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Chemical Components of *Dendrobium chrysotoxum*†

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Two new phenanthrenes, named chrysotoxols A and B (**1**, **2**) and 24 known compounds were isolated from the stems of the accession species in 2010 Chinese Pharmacopoeia of "Shihu", *Dendrobium chrysotoxum*. All structures were elucidated by spectroscopic (NMR, MS, UV and IR) methods and the comparison with reported data in the literatures. Compound **2** and six known phenols were assessed for cytotoxic activity against two human tumor cell lines (A549 and HL-60). The results indicated that erianin may have cytotoxic activity and the other six compounds lost activity at the concentration down to 10^{-5} mol•L^{-1}.

Keywords Orchidaceae, *Dendrobium chrysotoxum*, chrysotoxol, phenanthrene, cytotoxic activity

Introduction

The stems of several *Dendrobium* species (Orchidaceae) are used as "Shihu" in traditional Chinese medicine[1] for a long time for the purpose of being beneficial to the stomach and promoting the production of body fluid, nourishing yin and clearing heat. The supply of some of these species (such as *D. officinale*) is very limited nowadays. In contrast, *D. chrysotoxum* is very abundant and distributed mainly in the southwest of China, Malaysia, India and Myanmar.[2] *D. chrysotoxum* has been collected as an official "Shihu" species in the Chinese Pharmacopoeia 2010. There have been a few reports on the chemical components of this plant;[3-9] however, the chemical basis of this species for the claimed medical benefits has not yet been clarified. In order to make better use of this abundant species and provide chemical evidence to its beneficial effects in the treatment of several medical conditions, we decided to reinvestigate *D. chrysotoxum*. Chemical studies of this species resulted in the isolation of 26 compounds. Herein, we report the isolation and structure characterization of two new phenanthrenes named chrysotoxols A (**1**) and B (**2**), firstly isolated by us from *Dendrobium* species. Some antitumor activity of phenanthrenes and bibenzyls have been reported earlier,[10,11] thus, compounds **2**, three known bibenzyls and three known phenanthrenes were assessed for cytotoxic activities against two human tumor cell lines (A549 and HL-60).

Experimental

General experimental procedures

Melting points were measured on an XRC-1 micro-melting point apparatus (Beijing, P. R. China) and were uncorrected. Optical rotations were measured with a Horiba SEAP-300 polarimeter. UV spectra were measured on a Hitachi UV-3210 spectrometer (Shanghai, P. R. China). IR spectra were measured with a Bio-Rad FTS-135 IR spectrometer (Bio-Rad, Richmond, CA) with KBr pellets. MS spectra were obtained on a VG Auto Spec-3000 mass spectrometer (VG, Manchester, England), 70 eV for EI. 1D and 2D NMR spectra were recorded on Bruker AM-400 MHz and Bruker DRX-500 spectrometers (Karlsruhe, Germany), with trimethylsilane (TMS) as internal standard. Column chromatography was carried out on silica gel (200—300 mesh) and TLC was carried out on plates precoated with silica gel (10—40 μm, Qindao Marine Chemical Ltd., Qingdao, P. R. China). C_{18}-bonded silica column (LiChroprep, 40—63 μm, Merck, Darmstadt, Germany) was applied for reverse-phase chromatography. Sephadex LH-20 was purchased from Amersham Biosciences. Etoposide (⩾98%) as positive control for cytotoxic assay was purchased from Sigma (Catalog No. E1383, St. Louise, MO, USA).

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† Dedicated to Professor Jun Zhou on the occasion of his 80th birthday.

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Plant material

The stems of *D. chrysotoxum* for the first batch (3.6 kg) were collected in January 2004 from Xiaoshao of Kunming, and those for the second batch (4.0 kg) were collected in February 2006 from Xishuangbanna tropical botanical garden, Chinese Academy of Sciences (CAS). Both of them were identified by Professor Hong Yu of Yunnan University, Kunming, P. R. China. Voucher specimens (No. Zsh-2 and Zsh-5) were preserved at the State Key Laboratory of Phytochemistry and Plant Resource in West China, Kunming Institute of Botany, CAS, P. R. China.

