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Immunosuppressive flavones and lignans from *Bupleurum scorzonerifolium*

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Abstract

Two lignans, isochaihulactone and chaihunaphthone, together with eleven known compounds were isolated from the root of *Bupleurum scorzonerifolium*. Their structures were established on the basis of spectral evidence. In biological testing, eugenin and saikochromone potently inhibited CD28-costimulated activation of human peripheral blood T cells.

Keywords: *Bupleurum scorzonerifolium*; Umbelliferae; Flavones; Lignans; Isochaihulactone; Chaihunaphthone; Immunosuppressive effect

1. Introduction

Nan-Chai-Hu, the root of *Bupleurum scorzonerifolium* Willd. (Umbelliferae), is a traditionally important Chinese herb used in the treatment of influenza, fever, malaria, and menstrual disorders in China (Chang and But, 1987). In our search for immunosuppressive agents from Chinese herbs, we found that the crude extract of Nan-Chai-Hu specifically inhibited CD28-costimulated activation of human peripheral blood T cells. The acetone, MeOH and H_2O extracts of Nan-Chai-Hu showed a remarkable inhibitory activity at dose of 10 μg/ml resulting in 70, 76, and 55% inhibition of IL-2 secretion toward PMA and anti-CD28 monoclonal antibody costimulated activation human peripheral blood T cells, and 85, 68, and 82% inhibition at dose of 100 μg/ml, respectively. To investigate the active components, we isolated **1**–**13** including two lignans, isochaihulactone (**2**) and chaihunaphthone (**3**), and 11 known compounds from this plant. In this paper, the isolation of these compounds from Nan-Chai-Hu and their immunosuppressive effect toward human peripheral T-cell are reported.

2. Results and discussion

The EtOAc-soluble part of the MeOH extract of the *Bupleurum scorzonerifolium* root tissue was subjected to silica gel, Sephadex LH-20 and Lobar (RP-8) chromatography to yield two lignans, isochaihulactone (**2**) and chaihunaphthone (**3**), together with 11 known compounds including three flavones, five lignans, two chromones, and one xanthone. The known compounds were identified as nemerosin (**1**) (Feliciano et al., 1989), isoscutellarein-8-methyl ether (**4**) (Tomimori et al., 1982), oroxylin (**5**) (Buschi et al., 1991; Biekofsky et al., 1991), wogonin (**6**) (Harrison et al., 1994; Joshi et al., 1983), eugenin (**7**) (Ali et al., 1990; Tsui and Brown, 1996), saikochromone A (**8**) (Kobayashi et al., 1990), kaerophyllin (**9**) (Mikaya et al., 1981), isokaerophyllin (**10**) (Gonzalez et al., 1990), (−)-yatein (**11**) (Badheka et al., 1986), chinensinaphthol (**12**) (Harrowven, 1993; Iwasaki et al., 1995) and 1,2,3,7-tetramethoxyxanthone (**13**) (Ikeya et al., 1991; Ito et al., 1977), by comparison of spectroscopic data in the literature and/or authentic compounds. Among these, compounds, **4**–**12** are reported for the first time from this plant.

Compound **2** was obtained as white needles from MeOH, mp 137–138 °C and has a molecular formula of $C_{22}H_{22}O_7$ (HREIMS) this being the same as that of **1**. The UV spectrum (247, 298, and 327 nm) and the IR spectrum (1745, 1635, 1581 cm^{-1}), were similar to those

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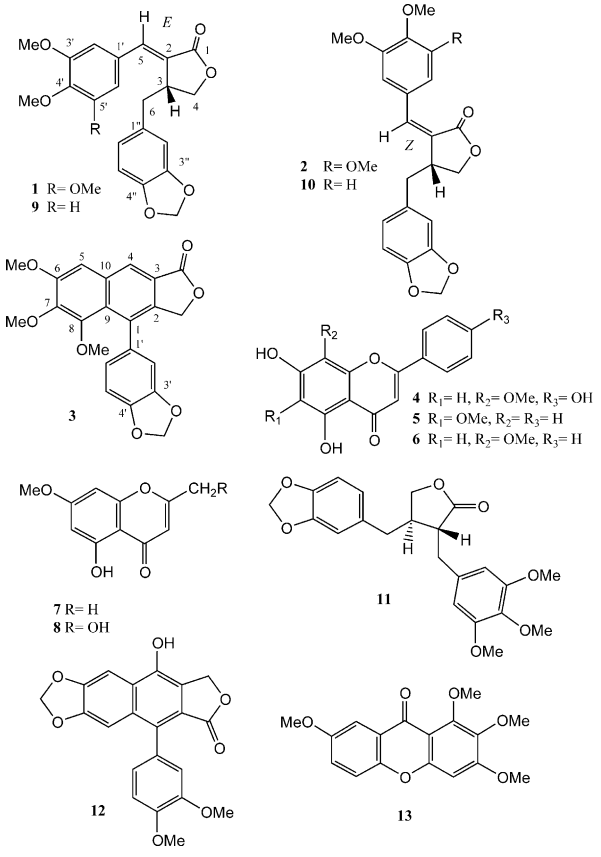

