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Note

Chemical constituents of *Dendrobium gratiosissimum* and their cytotoxic activities

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Two new bibenzyl derivatives, named dengraols A **1** and B **2**, are isolated from stems of *Dendrobium gratiosissimum* Rchb. (Orchidaceae), together with seven known compounds: 3,5,4′-trihydroxybibenzyl **3**, 3,4′-dihydroxy- 5-methoxybibenzyl **4,** 3, 4 - dihydroxy-5, 4′ - dimethoxy bibenzyl **5**, 3, 4′ - dihydroxy - 4, 5, 3′-trimethoxybibenzyl (moscatilin) **6** and 5, 4′- dihydroxy -3, 3′-dimethoxybibenzyl (gigantol) **7**, 5, 3′, 4′-trihydroxy - 3- methoxy-bibenzyl (tristin) **8** and 5, 3′- dihydroxy- 3-methoxybibenzyl (batatasin III) **9**. Among the isolated compounds, dengraols A **1** and B **2**, moscatilin **6** and gigantol **7** showed inhibitory activity of proliferation on HL-60 cells with IC_{50} values at 2.1, 6.4, 0.082 and 10.6 μM, respectively.

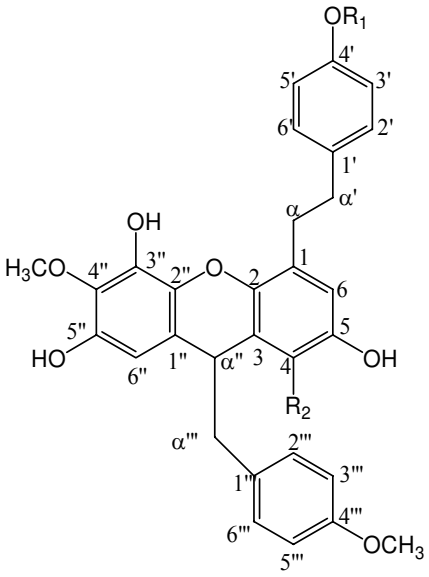
Keywords: Cytotoxic activity, bibenzyl derivative, *Dendrobium gratiosissimum*, dengraol A, B, HL-60 cell, Orchidaceae

The Chinese crude drug ‘Shi Hu’ (‘Sekkoku’ in Japanese) is prepared from the dried stems of *Dendrobium* species (Orchidaceae) and applied to tonic and antipyretic. The chemical constituents of this genus have been extensively investigated[1]. The isolation of bibenzyl derivatives, coumarins, lignans and phenanthrenes including new alkaloids from some species of *Dendrobium* were reported[2-6]. Although the stems of *D. gratiosissimum* Rchb. is also used as a source of ‘Shi Hu’ in China (2005 China Pharmacopoeia), the phytochemical study on *D. gartiosissimum* has not yet been conducted, which encouraged us to investigate the chemical constituents in the stems and the biological activity of isolated compounds. In this paper, we report the isolation and structure elucidation of bibenzyl derivatives from the stems of *D. gartiosissimum* and their inhibitory activity on HL-60 cell lines.

