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Cytotoxic Picrotoxane-type Sesquiterpenoid Lactones from *Dendrobium huoshanense*

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Abstract: Bioassay-guided led to the isolation of five compounds, including a new picrotoxane-type sesquiterpene lactone, aduncin C (**1**), together with four known ones (**2**-**5**) from *Dendrobium huoshanense*. Their structures were elucidated by means of extensive spectroscopic analysis. Biological evaluation of the isolates against four human cancer cell lines indicated broad-spectrum and cytotoxic activities with IC_{50} values ranging from 4.08 to 26.75 μM. Among them, α-dihydropicrotoxinin (**3**) exhibited significant cell proliferation inhibitory activity, especially for HL-60 (IC_{50} 5.81 μM), MCF- 7 (IC_{50} 6.49 μM), SW-480 (IC_{50} 6.80 μM), respectively.

Keywords: *Dendrobium huoshanense*; picrotoxane-type sesquiterpene; cytotoxicity.

1. Introduction

Dendrobium huoshanense C.Z.Tang & Cheng, is a member of important Orchidanceae family plant, which is a famous Chinese medicinal plant used as a precious tonic for thousands of years. Due to the harsh growth conditions, the natural distribution area of *D. huoshanense* is limited to the north of the Yangtze River, such as Huoshan and Jinzhai in Anhui, Yinshan in Hubei and Nanzhao in Henan [1]. According to the ancient Chinese literatures, it was recorded to improve a wide range of health problems such as yin-yang disharmony and weak eyesight [2]. It is present as an official drug in the Chinese Pharmacopoeia with its tonifying stomach/nourishing yin properties[3]. Reports indicated that its active ingredients possess anti-inflammatory, alcoholic gastric ulcer, anti-tumor activity, and so on [4-8]. Flavonoids were identified from the stems extract of *D. huoshanense* by a HPLC coupled with electrospray ionization multi-stage tandem mass (HPLC-ESI-Ms^n) analysis [9]. The present paper reported the isolation of bioassay-guided fractionation of petroleum ether and ethyl acetate extractions, a picrotoxane-type sesquiterpenoid lactone along with four ones as well as their cytotoxicity against four human cancer cell lines, using the MTT method.

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2. Materials and Methods

2.1. Plant Material

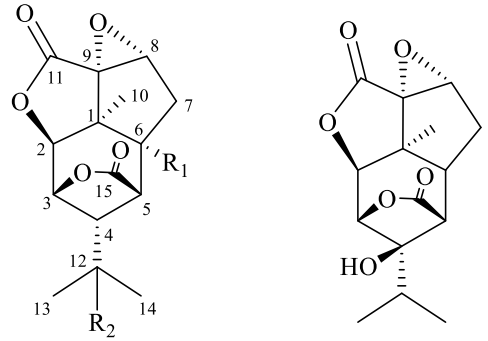
The stems of *D. huoshanense* were purchased from Changchong Traditional Chinese Medicine Development Co. Ltd, Huoshan County Anhui province, in July 2017, and identified by Prof. Shoujin Liu, Anhui University of Chinese Medicine. A voucher specimen (ACM2017070101) was deposited at the specimen center of Anhui University of Chinese Medicine.

2.2. General Experimental Procedures

IR spectra were recorded on a Nicolet iS10 spectrometer. The ^{1}H, ^{13}C and 2D NMR spectra were performed on Bruker ARX-500, ARX-600, and ARX-800 spectrometers, using TMS as an internal standard. HR-ESI-MS spectra were obtained on an Agilent 6210 TOF mass spectrometer. Preparative HPLC was carried out on an Agilent 1100 liquid chromatography with a YMC Pack ODS-A-column (250 × 10 mm, 5 μm, 120Å). Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden), MCI gel CHP 20P (75-150 μm, Mitsubishi Chemical Corp., Tokyo, Japan), RP-18 gel (40-75 μm, Fuji Silysia Chemical Ltd., Japan), and silica gel (200-300 mesh) used for column chromatography was supplied by Qingdao Marine Chemical Factory.

