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Hirtellanines A and B, a pair of isomeric isoflavonoid derivatives from *Campylotropis hirtella* and their immunosuppressive activities

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ABSTRACT

A pair of isomeric isoflavonoid derivatives, Hirtellanines A (**1**) and B (**2**), has been isolated from the roots of *Campylotropis hirtella* (Franch.) Schindl. and their structures were elucidated on the basis of spectroscopic methods, with special emphasis on 1D and 2D NMR techniques. The in vitro assay showed that Hirtellanine A had strong B lymphocyte suppression activity (IC_{50}: 0.06 μM) and T lymphocyte suppression activity (IC_{50}: 0.92 μM). Hirtellanine B showed moderate B lymphocyte suppression activity (IC_{50}: 3.00 μM) and T lymphocyte suppression activity (IC_{50}: 9.55 μM). Due to the potent immunosuppressive activities and lower cytotoxicity, Hirtellanine A could be a promising lead towards novel immunosuppressive agents.

Campylotropis hirtella (Franch.) Schindl., which belongs to the plant family Leguminosaea, traditionally was used in Chinese folklore as a herb for activating blood circulation to treat diseases such as irregular menstruation, dysmenorrhea, metrorrhagia, metrostaxis as well as gastric ulcer.[1] It was reported that the Chinese herbs for activating blood circulation usually possessed immunosuppressive activities.[2,3] Thus, *C. hirtella* was selected to initiate our studies for promising immunosuppressants.

So far, tannins, steroids, triterpenes, flavonoids, coumarins, lignans, C-glycosylfavones and aromatic glycosides have been isolated from this plant.[4–8] Our efforts on identifying novel natural products from the roots of *C. hirtella* led to the isolation of a pair of isomeric isoflavonoid derivatives Hirtellanine A (**1**) and B (**2**). This letter describes the structure elucidation of **1** and **2** on the basis of spectroscopic methods, with special emphasis on 1D and 2D NMR techniques. Their immunosuppressive activities on mitogen-induced splenocyte proliferations as well as their cytotoxicity on splenic lymphocytes were also described.

The EtOAc extract of the dried roots (2 kg) of *C. hirtella* was purified by repeated column chromatography over silica gel eluted with petroleum ether/acetone, petroleum ether/ethyl acetate and Pharmadex LH-20 eluted with methanol to afford Hirtellanines A (**1**) and B (**2**) (Fig. 1).[9]

Hirtellanine A (**1**)[10] was obtained as a white amorphous powder and had an empirical formula, $C_{21}H_{16}O_7$ (calcd 380.0896), which was supported by HR-EIMS ([M]$^+$ *m/z* 380.0890). The UV spectroscopic absorption at 270 nm and IR absorption at 1634 cm^{-1} suggested an isoflavone structure for **1**. Moreover the absence of a characteristic proton due to H-2 (usually appearing at δ ca 8) in the ^{1}H NMR spectrum was indicative of a coumaronochromone skeleton.[11] Three aromatic protons, each as singlet, were observed at δ_H 7.35, δ_H 7.05, and δ_H 6.91 in the ^{1}H NMR spectrum. One singlet (3H) was observed at δ_H 3.81, thus evidencing a methoxyl group. Two one-proton doublets (J = 10.0 Hz) at δ_H 6.66 and δ_H 5.91, and two methyl groups at δ_H 1.43 as a singlet indicated the presence of a dimethylchromene ring.[12]

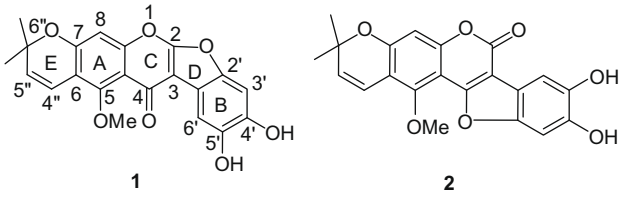

Figure 1. Structures of Hirtellanines A (**1**) and B (**2**).