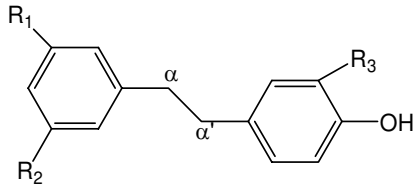
Results and Discussion

From the stems of *D. gartiosissimum*, 9 bibenzyl derivatives were isolated including two new compounds dengraols A **1** and B **2** and seven known compounds: 3, 5, 4′-trihydroxybibenzyl **3** (ref. 7), 3, 4′-dihydroxy-5-methoxybibenzyl **4** (ref. 8), 3, 4-dihydroxy-5, 4′-dimethoxybibenzyl **5** (ref. 9), 3, 4′-dihydroxy - 4, 5, 3′- trimethoxybibenzyl (moscatilin) **6** (ref. 10), 5, 4′-dihydroxy-3, 3′-dimethoxybibenzyl (gigantol) **7** (ref. 10), 5, 3′, 4′- trihydroxy-3-methoxybibenzyl (tristin) **8** (ref. 4) and 5, 3′-dihydroxy - 3 - methoxybibenzyl (batatasin III) **9** (ref 4, **Chart 1**). The structures of known compounds were determined by comparing their spectral data with those of authentic samples or previously reported data. Among these compounds, **3** has been firstly obtained from *Dendrobium*, **4** and **5** have been already isolated from *D. amoenum* and *D. moniliforme*. Compounds **6** and **7** were popular constituents in this genus[10]. Dengraols A **1** and B **2** are the new ones additions to the growing list of naturally occurring bibenzyl derivatives. The structural elucidation of two new compounds is described in detail.

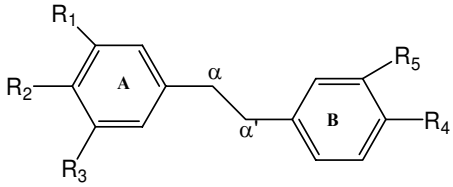
Dengraol A **1** was obtained as pale-yellow oil. Its molecular formula was established as $C_{30}H_{28}O_7$ by HREIMS giving a molecular ion at *m/z* 500.1284 $[M]^+$ (Calcd 500.1259). The UV absorptions at 213 and 282 nm were characteristic of a bibenzyl skeleton[11,12]. The presence of phenolic groups was indicated by the color reaction with $FeCl_3$ (violet) and its IR absorption bands [3415cm^{-1}(OH)]. The ^1H NMR spectrum exhibited 2 methoxyl signals at δ 3.70 (s) and 3.80 (s), 11 aromatic proton signals at δ 6.25 - 7.10 and 7 aliphatic proton signals. The ^{13}C NMR (**Table I**) and HMQC spectra revealed that **1** contained twelve quaternary, twelve methine, three methylene and two methoxyl carbons, respectively. Among the 11 aromatic protons, four protons at δ 7.10 (2H, d, J = 8.5 Hz) / 6.77 (2H, d, J = 8.5 Hz) and 6.66 (2H, d, J = 8.9 Hz) / 6.57 (2H, d, J = 8.9 Hz) were two pairs of typical AB splitting pattern of *ortho*-coupled doublet. Two aromatic protons at δ 6.56 (1H, d, J = 2.4 Hz) / 6.40 (1H, d, J = 2.4 Hz) were a typical AB splitting pattern of *meta*-coupled