Extraction and isolation

The air dried stems of the plant (7.6 kg) were powdered and extracted with 95% aqueous EtOH (38 L×3) under reflux. The EtOH extract (105 L) was evaporated under reduced pressure and fractionated successively into EtOAc soluble (250 g) and *n*-BuOH soluble (249 g) fractions. The *n*-BuOH extract (249 g) was subjected to silica gel column chromatography (ϕ 12×120 cm) and eluted with $CHCl_3$-MeOH (V∶V=15∶1—3∶1, 45 L) to afford six fractions (I—VI). Fraction II (20 g) was subjected to silica gel column chromatography (ϕ 4×70 cm, $CHCl_3$-MeOH, V∶V=12∶1, 5 L), and then purified further on Sephadex LH-20 (ϕ 2.0×130 cm, MeOH, 2 L) to afford compound **1** (13 mg). Fraction III (12 g) was subjected to column chromatography repeatedly over silica gel (ϕ 4×60 cm, $CHCl_3$-MeOH, V∶V=12∶1, 4 L), Sephadex LH-20 (ϕ 2×120 cm, MeOH, 2 L) and RP-C_{18} (ϕ 2×30 cm, MeOH-H_2O, V∶V=7∶3, 1 L) to yield compound **2** (3 mg).

Chrysotoxol A (**1**): brown amorphous powder (Me_2CO); m.p. 212—214 ℃; $[\alpha]_D^{14}$ −14.7 (*c* 0.11, acetone); UV (MeOH) λ_{max} (log ε): 204 (4.7), 279 (4.3), 295 (4.1) nm; NMR ($(CD_3)_2CO$, 500 MHz) δ: 2.58—2.67 (m, 4H, H-9,10), 2.61 (dd, J=15.5, 8.5 Hz, 1H, H-11β), 2.96 (dd, J=15.5, 5.5 Hz, 1H, H-11α), 3.83 (s, 3H, H-20), 4.06 (sextet, J=8.5, 8.5, 5.5 Hz, 1H, H-12), 4.56 (d, J=8.5 Hz, 1H, H-13), 6.37 (s, 1H, H-3), 6.66 (dd, J=8.4, 2.1 Hz, 1H, H-6), 6.69 (d, J=2.1 Hz, 1H, H-8), 6.79 (d, J=8.1 Hz, 1H, H-18), 6.88 (dd, J=8.1, 1.5 Hz, 1H, H-19), 7.04 (d, J=1.5 Hz, 1H, H-15), 8.19 (d, J=8.4 Hz, 1H, H-5); ^{13}C NMR ($(CD_3)_2CO$, 125 MHz) δ: 26.0 (t, C-10), 30.5 (t, C-9), 32.6 (t, C-11), 56.1 (q, C-20), 68.7 (d, C-12), 82.6 (d, C-13), 102.7 (d, C-3), 110.6 (s, C-1), 111.7 (d, C-15), 113.3 (d, C-6), 114.5 (d, C-8), 115.3 (d, C-18), 116.5 (s, C-4a), 121.4 (d, C-19), 126.0 (s, C-8a), 130.0 (d, C-5), 131.5 (s, C-14), 139.2 (s, C-10a), 139.4 (s, C-4b), 147.2 (s, C-17), 148.0 (s, C-16), 154.1 (s, C-2,4), 155.9 (s, C-7); IR (KBr) ν_{max}: 3406, 2930, 2840, 1699, 1606, 1517, 1465, 1429, 1364, 1273, 1234, 1140, 1029, 823, 671 cm^{-1}; EIMS *m/z* (%): 406 ($[M]^+$, 31), 241 (100), 166 (39), 137 (27); HR-ESIMS calcd for $C_{24}H_{22}O_6Na$ $[M+Na]^+$ 429.1314, found 429.1323.