2.3. Extraction and Isolation

The crude powder of *D. huoshanense* (9.8 kg) was extracted by percolation with ethanol at room temperature. The ethanol extract (1.6 kg) was obtained after removing the solvent under vacuum, and was suspended in H_2O and partitioned with petroleum ether (PE) and ethyl acetate (EtOAc). The PE-soluble fraction (400 g) was subjected to normal-phase silica gel CC using a CH_2Cl_2–MeOH gradient elution from 100: 0 to 80: 20 (v/v) to obtain eight fractions. *Fr.2* (78 g) was separated by a MCI reversed-phase chromatography column using MeOH-H_2O gradient elution from 60: 40 to 0: 100 (v/v) to obtain three fractions (*Fr.2.1 – Fr.2.3*). *Fr.2.2* (5.4 g) was separated by Sephadex LH-20 column chromatography and preparative HPLC to afford **2** (33 mg). *Fr.2.7* (2.1 g) was separated by silica gel column chromatography, preparative HPLC and Sephadex LH-20 column chromatography to obtain compounds **3** (5 mg) and **4** (7 mg).



1 R_1 = H, R_2 = OH
2 R_1 = OH, R_2 =OH
3 R_1 = OH, R_2 = H
4 R_1 = H, R_2 = H

5

Figure 1. Structures of compounds **1**-**5**

The EtOAc-soluble (150 g) performed on silica gel column chromatography (200~300 mesh) using CH_2Cl_2-MeOH (95: 5→80: 20, v/v) gradient elution to obtain *Fr.1— Fr.7*. *Fr.2* was separated by MCI reversed-phase column chromatography, eluting with MeOH-H_2O gradient, TLC detection to

obtain fractions *Fr.2.1 – Fr.2.4*. *Fr.2.2* was subjected to ODS reversed-phase column chromatography, Sephadex LH-20 column chromatography, silica gel column chromatography and Sephadex LH-20 column chromatography to obtain compound **1** (6 mg) and **5** (24 mg).

2.4. Spectroscopic Data

*Aduncin C(**1**)*: White needle crystals, $[\alpha]_D^{20}$ −13.4 (*c* 1.0, MeOH); IR (KBr): υ_{max} 3435, 1799, 1776, 1367, 1384 cm^{-1}; ESI-MS *m/z* 317 $[M + Na]^+$, 611 $[2M + Na]^+$; HRESIMS *m/z* 317.0994 $[M + Na]^+$ (calcd for $C_{15}H_{18}O_6Na$, 317.0996). ^1H NMR (500 MHz, MeOD) and ^{13}C NMR (125 MHz, MeOD) data, see Table 1.

2.5. Cytotoxicity Assays

The cytotoxicity assay was performed according to the MTT method [10] with four human cancer cell lines: human myeloid leukemia HL-60, hepatocellular carcinoma SMMC-7721, breast cancer MCF-7 and colon cancer SW480. 5-FU was used as a positive control. Briefly, 1×10^4/mL cells were seeded in 96-well plates (100 μL/well) were cultured in serum-free IMDM (HL-60) or RPMI-1640 medium (SMMC-7721) or L-15 medium (SW-480) or MEM medium (MCF-7), supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 until 90% confluent, and then incubated for 2 h to synchronize. The cells were treated and incubated (200 μL/well) with six concentrations of drugs in three replicates for 72 h. Then, 20 μL of MTT (5 mg/mL) was added to each well after removal of 100 μL medium, and incubcated for another 4 h. The OD value of each well was recorded on a Berthold LB941 (Berthold Co. Ltd) reader at 570 nm and IC_{50} values were calculated by Reed and Muench's method[11].

3. Results and Discussion

3.1. Structure Elucidation

Compound **1** was isolated as white needles. Its quasi-molecular ion at *m/z* 317.0994 $[M + Na]^+$ suggested a molecular formula of $C_{15}H_{18}O_6$ (calcd for $C_{15}H_{18}O_6Na$, 317.0996) from the HR-ESI-MS spectrum. The infrared (IR) spectrum showed absorption bands at 3435 cm^{-1} (hydroxyl group) and 1799, 1776 cm^{-1} (carbonyl group), 1384 and 1367 cm^{-1} (geminal dimethyl group).