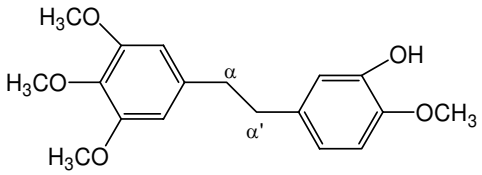
1 R_1= H, R_2=H
2 R_1=CH_3, R_2=OCH_3



3 R_1=R_2=R_3=OH
4 R_1=R_3=OH, R_2=OCH_3
7 R_1=R_3=OCH_3, R_2=OH
8 R_1=R_2=OH, R_3=OCH_3



5 R_1=R_2=R_4=OH, R_3=OCH_3, R_5=H
6 R_1=R_3=R_5=OCH_3, R_2=R_4=OH
9 R_1=OCH_3, R_2=R_4=H, R_5=OH



erianin

Chart 1 — Structures of Compounds **1-9** and erianin

doublet. Among the seven aliphatic protons, the signals at δ 2.80-2.90 (4H, m) which were corresponding to carbon signals at δ 34.9 and 37.2 determined by HMQC spectrum were assigned to two methylene groups of a bibenzyl derivative (Ar-CH_2-CH_2-Ar')[13]. The other three aliphatic protons were assigned to one methine [δ 4.18 (1H, dd, J = 5.5 Hz)] and a methylene group δ 2.70 (2H, m) by the analysis of DEPT and HMQC spectra, and were reminiscent of a dimeric structure of two bibenzyl moieties linked by C-C (sp^2-sp^3) bond[14]. The ^{1}H-^{1}H COSY spectrum showed the correlation between the methine proton at δ 4.18 (H-α″) and the methylene protons at δ 2.70 (H-α‴). The ^{13}C NMR, DEPT and HMQC spectra displayed the C-α″ and C- α‴ resonances at δ 39.0 and 45.6. From these spectral data, it could be inferred that a second unit of **1** was also a bibenzyl moiety. In the HMBC spectrum, the significant long-range coupling relations of proton and carbon signals were observed (**Figure 1**) between δ 4.18 (H-α″) and δ 101.9 (C-4), 154.9 (C-2), 141.9(C-1″), 109.8 (C-6″) and 140.1 (C-2″), which confirmed that these two bibenzyl units were connected at C-3 and C-α″ through C-C linkage. The linkage was also found in the structure of a known compound Artomezianol[15]. The other important three-bond coupling correlations in the HMBC spectrum were obtained as follows: δ 2.80 (H-α) / δ 112.1 (C-6), 154.9 (C-2); δ 2.90 (H-α′) / δ 130.2 (C-2‴, C-6‴); δ 6.25 (H-6″) / δ 140.1 (C-2″), 136.8 (C-4″); δ 6.57 (H-6‴) / δ 159.2 (C-4‴). 131.4 (C-2‴). The negative ESI-MS spectra showed main peaks at 499.2 [M-1]$^-$ and 378.0 [M - methoxybenyl - 1]$^-$. Therefore, by these spectral data analyses, the structure of **1** was deduced as bibenzyl dimmer and named Dengraol A.

Dengraol B **2** was obtained as yellow oil. The UV spectrum showed the absorption maxima at 218 and 284 nm, which was similar to those of **1**. The IR spectrum exhibited absorptions at 3420 (OH), 1657, 1531, 780 cm^{-1} and 680 cm^{-1} (aromatic rings). HR-

Table I — ^{1}H- (500 MHz) and ^{13}C- (125 MHz) NMR spectral data for dengraol A (**1**, $CDCl_3$) and dengraol B (**2**, CD_3OD).

	dengraol A **1**		dengraol B **2**	
Carbon	^{1}H, δ (*J*, Hz)	^{13}C, δ	^{1}H, δ (*J*, Hz)	^{13}C, δ
1		133.5		133.6
2		154.9		132.2
3		117.8		118.1
4	6.40(d,2.4)	101.9		145.6
5		157.3		139.3
6	6.56(d,2.4)	112.1	6.47(s)	107.3
1′		115.9		129.0
2′	7.10(d,8.5)	130.2	7.07(d,8.6)	129.3
3′	6.77(d,8.5)	116.0	6.80(d,8.6)	113.8
4′		156.8		158.0
5′	6.77(d,8.5)	116.0	6.80(d,8.6)	113.8
6′	7.10(d,8.5)	130.2	7.04(d,8.6)	129.3
a	2.80(m)	34.9	2.80(m)	33.7
a′	2.90(m)	37.2	2.90(m)	36.9
1″		141.9		118.0
2″		140.1		138.7
3″		137.6		135.0
4″		136.8		134.7
5″		141.7		140.8
6″	6.25(s)	109.8	6.10(s)	109.8
1‴		131.6		130.4
2‴	6.57(d,8.9)	131.4	6.55(d,8.7)	130.6
3‴	6.66(d,8.9)	113.9	6.66(d,8.7)	113.3
4‴		159.2		158.1
5‴	6.66(d,8.9)	113.9	6.66(d,8.7)	113.3
6‴	6.57(d,8.9)	131.5	6.55(d,8.7)	130.6
a″	4.18(dd,5.5)	39.0	3.91(d,5.2)	39.0
a‴	2.70(m)	45.6	2.70(m)	44.6
4-OCH_3			3.88(s)	56.4
4′-OCH_3			3.79(s)	55.3
4″-OCH_3	3.80(s)	55.4	4.01(s)	61.5
4‴-OCH_3	3.70(s)	61.4	3.75(s)	55.2