Chrysotoxol B (**2**): brown amorphous powder (MeOH); m.p. 193—195 ℃; $[\alpha]_D^{14}$ 16.1 (*c* 0.60, MeOH); UV (MeOH) λ_{max} (log ε): 208 (4.7), 278 (4.2) 298 (4.1) nm; NMR (CD_3OD, 500 MH_Z) δ: 2.57 (dd, J=15.5, 8.5 Hz, 1H, H-11β), 2.62—2.67 (m, 4H, H-9,10), 2.93 (dd, J=15.5, 5.5 Hz, 1H, H-11α), 3.84 (s, 6H, H-20, 21), 4.05 (sextet, J=8.5, 8.0 Hz, 5.5, 1H, H-12), 4.59 (d, J=8.0 Hz, 1H, H-13), 6.33 (s, 1H, H-3), 6.62 (d, J=8.5 Hz, 1H, H-6), 6.63 (s, 1H, H-8), 6.72 (s, 2H, H-15, 19), 8.13 (d, J=8.5 Hz, 1H, H-5); ^{13}C NMR (C_5D_5N, 125 MHz) δ: 26.1 (t, C-10), 30.2 (t, C-9), 33.5 (t, C-11), 56.3 (q, C-20,21), 68.6 (d, C-12), 83.6 (d, C-13), 103.2 (d, C-3), 106.6 (d, C-15, 19), 110.6 (s, C-1), 114.0 (d, C-6), 115.2 (d, C-8), 117.2 (s, C-4a), 126.2 (s, C-8a), 130.2 (s, C-14), 130.4 (d, C-5), 139.0 (s, C-10a), 139.5 (s, C-4b), 137.7 (s, C-17), 149.0 (s, C-16,18), 154.4 (s, C-2), 155.0 (s, C-4), 157.0 (s, C-7); IR (KBr) ν_{max}: 3425, 2930, 1609, 1518, 1464, 1427, 1328, 1237, 1116, 1032, 833, 669 cm^{-1}; FABMS (negative ion) *m/z*: 435 ($[M-H]^-$), 339, 325, 311, 279; HR-FABMS (negative ion) calcd for $C_{25}H_{23}O_7$ $[M-H]^-$ 435.1443, found 435.1449.

Cytotoxic assay

Compounds **2**, three known bibenzyls, erianin, gigantol and tristin, together with three known phenanthrenes, confusarin, moscatin and epheranthol B (≥90%) were tested for their cytotoxic effects against human lung carcinoma A549 and human leukemia HL-60 cell lines, and the sulforhodamine B (SRB) assay[12] and the methyl-thiazol-tetrozolium (MTT) assay[13] were used in the experiments respectively. Etoposide was used as the positive control. The cell growth inhibition rates of these eight compounds are shown in Table 1. The identical results for the positive control with literature data[12,13] indicated its credibility for cytotoxic assay.

Results and Discussion

After repeated column chromatographic purification on silica gel, Sephadex LH-20 and RP-C_{18} gel, the *n*-BuOH extract of the stems of *D. chrysotoxum* afforded two new compounds **1**, **2** (Figure 1).

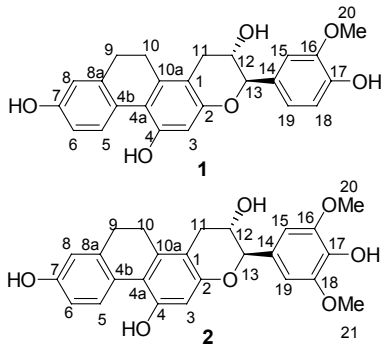

Figure 1 Structures of compounds **1**, **2**.

Compound **1** was obtained as a brown amorphous powder with active optical rotation (−14.7), its mo-

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Table 1 The cell growth inhibition rates (%) of compound **2** at different concentration (mol•L^{-1}) and six known phenols (etoposide was used as positive control)