of **1**, indicated the presence of a γ-butyrolactone lignan (Gonzalez et al., 1990). The ^{13}C NMR and ^{1}H NMR spectra of **2** showed a close resemblance to that of **1** except that the H-2′, 6′ (δ 7.24) and C-3 (δ 44.43), C-5 (δ 140.60), and C-6 (δ 40.72) signals were further downfield in **2**, and the γ-lactone carbonyl (C=O, δ 169.29) is further upfield in **2**. Thus, **2** is a geometric isomer of **1** with a *Z* configuration on C-2 (**5**). This geometric structure was further supported by the NOESY spectral data exhibiting H-6 (δ 2.78, 2.92; *dd*) NOEs to H-5 (δ 6.60). The negative optical activity of **2** is similar to that of kaerophyllin and other lignans which have structures with H-3β (Gonzalez et al., 1990). Compound **2** is named isochaihulactone (**2**).

Compound **3** was obtained as a yellow amorphous solid, mp 164–166 °C with a molecular formula of $C_{22}H_{18}O_7$ (HREIMS). The spectrum (228, 258, and 318 nm) indicated the presence of an arylnaphthalene lignan (Klemm et al., 1966). The IR {1759 cm^{-1} (C=O)} and the ^{13}C NMR {δ 171.38 *s* (C=O)}spectrum indicated the presence of a γ-butyrolactone (Harrowven, 1993; Iwasaki et al., 1995). The ^{1}H NMR spectrum ($CDCl_3$) of 3 showed one ABX system and two singlets in the aromatic region, and one methylenedioxy, three methoxy, and methylene signals in the aliphatic region which may be due to one methylenedioxyphenyl and one trimethoxy-3H-naphtho [2,3-c]furan-1-one groups (Table 3). The methoxy signal at δ 3.36, being further upfield than

Table 1
^{1}H NMR spectral data for **1**, **2**, and **9**–**10**

Position	δ H mult (*J* Hz)			
	1	**2**	**9**	**10**
3	3.80 *m*[a]	3.29 *m*[a]	3.78 *m*[a]	3.28 *m*[a]
4α	4.22 *dd* (9.1, 1.6)	4.10 *dd* (9.0, 3.8)	4.24 *m*[a]	4.09 *dd* (9.0, 3.8)
4β	4.27 *dd* (9.1, 6.7)	4.31 *dd* (9.0, 7.3)	4.24 *m*[a]	4.30 *dd* (9.0, 7.3)
5	7.47 *d* (1.5)	6.60 *d* (1.5)	7.50 *s*	6.61 *d* (1.6)
6α	2.63 *dd* (14.1, 9.9)	2.78 *dd* (13.7, 9.0)	2.58 *dd* (14.4, 10.3)	2.77 *dd* (13.8, 9.0)
6β	2.99 *dd* (14.1, 4.7)	2.92 *dd* (13.7, 6.7)	3.01 *dd* (14.4, 4.1)	2.92 *dd* (13.8, 6.7)
2′	6.74 *s*	7.24 *s*	7.02 *d* (1.5)	8.07 *d* (2.0)
5′			6.91 *d* (8.4)	6.82 *d* (8.4)
6′	6.74 *s*	7.24 *s*	7.19 *dd* (8.4, 1.5)	7.15 *dd* (8.4, 2.0)
2″	6.57 *m*[a]	6.67 *d* (1.4)	6.62 *d* (1.5)	6.68 *d* (1.6)
5″	6.66 *d* (7.8)	6.74 *d* (7.8)	6.69 *d* (7.8)	6.74 *d* (7.9)
6″	6.56 *m*[a]	6.61 *dd* (7.8, 1.4)	6.59 *dd* (7.8, 1.5)	6.62 *dd* (7.9, 1.6)
3′-OMe	3.85 *s*	3.87 *s*	3.88 *s*	3.89 *s*
4′-OMe	3.86 *s*	3.87 *s*	3.91 *s*	3.92 *s*
5′-OMe	3.85 *s*	3.87 *s*		
OCH_2O	5.88 *d* (1.4) 5.89 *d* (1.4)	5.93 *d* (1.3) 5.94 *d* (1.3)	5.90 *d* (1.5)	5.93 *d* (1.4) 5.94 *d* (1.4)