ESIMS exhibited a molecular ion peak at *m/z* 545.2076 $[M+1]^+$ (calcd 545.2111). The ^{1}H NMR spectrum showed two pairs of *ortho*-coupled protons signals at δ 7.10 (2H, d, *J* = 8.5 Hz) / 6.77 (2H, d, *J* = 8.5 Hz) and 6.55 (2H, d, *J* = 8.9 Hz) / 6.66 (2H, d, *J* = 8.9 Hz), and two single aromatic proton signal at δ 6.47 (1H, s) and 6.10 (1H, s). The appearance of 423.1[M - methoxybenzyl]$^+$ in ESI/MS spectrum and similitude of NMR spectral data between **1** and **2** indicated that **2** was also a bibenzyl dimmer. In the HMBC spectrum, the following correlations were observed as follows: δ 6.47 (H-6) / δC 33.7 (C-α), 132.2 (C-2) and 145.5 (C-4); δ 6.10 (H-6″)/ δC 138.7 (C-2″), 134.7 (C-4″) and 44.6 (C-6″). So, two single aromatic proton signals were assigned to H-6 and H-6″, respectively. By extensive analysis of HMBC spectrum (**Figure 1**), main correlations were further observed between proton signals at δ 7.07 (H-2′, 6′) and carbon signals at 33.7 (C-α) and 158.0 (C-4′); between four methoxyl protons at δ 3.75, 3.79, 3.88 and 4.01 and carbon signals at 158.1 (C-4‴), 158.0 (C-4′), 145.6 (C-4) and134.7 (C-4″), respectively.

In addition, correlations in the NOESY spectrum were observed between the methoxyl protons at C-4′ (δ 3.79) and H-3′, H-5′ (δ 6.80), which implied that the substitution at C-4′ was a methoxyl group. Then the structure of **2** was characterized and named dengraol B.

Although the pronounced anti-mitotic property of several bibenzyl derivatives was reported[17], the cytotoxic activity of bibenzyl derivatives has scarcely been reported[18,19]. A bibenzyl compound Erianin (from *Dendrobium* genus) showed significant activity against human leukemia HL-60 cell with IC_{50} 0.038 μM[20] has been found. In this paper, the inhibitory activity of selected compounds from this plant and erianin as positive control on HL-60 cells were measured with colorimetric MTT assay[18]. The results are shown as IC_{50} values (μM) in **Table II**. Dengraols A **1** and B **2**, moscatilin **6** and gigantol **7** showed appreciable inhibitory activity against HL-60 with IC_{50} values at 2.1, 6.4, 0.082 and 10.6 μM, respectively, while the others showed no significant activity. Among the isolated benzyl monomers, comparison of the structure and the activity in **3**, **4**, **5** and **8**, compounds **5** and **8** showed weak inhibitory activity at 25.6 and 45.6 μM, while **3** and **4** showed very weak activity, which suggested that *p*-hydroxyl group in B benzyl ring may play an important role in the inhibitory effect. The differences of structure and cytotoxic activity among **6**, **7**, **9** and erianin indicated that the number and the position of hydroxyl and methoxyl groups in the bibenzyl are important for the cytotoxic activity against HL-60, which can be found in the inhibition against K_{562} cell lines of the known bibenzyl derivatives: chrysotobibenzyl, chrystoxine and erianin[18]. In the all bibenzyl derivatives isolated, dengraols A **1** and B **2**, which are asymmetric dimeric bibenzyl derivatives, had more potential activity than those of monomeric bibenzyl derivatives.