Table 1. ^1H and ^{13}C NMR data for compound **1** and **4** in CD_3OD

Position	**1**[a]		**4**[b]	
	δ_C	δ_H	δ_C	δ_H
1	45.8 (s)	-	44.7 (s)	-
2	82.5 (d)	4.89 (1H, d, 3.5 Hz)	82.5 (d)	4.65 (1H, d, 3.5 Hz)
3	79.3 (d)	5.00 (1H, dd, 5.0, 3.5 Hz)	79.3 (d)	4.92 (1H, dd, 5.7, 3.5 Hz)
4	54.0 (d)	2.70 (1H, like-t, 4.8 Hz)	51.7 (d)	2.62 (1H, like-t, 5.4 Hz)
5	44.8 (d)	2.67 (1H, like-t, 4.5 Hz)	44.7 (d)	2.43 (1H, m)
6	51.9 (d)	2.80 (1H, t, 6.4 Hz)	51.7 (d)	2.49 (1H, t, 6.2 Hz)
7	35.5 (t)	2.13 (1H, dd, 15.0, 7.2 Hz)	35.1 (t)	2.19 (1H, dd, 15.2, 7.3 Hz)
		2.33 (1H, dd, 15.0, 3.4 Hz)		2.37 (1H, dd, 15.2, 3.5 Hz)
8	68.6 (d)	3.81 (1H, d, 3.4 Hz)	69.0 (d)	3.80 (1H, d, 3.4 Hz)
9	74.9 (s)	-	74.9 (s)	-
10	20.0 (q)	1.32 (3H, s)	21.5 (q)	1.35 (3H, s)
11	173.0 (s)	-	173.0 (s)	-
12	69.1 (s)	-	25.8 (d)	1.57 (1H, m)
13	30.1 (q)	1.34 (3H, s)	20.8 (q)	0.99 (3H, d, 6.5 Hz)
14	29.7 (q)	1.30 (3H, s)	20.8 (q)	1.05 (3H, d, 6.5 Hz)
15	179.2 (s)	-	179.2 (s)	-

[a]Data were measured on δ_H 800 MHz and δ_c 125 MHz, and [b] on δ_H 600 MHz and δ_c 150 MHz, *J* in Hz. Assignments were based on 2D-NMR experiments.

The ^{1}H-NMR spectrum (500 MHz, CD_3OD) (Table 1) presented three sets of methyl signals at δ_H 1.34 (3H, s), 1.30 (3H, s) and 1.32 (3H, s); a set of methylene signals at δ_H 2.33 (1H, dd, J = 15.0, 3.4 Hz, H-7β) and δ_H 2.13 (1H, dd, J = 15.0, 7.2 Hz, H-7α); six sets of methine signals (including three sets of oxymethines) at δ_H 5.00 (1H, dd, J = 5.0, 3.5 Hz), 4.89 (1H, d, J = 3.5 Hz), 3.81 (1H, d, J = 3.4 Hz), 2.80 (1H, d, J = 6.4 Hz), 2.70 (1H, d, J = 4.8 Hz) and 2.67 (1H, t, J = 4.5 Hz). Its ^{13}C and DEPT NMR spectra (125 MHz, CD_3OD) (Table 1) exhibited 15 carbon signals were ascribed to three methyls, a methylene, six methines and five quaternary carbons (including two oxygenated at δ_C 74.9 and 69.1, two ester carbonyl at δ_C 179.2 and 173.0). The NMR data were similar to those of **2**, except that a hydroxyl group attached to C-6 disappeared in **1**, which supported by the ^{13}C-NMR signals for C-6, C-7 and C-5 in **1** shifted upfield 35.2, 8.9 and 6.9 ppm compared with those in **2**, respectively. Detailed analysis of the correlations of 2D-NMR (Figure 2), two proton spin systems of H-3/H-4 and H-5/H-6/H-7/H-8 in the ^{1}H-^{1}H COSY spectrum, and the expected HMBC cross-peaks from H-2 to C-1, C-9, C-10 and C-11, from H-3 to C-1 and C-15, from H-6 to C-8, C-9, C-10 and C-15, and from H-13 to C-4, C-12 and C-14. The relatively configuration of **1** was established by a ROESY experiment and comparison of its spectroscopic data with those of (-)-picrotin [12]. The correlations of H-2 ↔ H-10, H-6 ↔ H-10, and of H-3 ↔ H-5, H-3 ↔ H-13, H-5 ↔ H-14, and of H-7α↔ H-10 suggested that H-2, H-3, H5, H-6, H-10, H_3-13 and H_3-14 be all in α-oriented, whereas H-4 and H-8 be in β-position. Therefore, the structure of **1** was determined, and named as aduncin C, as shown in Figure 1.