[a] Data with multiplicity "*m*" were overlapped or poorly resolved signals whose chemical shifts were assigned from COSY-45 or HMQC spectra.

Table 2
^{13}C NMR spectral data for 1, 2, and 9–10

Position	δ C mult.[a]			
	1	**2**	**9**	**10**
1	172.28 *s*	169.29 *s*	172.56 *s*	169.61 *s*
2	126.99 *s*	126.36 *s*	125.58 *s*[b]	124.66 *s*[b]
3	39.42 *d*	44.43 *d*	39.60 *d*	44.42 *d*
4	69.66 *t*	69.82 *t*	69.46 *t*	69.46 *t*
5	137.61 *d*	140.60 *d*	137.39 *d*	140.68 *d*
6	37.68 *t*	40.72 *t*	37.53 *t*	40.82 *t*
1′	129.37 *s*	128.83 *s*	126.82 *s*[b]	126.81 *s*[b]
2′	107.28 *d*	108.65 *d*	112.87 *d*[b]	113.60 *d*[b]
3′	153.32 *s*	152.62 *s*	150.65 *s*	150.54 *s*
4′	139.77 *s*	139.61 *s*	149.05 *s*	148.36 *s*
5′	153.32 *s*	152.62 *s*	111.25 *d*[b]	110.22 *d*[b]
6′	107.28 *d*	108.65 *d*	123.54 *d*[b]	125.71 *d*[b]
1″	131.17 *s*	131.31 *s*	131.43 *s*[b]	131.46 *s*[b]
2″	108.96 *d*	109.29 *d*	108.91 *d*[b]	109.28 *d*[b]
3″	147.91 *s*	147.94 *s*	147.91 *s*	147.92 *s*
4″	146.52 *s*	146.49 *s*	146.49 *s*	146.44 *s*
5″	108.38 *d*	108.39 *d*	108.41 *d*[b]	108.36 *d*[b]
6″	121.80 *d*	122.29 *d*	121.89 *d*	122.27 *d*
3′-OMe	56.20 *q*	56.18 *q*	55.93 *q*	55.84 *q*
4′-OMe	60.93 *q*	60.90 *q*	55.96 *q*	55.93 *q*
5′-OMe	56.20 *q*	56.18 *q*		
OCH_2O	101.04 *t*	101.03 *t*	101.02 *t*	101.82 *t*

[a] Multiplicities were obtained from DEPT experiment.
[b] Revised signals (Gonzalez et al., 1990).

other methoxy signals, was shielded by the methylenedioxyphenyl group. The NOESY experiment displayed NOE interaction: H-4 (δ 8.29 *s*)/H-5 (δ 7.14 *s*), H-5/6-OMe (δ 4.01 *s*), and 7-OMe (δ 3.92 *s*)/8-OMe (δ 3.36 *s*) indicated the presence of 5,6,7-trimethoxy-3H-naphtho[2,3-c]furan-1-one. Moreover, there were also NOE interactions between H-2′ and H-6/2-CH_2 (δ 5.02, 5.10) and 8-OMe (δ 3.36), respectively. Thus, its structure was determined as 4-benzo[1,3]-dioxol-5-yl-5,6,7-trimethoxy-3H-naphtho[2,3-c]furan-1-one (**3**), which was previously obtained as a synthetic compound (Klemm et al., 1966), but is now described for the first time from a natural source and named chaihunaphthone (**3**).

Analyses of HMBC, HMQC and COSY-45 data, furnished the complete ^{1}H and ^{13}C NMR assignment (Tables 1–3) for **1–3**, **9–10**, and **12**. Therefore, the chemical shifts of ^{13}C NMR of **9** and **10** were partially revised (Table 2).