	A549					HL-60				
	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
2	81.1	0	0	0	0	65.0	21.4	0	0	0
Confusarin	97.3	1.6	0	0	0	55.7	23.1	4.1	0	8.7
Moscatin	93.4	14.2	0.8	0	1.0	47.2	0	0	0	4.1
Epheranthol B	62.4	0	0	0	0	76.3	0	0	0	0
Erianin	73.2	46.8	35.9	21.4	0	68.3	45.9	28.0	28.5	23.6
Gigantol	61.5	0	0	0	0	32.4	3.3	0	0	9.9
Tristin	38.2	0	0	0	0	32.6	3.9	0	0	0
Etoposide	88.4	60.0	27.9	11.7	7.7	89.8	91.9	91.5	82.3	66.3

lecular formula was assigned as $C_{24}H_{22}O_6$ from the HR ESIMS (*m/z* 429.1323 $[M+Na]^+$, calcd for 429.1314) and its NMR data, which indicated 14 degrees of unsaturation. The UV spectrum [λ_{max} (log ε): 278 (4.2), 298 (4.1) nm] indicated an extended conjugated system. The ^{1}H NMR and ^{13}C NMR spectra of **1** showed the existence of one methoxy group, three aliphatic methylenes, two aliphatic methines and three aromatic rings. Two methylene carbons at δ_C 30.5 (C-9) and 26.0 (C-10) correlated four benzylic protons at δ_H 2.58—2.67 (m, 4H, H-9 and H-10) in the HSQC spectrum indicated a dihydrophenanthrene core,[14] which was confirmed by the correlations [H-9, H-10/C-1, C-4a, C-8, C-8a, C-10, C-10a, C-11] in the HMBC spectrum (Figure 2.) of **1**. Three aromatic protons at δ_H 8.19 (d, J=8.4 Hz, 1H, H-5), 6.66 (dd, J=8.4, 2.1 Hz, 1H, H-6), 6.69 (d, J=2.1 Hz, 1H, H-8) displayed a 1,3,4-substituted phenyl and the singlet signal at δ_H 6.37 (s, 1H, H-3) indicated a 1,2,3,4,5-pentasubstituted phenyl for the dihydrophenanthrene core. The rest three aromatic protons at δ_H 7.04 (d, J=1.5 Hz, 1H, H-15), 6.79 (d, J=8.1 Hz, 1H, H-18), 6.88 (dd, J=8.1, 1.5 Hz, 1H, H-19) indicated a 1,3,4-substituted aromatic ring. Based on the coupling constants of four protons at δ_H 2.61 (dd, J=15.5, 8.5 Hz, 1H, H-11β), 2.96 (dd, J=15.5, 5.5 Hz, 1H, H-11α), 4.06 (sextet, J=8.5, 8.5, 5.5 Hz, 1H, H-12), 4.56 (d, J=8.5 Hz, 1H, H-13), a structural moiety of —CH_2CHOHCHO— (C-11, C-12, C-13) was proposed, which was then confirmed by correlations [H-12/H-11α, H-11β, H-13] in the COSY spectrum. Then the six-membered heterocycle was established by the correlations [H-11/C-1, C-2, C-10a, C-12, C-13; H-12/C-13, C-14; H-13/C-2, C-11, C-12, C-14, C-15, C-19] in the HMBC spectrum. The coupling constant (J=8.5 Hz) between H-12 and H-13 suggested a *trans* relationship of these two pseudo-axial protons as shown in Figure 1.[15] Thus, compound **1** was characterized as *trans*-2-(4-hydroxy-3-methoxyphenyl)-2,3,5,6-tetrahydrophenanthro[2,1-*b*]pyran-3,8,11-triol, named chrysotoxol A.

Compound **2** was obtained as a brown amorphous powder with active optical rotation (16.1), possessing a molecular-ion peak at *m/z* 435 in its negative FABMS. Its molecular formula was assigned as $C_{25}H_{24}O_7$ from the negative HR-FABMS (435.1449 $[M-H]^-$, calcd for 435.1443) and its NMR data, indicating 14 degrees of

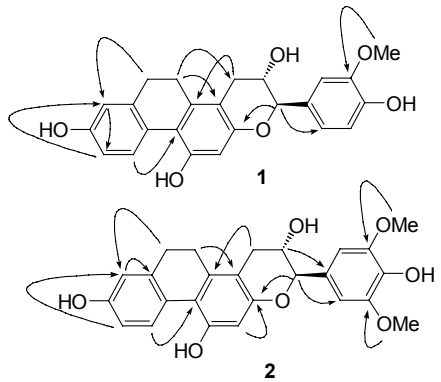

Figure 2 Key HMBC (H to C) correlations of compounds **1**, **2**.