In addition, the ^{13}C NMR and DEPT experiments of **1** showed 21 carbon resonances containing three methyl groups (δ_C 28.4, 28.4, and 63.2), five methines (δ_C 99.5, 101.6, 106.5, 115.6, and 132.2), and 13 quaternary carbons (δ_C 78.5, 112.3, 113.7, 114.1, 114.1, 142.8, 144.5, 144.9, 155.5, 155.9, 157.3, 163.0, 172.3). The spectroscopic data supported the presence of a coumaronochromone skeleton with a dimethylchromene ring moiety. In the HMBC experiments (Fig. 2), the proton signal at δ_H 6.91 showed correla-

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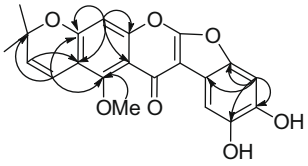

Figure 2. Key HMBC (H-C) correlations of Hirtellanine A (**1**).

tions with the carbon resonances at δ_C 112.3 (C-6), δ_C 157.3 (C-7), δ_C 155.5 (C-9), δ_C 113.7 (C-10) allowing us to assign δ_H 6.91 at the position of H-8. The signal at δ_H 6.66 (H-4″) revealed correlations to δ_C 155.9 (C-5), δ_C 157.3 (C-7), and δ_C 78.5 (C-6″), while the signal at δ_H 5.91 (H-5″) displayed a correlation to δ_C 113.7 (C-6), which supported that the dimethylchromene ring was fused to C-6 and C-7. The methoxyl group attached to C-5 accounted for the HMBC correlation between δ_H 3.81 and δ_C 155.9. Two isolated aromatic proton signals at δ_H 7.35, δ_H 7.05 as well as the signals of two adjacent hydroxyl groups at δ_H 9.28 suggested 4′-and 5′-substitutions on ring B. Thus, the structure of compound **1** was established as 4′,5′-dihydroxy-5-methoxy-[6″,6″-dimethylpyrano(2″,3″:7,6)] coumaronochromone. All the ^{1}H and ^{13}C NMR spectroscopic signals of **1** (Table 1) were assigned on the basis of ^{1}H–^{1}H COSY, HSQC, HMBC spectra.

Hirtellanine B (**2**),[13] obtained as a white amorphous powder, showed a $[M+Na]^+$ peak at *m*/*z* 403.0894 (calcd 403.0794), in the high-resolution electrospray-ionization time of flight mass spectroscopy (HR-ESI-TOF-MS), corresponding to a molecular formula $C_{21}H_{16}O_7$. The IR absorptions due to δ-lactone carbonyl (1718 cm^{-1}), aromatic ring (1618, 1458 cm^{-1}), and aromatic C-O (1269 cm^{-1}) as well as the characteristic UV absorptions at 245 and 360 nm indicated the coumestan nature of compound **2**.[14–16] The NMR spectroscopic data (Table 1) of **2** was closely related to that of **1**. The signals of a dimethylchromene ring, a methoxyl group and three isolated aromatic protons were also observed in the ^{1}H NMR. However, the carbon signals of A and C rings shifted upfield, especially C-3, C-4, and C-10 shifted from δ_C 114.1, 172.3, 112.3 to δ_C 103.2, 157.3, 101.6; signal at C-2′ shifted downfield from δ_C 142.8 to δ_C 149.2, which accounted for the presence of coumestan instead of coumaronochromone. Furthermore, the structure was confirmed by HMBC correlations and concluded to be 4′,5′-dihydroxy-5-methoxy-[6″,6″-dimethylpyrano(2″,3″:7,6)] coumestan.

Table 1
NMR spectroscopic data of compounds Hirtellanine A (**1**) and B (**2**) (δ values in ppm, *J* values in Hz)

No.	Hirtellanine A (**1**)[a]		Hirtellanine B (**2**)[b]	
	^{1}H	^{13}C	^{1}H	^{13}C
2		163.0		157.1
3		114.1		103.2
4		172.3		157.3
5		155.9		150.6
6		113.7		112.0
7		157.3		155.8
8	6.91 s	101.6	6.79 s	100.8
9		155.5		153.7
10		112.3		101.6
1′		114.1		113.5
2′		142.8		149.2
3′	7.05 s	99.5	7.20 s	98.8
4′		144.5		145.9
5′		144.9		144.5
6′	7.35 s	106.5	7.27 s	104.6
4″	6.66 (d, 10.0)	115.6	6.68 (d, 10.0)	114.9
5″	5.91 (d, 10.0)	132.2	5.95 (d, 10.0)	131.5
6″		78.5		77.7
6″-Me	1.43 s	28.4	1.45 s	27.9
6″-Me	1.43 s	28.4	1.45 s	27.9
5-OMe	3.81 s	63.2	3.96 s	63.1

[a] ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR in DMSO.
[b] ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR in DMSO.

Table 2
Summary of cytotoxicity and suppressive activities[a]

Compounds	Cytotoxicity	Proliferative responses of lymphocytes			
		ConA		LPS	
	CC_{50} (μM)	IC_{50} (μM)	SI[b]	IC_{50} (μM)	SI
Hirtellanine A	3.03	0.92	3.29	0.06	50.50
Hirtellanine B	26.32	9.55	2.76	3.00	8.77
CsA	0.88	0.01	88.00	0.07	12.39

[a] The assay conditions were described in references and notes.
[b] SI: safety index, the ratio of CC_{50}/IC_{50}.