In biological testing, the major isolated compounds **1**, **2**, and **5–9** were examined on CD28-costimulated activation of human peripheral T cells. Eugenin (**7**) and saikochromone (**8**), which have 50% inhibitory activities of IL-2 secretion at dose less than of 5 μg/ml, showed the most potent immunosuppressive activities toward PMA + anti-CD28-costimulated T cells without significant cytotoxic effect on T cells survival (Table 4 and 5). Compounds **1**, **2**, **5**, **6** and **9** exhibited potent inhibitory activities on CD28-costimulated T cells at dose less than 2 μg/ml, but also showed significant cytotoxic effect on T cells survival (Tables 4 and 5).

Table 3
^{1}H and ^{13}C NMR spectral data for **3** and **12**[a]

Position	**3**		**12**	
	δ H mult (*J* Hz)	δ C mult[b]	δ H mult (*J* Hz)	δ C mult[b]
1		131.73 *s*		130.38 *s*
2		139.16 *s*		122.44 *s*
3		122.39 *s*		119.17 *s*
4	8.29 *s*	124.93 *d*		145.36 *s*
5	7.14 *s*	104.54 *d*	7.60 *s*	98.10 *d*
6		153.46 *s*		148.76 *s*
7		145.01 *s*		148.53 *s*
8		149.65 *s*	6.84 *s*	102.61 *d*
9		126.26 *s*		131.15 *s*
10		132.07 *s*		124.73 *s*
C=O		171.38 *s*		169.55 *s*
CH_2	5.02 *d* (15.0)	69.84 *t*	5.34 *s*	66.77 *t*
	5.10 *d* (15.0)			
1′		133.19 *s*		127.57 *s*
2′	6.75 *d* (1.6)	108.88 *d*	6.81 *d* (1.2)	114.29 *d*
3′		147.21 *s*		148.12 *s*
4′		146.61 *s*		148.25 *s*
5′	6.86 *d* (7.8)	107.78 *d*	7.04 *d* (8.0)	111.23 *d*
6′	6.71 *dd* (7.8, 1.6)	120.73 *d*	6.76 *dd* (8.0, 1.2)	122.61 *d*
OMe	4.01 *s* (6-OMe)	55.97 *q*		
OMe	3.92 *s* (7-OMe)	61.09 *q*	3.69 *s* (3′-OMe)	55.53 *q*
OMe	3.36 *s* (8-OMe)	60.89 *q*	3.83 *s* (4′-OMe)	55.46 *q*
OCH_2O	6.02 *d* (1.4)	101.11 *t*	6.14 br *s*	102.01 *t*
	6.03 *d* (1.4)			

[a] Compound **3** in $CDCl_3$; compound **12** in DMSO-d_6.
[b] Multiplicities were obtained from DEPT experiment.

Table 4
Cytotoxic activity of compounds **1**, **2**, and **5–9** on human peripheral blood T cells

Dose (μg/ml)	T cell survival rate (%)[a]					
Compounds	0.4	1.0	2.0	5.0	10.0	25.0
Nemerosin (**1**)	–	64	69	–	63	–
Isochaihulactone (**2**)	66	–	73	76	–	–
Oroxylin (**5**)	–	61	72	67	–	–
Wogonin (**6**)	60	–	69	–	60	–
Eugenin (**7**)	–	–	–	98	91	68
Saikochromone A (**8**)	–	–	–	90	85	72
Kaerophyllin (**9**)	–	–	71	–	65	–

[a] Purified human peripheral blood T cell at 5×10^5/ml were treated with vehicles or compounds. The surviving cells were assayed using MTT colorimetric method. The percent cytotoxic activity was calculated as (sample value–medium control)/(high control–medium control) × 100% (Lai et al., 1999).

3. Experimental

3.1. Generals

Mps: uncorr; ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz) and 2D NMR (500 MHz): $CDCl_3$ and DMSO-d_6 using the solvent peak as int. standard; MS: direct inlet system; UV: MeOH or $CHCl_3$; IR: KBr disc.

3.2. Plant material

Bupleurum scorzonerifolium Willd. root were supplied from Chung-Yuan Co., Taipei, and the plant was identified by Prof H.C. Lin of the National Defense Medicinal Center, where a voucher specimen was deposited (NDMCP No. 900801).