unsaturation. Compared to compound **1**, the ^{1}H NMR and ^{13}C NMR spectra of **2** (Table 2) indicated the presence of an additional methoxyl group in **2**, which was confirmed by its molecular weight. The methoxyl group should be linked to C-18 based on the [H-21/C-18] correlation data in the HMBC spectrum. The coupling constant (J=8.0 Hz) of H-12 with H-13 corresponded to a *trans* relationship of these two pseudo-axial protons.[15]

To further confirm the relative configuration of **2**, we calculated its CD spectrum. The absolute configuration of compound **2** was established by comparison of the experimental circular dichroism (CD) spectrum with time-dependent density functional theory (TD-DFT)-calculated ECD spectrum.[16] Two possible conformers for both structure 12*S*,13*R* were found. The corresponding minimum geometries were further fully optimized using DFT at the B3LYP/6-31G(d,p) level as implemented in the Gaussian 03 program package.[17] The ECD calculations for the conformers were then conducted with the B3LYP/6-31G(d,p) basis set in the gas phase. The calculated ECD spectrum, weighted based on the Gibbs free energy, for the 12*S*,13*R* of **2** is in agreement with the experimental ECD (Figure 8 in supporting information). Therefore, the absolute configuration of **2** was established as 12*S*,13*R* on the basis of the evidence from the calculated ECD spectrum. Thus, compound **2** was elucidated as (12*S*,13*R*)-2-(4-hydroxy-3,5-dimethoxyphenyl)-2,3,5,6-tetrahydrophenanthro[2,1-*b*]pyran-3,8,11-triol, which was named chrysotoxol B. From a biogenetic point of view, com-

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pound **1** may share the same absolute configuration (as 12*S* and 13*R*) as **2**, opposite optical rotation (−14.7 for compound **1** and 16.1 for compound **2**) may be caused by methoxy at C-18.

24 known compounds were also isolated in this study (Supporting Information) and their structures elucidated by comparing their spectra data with those reported in literatures and/or by direct comparison on TLC with authentic samples. Those 24 compounds were identified as follows: trigonopol B,[18] chrysotobibenzyl,[5] erianin,[6] chrysotoxine,[4] moscatilin,[19] gigantol,[6,19] batatasin,[19] tristin,[19] confusarin,[5] chrysotoxene,[3] epheranthol B,[20] moscatin,[18] 4,5-dihydroxy-2,6-dimethoxy-9,10-dihydrophenanthrene,[21] episyringaresinol,[19] 3-hydroxymethyl-5-(3-hydroxy-propyl)-2-(4-hydroxy-3-methoxyphenyl)-7-methoxybenzodihydrofuran,[22] syringoside,[23] 3,6,9-trihydroxy-3,4-dihydroanthracen-1(2*H*)-one,[24] dendroflorin,[5] dengibsin,[5] 5,6-dihydroxy-4'-methoxy-flacanone,[25] antiarol,[26] salidrosol,[27] *β*-sitosterol and daucosterol.

The majority of the compounds isolated from *D. chrysotoxum* in this study belong to phenanthrenes and bibenzyls, especially bibenzyls, such as erianin and gigantol. These two main compositions could be used as quality specification for this TCM. *D. chrysotoxum* may be in rich amount of bibenzyls, while alkaloids and terpenes have not yet been isolated from this species. This is differ from chemical constituents of *D. nobile* which was used as a mainly source of "Shihu". A number of biological activities have been described for phenanthrenes and bibenzyls such as antimicrobial, spasmolytic, antiplatelet aggregation, antiallergic and anti-inflammatory activities, including antitumor activity.[10,11] We have subjected compounds **2**, three known bibenzyls and three known phenanthrenes to the testing of cytotoxic activity against two human tumor cell lines, A549 and HL-60 (Table 1). Though most compounds displayed inhibition effect against both cell lines at concentration of 10^{-4} mol•L^{-1}, none of them showed significant inhibition at 10^{-5} mol•L^{-1} except erianin which was reported to be an anti-angiogenic agent.[10]

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