaroyltyramine, and *N-trans*-feruloytyramine were subjected to anti-HIV[22] and cytotoxicity[28] evaluations. Among them, only dehydrooxoperezinone (**3**) displayed moderately anti-HIV activity in acutely infected H-9 lymphocyte cells with IC_{50} and EC_{50} values of 25.1 and 17.5 μg/mL, respectively, and a therapeutic index (IC_{50}/EC_{50}, TI) of 1.43. However, no active compound was found when we subjected them to cytotoxicity testing against two human cancer cell lines (breast carcinoma (MCF-7) and lung carcinoma (A549)). In conclusion, since the aristolochic acids are known to have toxic properties, it is safe to use *Aristolochia* species as Chinese medicinal herbs only after removing any aristolochic acids that may be present.

Experimental Section

General Experimental Procedures. Melting points were recorded on a Yanagimoto MP-S3 melting point apparatus and are uncorrected. UV spectra were recorded on a Hitachi UV-3210 spectrophotometer. IR spectra were measured on a Shimazu FTIR-8501 spectrometer as solid dispersions on KBr. NMR spectra were recorded on a Bruker AC-200, Avance-300, AMX-400, or a Varian-Unity Plus 400 FT-NMR spectrometer; all chemical shifts are expressed in ppm with respect to tetramethylsilane as internal standard. Mass spectra were obtained on a VG 70-250S spectrometer by a direct inlet system.

Plant Material. The stems of *Aristolochia manshuriensis* were purchased from market (Kaiser Pharmaceutical Company) in Tainan, Taiwan, in June 1998. The plant identification was made by Professor C. S. Kuoh, Department of Biology, National Cheng Kung University. A voucher specimen (no. Wu980031) was deposited in the Herbarium of National Cheng Kung University, Tainan, Taiwan.

Extraction and Isolation. The stems of *A. manshuriensis* (10 kg) were powdered and extracted under reflux with MeOH 10 times. The combined extracts were concentrated under reduced pressure to give a dark brown syrup. The syrup was then suspended in H_2O and partitioned with $CHCl_3$. The concentrated $CHCl_3$ layer (540 g) was fractionated on a silica gel column and eluted with a gradient of hexane−EtOAc (from pure hexane to pure EtOAc) to yield eight fractions. Fraction 1 was subjected to chromatography on a silica gel column eluting with a gradient of hexane−EtOAc (19:1 to pure EtOAc) to give aristofolin E (25 mg, 0.00025%). Similarly, fraction 2 was chromatographed with a gradient solvent of hexane−EtOAc (19:1 to pure EtOAc) to give aristolochic acid-II methyl ester (2 mg, 0.00002%), aristolactam-I (64 mg, 0.00064%), aristolide-B (15 mg, 0.00015%), aristolochic acid-I methyl ester (0.34 g, 0.0034%), demethylaristofolin E (**1**) (83 mg, 0.00083%), and a mixture of β-sitosterol and stigmasterol (80 mg, 0.00080%), successively. Fraction 3 was further chromatographed on a column packed with silica gel and eluted with a gradient of $CHCl_3$−MeOH (49:1 to pure MeOH) to yield aristolactam-II (83 mg, 0.00083%), methyl vanillate (49 mg, 0.00049%), and glyceryl-α-linocerate (2 mg, 0.00002%). Fraction 5 was chromatographed on a silica gel column eluting with a gradient of $CHCl_3$−MeOH (49:1 to pure MeOH) to give dehydrooxoperezinone (**3**) (69 mg, 0.00069%), 9-methoxy-aristolactam-I (71 mg, 0.00071%), aristolactam AII (2 mg, 0.00002%), and aristolochic acid-IVa methyl ester (80 mg, 0.00080%). Fraction 6 was subjected to repeated column

chromatography on silica gel eluting with a gradient of $CHCl_3$−MeOH (19:1 to pure MeOH) followed by preparative TLC to yield aristolochic acid-II (4 mg, 0.00004%), *N-trans*-feruloylmethoxytyramine (55 mg, 0.00055%), *N-p*-coumaroyltyramine (43 mg, 0.00043%), *N-trans*-feruloytyramine (76 mg, 0.00076%), ferulic acid (70 mg, 0.00070%), *p*-hydroxybenzoic acid (1 mg, 0.00001%), and *β*-sitosteryl-*β*-D-glucoside (0.27 g, 0.0027%). Using the same procedure, fractions 7 and 8 gave demethylsonodione (3 mg, 0.00003%) and sodium aristolochate-I (3 mg, 0.00003%), aristolochic acid-I (3.87 g, 0.0387%), respectively.

The concentrated H_2O layer (530 g) was subjected to column chromatography on Diaion LH-20 by eluting with a gradient of H_2O−MeOH (from pure H_2O to pure MeOH) to give seven fractions. Purification of fraction 5 on a silica gel column with $CHCl_3$−MeOH−H_2O (5:1:0.1) as eluent gave aristomanoside (**2**) (61 mg, 0.00061%), *p*-coumaric acid (56 mg, 0.00056%) and vanillic acid (60 mg, 0.00060%). Further separation of fraction 7 on a silica gel column eluting with a gradient of $CHCl_3$−MeOH−H_2O (9:1:0.1 to pure MeOH) yielded aristoloside (94 mg, 0.00094%).