Hirtellanines A and B described above were investigated for their immunosuppressive activities on ConA as well as LPS-induced splenocyte proliferation in vitro.[17] The results (Table 2) showed that Hirtellanine B exhibited moderate activity with an IC_{50} value of 3.00 μM (B lymphocyte suppression), and an IC_{50} value of 9.55 μM (T lymphocyte suppression), while Hirtellanine A had very potent activity with an IC_{50} value of 0.06 μM (B lymphocyte suppression), and an IC_{50} value of 0.92 μM (T lymphocyte suppression). The cytotoxicity on splenic lymphocytes was tested also, and both of them showed lower cytotoxicity with the CC_{50} values of 3.03 μM and 26.32 μM, respectively. It is worth noting that Hirtellanine A displayed stronger B lymphocyte suppressive activity and higher SI value than cyclosporin A. Thus, Hirtellanine A could be a lead for new immunosuppressants.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.05.043.

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9. *Isolation of Hirtellanines A and B*: The roots of *Campylotropis hirtella* (Franch.) Schindl. were collected from Yunnan province, China, and authenticated by Professor Zhou Xiujia of Shanghai University of TCM. A voucher specimen had been deposited in the herbarium of the Shanghai University of TCM. The air-dried and comminuted roots of *C. hirtella* (2 kg) were extracted with 95% EtOH. The EtOH extracts were evaporated under reduced pressure to give a residue (387 g), which was suspended in distilled water and partitioned successively with petroleum ether, EtOAc, and *n*-BuOH. The EtOAc extract was applied to a silica gel column, eluting with petroleum ether containing increasing amounts of EtOAc. After repeated column chromatography over silica gel eluted with

petroleum ether/acetone, petroleum ether/ethyl acetate, and Pharmadex LH-20 eluted with methanol, Hirtellanines A (16 mg), and B (12 mg) were obtained.
10. Data for Hirtellanine A (**1**): white amorphous powder, UV λ_{max} (MeOH): 270, 305 nm; IR ν_{max} (KBr): 3211, 1634, 1597, 1518, 1456, 1333, 1288, 1120, 873; ^{1}H and ^{13}C NMR data: see Table 1; HR-EIMS *m*/*z* 380.0890 $[M]^+$ (calcd 380.0896 for $C_{21}H_{16}O_7$); EIMS *m*/*z* (rel. int.): 380 (100), 365 (90), 351 (15), 336 (15), 322 (15), 306 (11), 294 (19), 183 (25), 139 (40), 91 (51), 55 (55); ESI-MS *m*/*z* 381 $[M+H]^+$.
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13. Data for Hirtellanine B (**2**): white amorphous powder, UV λ_{max} (MeOH): 245, 360 nm; IR ν_{max} (KBr): 3317, 2922, 1718, 1618, 1458, 1369, 1350, 1269, 1150, 1122, 999, 835; ^{1}H and ^{13}C NMR data: see Table 1; HR-ESI-TOF-MS *m*/*z* 403.0894 $[M+Na]^+$ (calcd 403.0794 for $C_{21}H_{16}O_7Na$); ESI-MS *m*/*z* 381 $[M+H]^+$.
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17. The two compounds were dissolved in pure dimethyl sulfoxide (DMSO) as a stock solution, and stored at 4 °C. The stock solution was diluted to the needed concentrations with RPMI-1640 supplemented with 10% FBS. The final concentration of DMSO in the culture medium was less than 0.01%, which had no influence on the assays. Splenic lymphocytes were cultured for 48 h with 5 μg/mL of ConA or 10 μg/mL of LPS plus Hirtellanines A and B and Cyclosporin A. Cells were pulsed with 0.5 μCi/well of [3H]-thymidine for 8 h and harvested onto glass filters. The incorporated radioactivity was then counted using a Beta Scintillation Counter (MicroBeta Trilux, PerkinElmer Life Sciences, Boston, MA). Cytotoxicity was assessed by the MTT assay. Briefly, splenic lymphocytes were cultured for 48 h with Hirtellanines A and B and Cyclosporin A. The cells cultured with media alone were used as controls. MTT (5 mg/mL) reagent was added 4 h before the end of culture, and the supernatants were discarded, then cells were lysed with dimethyl sulfoxide (DMSO). O.D. values were read at 570 nm, and the percentage of cell death was calculated.