Experimental Section

Melting points were measured on a Kofle X-4 micro hot stage melting point apparatus. Optical rotations were measured with a DIP-360 automatic polarimeter (Jasco Co., Tokyo). UV spectra were measured with a SHIMADZU UV-2200 recording

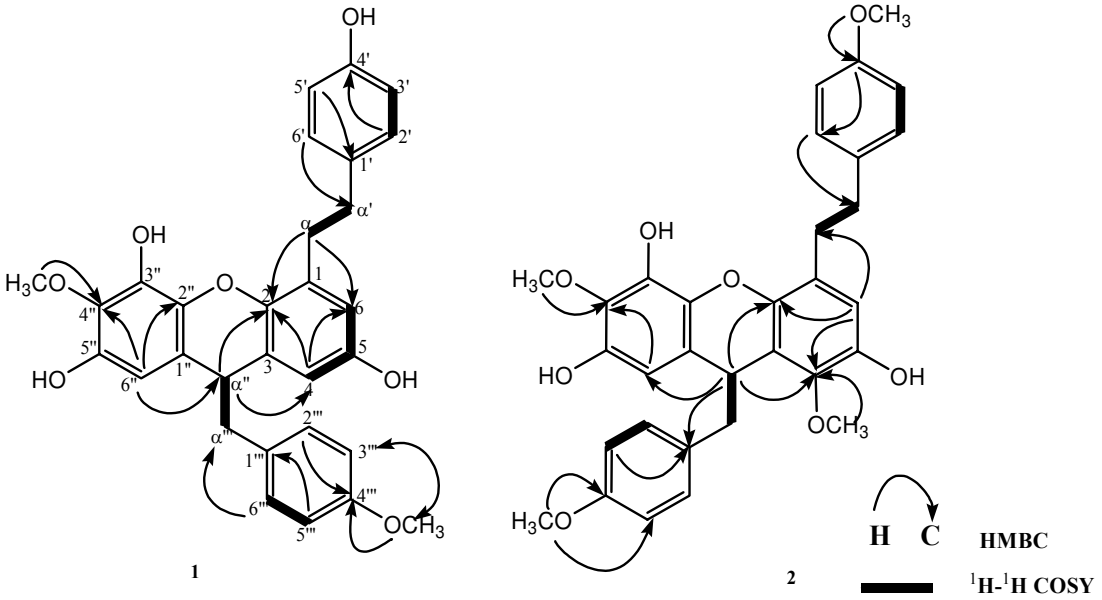


Figure 1 — Main correlations in HMBC and ^{1}H-^{1}H COSY spectra for dengraols A **1** and B **2**

Table II —Cytotoxic activities of 9 compounds from *D. gratiosissimum* against tumor cells HL-60

Compds	IC_{50} (μM)
Dengraol A **1**	2.1
Dengraol B **2**	6.4
3, 5, 4′ -Trihydroxybibenzyl **3**	>100
3, 4′ -Dihydroxy-5-methoxybibenzyl **4**	89.8
3, 4-Dihydroxy-5, 4′ -dimethoxybibenzyl **5**	25.6
Moscatilin **6**	0.082
Gigantol **7**	10.6
Tristin **8**	45.6
Batatasin III **9**	>100
Erianin (positive control)	0.041

spectro photometer (Shimadzu Co., Kyoto) and IR spectra with a SHIMADZU FT/IR infrared spectrometer (Shimadzu Co., Kyoto). ^{1}H- (500 MHz) and ^{13}C- (125 MHz) NMR spectra were run on a Bruker ACF-500 spectrometer in $CDCl_3$ (**1**) and CD_3OD (**2**), the chemical shifts are represented as δ (ppm) with tetramethylsilane (TMS) as an internal standard. ESIMS and HRESIMS were run on Agilent 1100 LC-ESI/MS and G3250AA LC-MSD TOF-ESI/MS spectrometers (Angilent Co., USA), respectively. Column chromatographic separation was carried out using silica gel H-60 (Qingdao Oceanic Chemical Group Corporation, Qingdao, China), Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden) and Lichroprep RP-18 (40-63 μm), GF_{254} silica gel TLC plate (Qingdao Oceanic Chemical Group Corporation, Qingdao, China) were used for analytical TLC. Medium pressure liquid chromatography (MPLC) was carried out on Lichroprep RP-16 (Merck Co., Darmstadt, Germany).