The known isolates were identified as (-)-picrotin (**2**) [12-13], α-dihydropicrotoxinin (**3**) [14], 4-deoxyaduncin (**4**)[15], and aduncin (**5**) [16-17] based on comparison with NMR and MS data in the references.

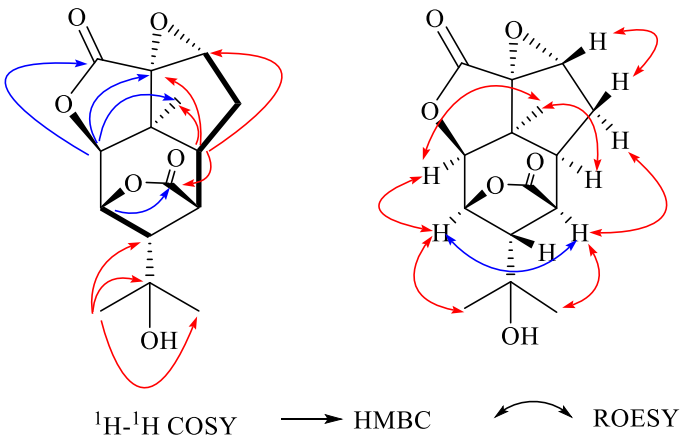


Figure 2. Key ^{1}H-^{1}H COSY, HMBC and ROESY correlations of compound **1**

3.2. Cytotoxicity Activity

The cytotoxicity of ethanol extraction, PE-soluble fraction, EtOAc-soluble fraction and all the isolates was evaluated in vitro against HL-60, MCF-7, SMMC-7721 and SW-480 human cancer cell lines. Cell inhibition rate of ethanol extraction and PE-soluble fraction were cytotoxicity for SW480 human cancer cell at 47.09±1.10% and 49.96±1.00%, respectively. All the extractions showed moderate or weak activity against other cancer cell lines. Compound **3** was cytotoxicity for all the test cell lines with the IC_{50} value of 5.81, 6.49, 9.65 and 6.80 μM, respectively. Compound **2** exhibited activity against the SW480 cell lines, having IC_{50} values of 8.25 μM. Compound **4** showed moderate cytotoxity against HL-60 and SMMC-7721 cell lines having IC_{50} values of 7.62 and 8.49 μM, respectively. Whereas, the other compounds exhibited weak cytotoxicity (Table 2).

Table 2. Cytotoxicity of compounds isolated from the stems of *D. huoshanense* (IC_{50}[a] values in μM; n = 3)

Compounds	IC_{50} (μM)			
	HL-60	**MCF-7**	**SMMC-7721**	**SW-480**
1	10.67 ± 0.86	17.11 ± 1.38	22.83 ± 2.17	26.75 ± 2.32
2	10.62 ± 0.61	9.43 ± 0.67	12.86 ± 1.17	8.25 ± 0.74
3	5.81 ± 0.39	6.49 ± 0.45	9.65 ± 0.74	6.80 ± 0.52
4	7.62 ± 0.43	10.50 ± 0.88	8.42 ± 0.69	12.03 ± 1.01
5	9.37 ± 0.57	12.62 ± 0.81	10.88 ± 0.92	11.26 ± 0.92
5-FU[b]	6.43 ± 0.46	9.18 ± 0.73	4.08 ± 0.35	5.80 ± 0.43

[a] IC_{50} is defined as the concentration that resulted in a 50% decrease in cell number.
[b] 5-FU was used as a positive control.

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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