Demethylaristofolin E (1): yellow crystalline solid ($CHCl_3$/$(CH_3)_2CO$); mp 256−258 °C; UV (CH_3OH) λ_{max} (log ϵ) 221 (4.38), 240 (sh, 4.31), 260 (4.45), 284 (4.17), 323 (3.92), 353 (3.42), 370 (3.38) nm; IR (KBr) ν_{max} 3000 (br), 1670, 1591 cm^{-1}; ^1H NMR (DMSO-d_6) δ 6.44 (2H, s, OCH_2O), 7.68 (2H, m, H-6 and -7), 7.79 (1H, d, J = 9.6 Hz, H-9), 7.88 (1H, s, H-2), 7.97 (1H, m, H-8), 8.80 (1H, d, J = 9.6 Hz, H-10), 9.05 (1H, m, H-5), 13.13 (1H, br s, OH); ^{13}C NMR (DMSO-d_6) δ 102.5 (OCH_2O), 112.1 (C-2), 115.8 (C-4a), 122.3 (C-1), 124.1 (C-10), 126.8 (C-5), 127.0 (C-6 and -9), 127.7 (C-7 and -10a), 128.1 (C-4b and -8), 131.4 (C-8a), 144.6 (C-3), 146.7 (C-4), 168.4 (C=O); EIMS m/z 266 (M^+, 100), 249 (37), 221 (47), 193 (42), 163 (90), 151 (85); HREIMS m/z 266.0579 $[M]^+$ (calcd for $C_{16}H_{10}O_4$, 266.0579).

Aristomanoside (2): yellow amorphous powder ($CHCl_3$/CH_3OH); mp 195−197 °C; UV (CH_3OH) λ_{max} (log ϵ) 219 (4.30), 228 (4.30), 284 (4.29), 313 (4.24) nm; IR (KBr) ν_{max} 3200 (br), 1651, 1514 cm^{-1}; ^1H NMR (acetone-d_6) δ 2.69 (2H, t, J = 7.6 Hz, H-β'), 3.41 (2H, t, J = 7.6 Hz, H-α'), 3.1−3.7 (12H, m, 12 × CH of glucoside), 3.73 (3H, s, OCH_3-3′), 3.78 (3H, s, OCH_3-3), 4.49 (2H, m, 2 × OH), 4.82 (1H, d, J = 7.2 Hz, H-1‴), 4.93 (1H, d, J = 7.6 Hz, H-1″), 4.95 (1H, d, J = 4.8 Hz, OH), 4.98 (1H, d, J = 4.8 Hz, OH), 5.01 (1H, d, J = 4.8 Hz, OH), 5.05 (1H, d, J = 4.8 Hz, OH), 5.14 (1H, d, J = 4.8 Hz, OH), 5.22 (1H, d, J = 4.8 Hz, OH), 6.50 (1H, d, J = 15.2 Hz, H-α), 6.68 (1H, dd, J = 8.4, 2.0 Hz, H-6′), 6.82 (1H, d, J = 2.0 Hz, H-2′), 6.97 (1H, d, J = 8.4 Hz, H-5′), 7.04 (1H, dd, J = 8.4, 1.9 Hz, H-6), 7.07 (1H, d, J = 8.4 Hz, H-5), 7.15 (1H, d, J = 1.9 Hz, H-2), 7.33 (1H, d, J = 15.2 Hz, H-β), 8.02 (1H, br s, NH); ^{13}C NMR (acetone-d_6) δ 34.9 (C-β'), 39.3 (C-α′), 55.8 (OCH_3-4 and -4′), 60.8 (C-6″ and -6‴), 69.8 (C-4″ and -4‴), 73.3 (C-2″ and -2‴), 77.0−77.3 (C-3″, -3‴, -5″, and -5‴), 99.9 (C-1‴), 100.4 (C-1″), 110.9 (C-2), 113.3 (C2′), 115.3 (C5), 115.5 (C-5′), 120.6 (C-6′ and -α), 121.2 (C-6), 129.0 (C-1), 133.3 (C-1′), 138.6 (C-β), 145.2 (C-4′), 147.8 (C-4), 148.9 (C-3′), 149.2 (C-3), 165.3 (C=O); FABMS m/z 668 ($[M + H]^+$, 1), 460 (10), 391 (42), 307 (100), 289 (43); HRFABMS m/z 668.2552 $[M + H]^+$ (calcd for $C_{31}H_{41}NO_{15}$, 668.2554).

Dehydrooxoperezinone (3): orange needles ($CHCl_3$/CH_3OH); mp >280 °C; UV (CH_3OH) λ_{max} (log ϵ) 218 (4.63), 265 (4.71), 295 (4.51), 362 (4.35) nm; IR (KBr) ν_{max} 3163 (br), 1686, 1599 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.69 (6H, s, 2 × CH_3-2), 1.77 (3H, s, CH_3-8), 2.52 (3H, s, CH_3-5), 6.73 (1H, s, H-4), 11.50 (1H, br s, OH-3); ^{13}C NMR (DMSO-d_6) δ 8.0 (CH_3-8), 20.2 (CH_3-5), 25.6 (2 × CH_3-2), 95.9 (C-2), 107.4 (C-8), 116.4 (C-5), 120.1 (C-4), 130.5 (C-2a), 135.9 (C-8b), 146.1 (C-5a), 157.0 (C-3), 167.5 (C-8a), 177.1 (C-6), 180.0 (C-7); EIMS m/z 258 (M^+, 100), 243 (83), 229 (66), 215 (70), 186 (59), 159 (20), 141 (17), 115 (23); HREIMS m/z 258.0893 $[M]^+$ (calcd for $C_{15}H_{14}O_4$, 258.0893).

Anti-HIV Assay. The anti-HIV assay was carried out according to a procedure described in the literature.[22]

Cytotoxicity Assay. The cytotoxicity assay was carried out according to a procedure described in the literature.[28]

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