Plant Material: Stems of *D. gartiosissimum* were collected in Yunnan Province, China, in May 2004, and authenticated by Professor Luo-Shan Xu. A voucher specimen of the plant (No. BQ-2004-05) has been deposited at the Department of Pharmacognosy, China Pharmaceutical University.

Isolation Procedure: The fresh stems of *D. gartiosissimum* (50 kg) were cut into pieces, air-dried and extracted with hot EtOH (3 × 25 L), followed by solvent evaporation under reduced pressure to yield a dark residue (850 g). The residue was suspended in water and partitioned with EtOAc and *n*-BuOH successively to give three fractions (EtOAc phase 260 g, *n*-BuOH phase 160 g, and aqueous phase 120 g). An aliquot of the EtOAc-soluble fraction (240 g) was subjected to silica gel column chromatography eluted with petroleum ether-EtOAc (100:1, 95:5, 90:10, 80:20, 70:30, 60:40, 50:50) to yield seven fractions. The fractions IV-VI were further subjected to repeated column chromatography on silica gel eluted with petroleum-EtOAc (1:0-0:1) and on a Sephadex LH-20 column eluted with $CHCl_3$-MeOH (1:1). Repeated column chromatography of Fr. III on silica gel (petroleum ether-acetone, 50:1, 25:1, 10:1),

followed by MPLC on Rp-silica gel (80% MeOH-H_2O) afforded compounds **1** (21 mg) and **2** (18.2 mg), respectively. Compounds **5** (16.2 mg), **6** (21.5 mg) and **7** (40 mg) were obtained from the fraction V after repeated silica gel chromatography (petroleum ether-EtOAc, 25:1, 15:1) and Sephadex LH-20 chromatography with $CHCl_3$-MeOH (1:1). The fraction VI was chromatographed on Sephadex LH-20 chromatography with $CHCl_3$-MeOH (1:1) and repeated silica gel chromatography (petroleum ether-EtOAc, 15:1, 10:1) to yield **3** (205.2 mg), **4** (108.9 mg), **8** (4.5 mg) and **9** (26.7 mg), respectively.

Dengraol A **1**: Pale-yellow oil, $[\alpha]_D^{27}$ −8.1° (*c* = 0.65, MeOH). HRESIMS found *m/z* 500.1284 $[M]^+$, calculated for $C_{30}H_{28}O_7$ *m/z* 500.1259. UV λ_{max} (MeOH) nm: 213, 282. IR (KBr, cm^{-1}): 3415, 1653, 1521, 1438-1475, 842, 795, 710, 684. ^{1}H- and ^{13}CNMR spectral data are shown in **Table I**.

Dengraol B **2**: Yellow oil, $[\alpha]_D^{27}$ -12.1° (*c* = 0.89, MeOH). HREIMS found *m/z* 545.2076 $[M+1]^+$, calculated for $C_{32}H_{32}O_8$ *m/z* 545.2111. UV λ_{max} (MeOH) nm: 218, 284. IR (KBr, cm^{-1}): 3420, 1657, 1531, 1430-1470, 851, 780, 715, 680. ^{1}H- and ^{13}CNMR spectral data see **Table I**.

Evaluation of HL-60 Cytotoxic Activity: The isolated compounds were dissolved in 50% DMSO before investigation of inhibitory activity on HL-60. The tumor cells HL-60 were maintained in RPMI-1640 supplemented with 10% fetal bovine serum. The cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2. The cell growth was evaluated by MTT assay as previously reported[20]. The tumor cells were treated with above seven selected compounds and erianin (positive control) for 72 hr. The results are expressed as the IC_{50} in μM.

Acknowledgements

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