Table 5
Immunosuppressive activities of compounds **1**, **2**, and **5–9** on CD28-costimulated IL-2 secretion

Dose (μg/ml)	Inhibition of IL-2 secretion[a]					
Compounds	0.4	1.0	2.0	5.0	10.0	25.0
Nemerosin (**1**)	–	42%	53%	–	48%	–
Isochaihulactone (**2**)	52%	–	54%	50%	–	–
Oroxylin (**5**)	–	45%	49%	47%	–	–
Wogonin (**6**)	50%	–	77%	–	99%	–
Eugenin (**7**)	–	–	–	59%	72%	95%
Saikochromone A (**8**)	–	–	–	63%	71%	95%
Kaerophyllin (**9**)	–	–	49%	–	67%	–

[a] Purified human peripheral blood T cells at 5×10^5 /ml were treated in triplicate with various concentration of compounds for 24 h and then stimulated with anti-CD28 + PMA for another 24 h. The supernatants were collected for IL-2 measurements. Control value was 771 ± 128 pg/ml concentration of IL-2 secretion. Data are expressed as % of inhibition (average) from at least three different donors (Lai et al., 1999).

3.3. Extraction and isolation

The dried and powdered roots (18.5 kg) of *B. scorzonerifolium* were extracted with MeOH (25 l × 5) at room temperature for 4 h. The combined MeOH extracts were concentrated in vacuo to yield a residue (1.85 kg), which was dissolved in H_2O–MeOH (5:95) solution (1 l), and then partitioned (1: 1) with *n*-hexane to give the *n*-hexane-soluble fraction (154.5 g). The H_2O–MeOH (5:95) layer was evaporated to remove residual MeOH, and then distilled H_2O (500 ml) was added. This aqueous solution was partitioned with EtOAc to obtain EtOAc-soluble (315.3 g) and H_2O-soluble fractions (1380.20 g). The EtOAc-soluble fraction (315.3 g) was subjected to chromatography over silica gel and eluted with $CHCl_3$–MeOH (10:1), $CHCl_3$–MeOH (9:1), MeOH, successively, to afford five fractions. The third fraction (70.1 g) was applied to silica gel using CH_2Cl_2–MeOH (99:1) as eluents to yield nemerosin (**1**; 6.2 g), isochaihulactone (**2**; 50.0 mg), oroxylin (**5**; 65.0 mg), wogonin (**6**; 100.0 mg), eugenin (**7**; 30.0 mg), and kaerophyllin (**9**; 2.5 g). Further separation of the mother liquid by Sephadex LH-20 (eluent: MeOH) and a Lobar RP-8 column (B type) eluted with % MeOH/H_2O (4:1) yielded chaihunaphthone (**3**; 6.4 mg), isokaerophyllin (**10**; 11.2 mg), (−)-yatein (**11**; 112.0 mg), chinensinaphthol (**12**; 7.0 mg), and 1,2,3,7-tetramethoxyxanthone (**13**; 13.0 mg). The fourth fraction (35.9 g) was applied to silica gel using CH_2Cl_2–MeOH (95: 5) as eluents to yield saikochromone (**8**; 50.0 mg), and further separation through Sephadex LH-20 chromatography afforded isoscutellarein-8-methyl ether (**4**; 5.5 mg).

3.4. Isochaihulactone (*2*)

White needle crystals; mp 137–138 °C; $[\alpha]_D^{25}$ –29.0° (*c* 0.5, $CHCl_3$); IR (KBr) ν_{max} cm^{-1}: 1745, 1635, 1581, 1335, 1153; UV ($CHCl_3$) λ_{max} nm (log ϵ): 247 (4.08), 298 (4.17), 327 (4.08); for ^1H and ^{13}C NMR ($CDCl_3$), see Tables 1 and 2; HREIMS *m/z* 398.1374 (calcd for $C_{22}H_{22}O_7$ $[M]^+$ 398.1365); EIMS, 70 eV, *m/z* (rel. int.): 398 ($[M]^+$, 18), 263 (100), 207 (16), 135 (35).

3.5. Chaihunaphthone (*3*)

White needle crystals; mp 164–166 °C; IR (KBr) ν_{max} cm^{-1}: 1759, 1608, 1226, 1029; UV ($CHCl_3$) λ_{max} nm (log ϵ): 228 (4.40), 258 (4.48), 318 (3.84); ^1H and ^{13}C NMR ($CDCl_3$), see Table 3; HREIMS *m/z* 394.1039 (calcd for $C_{22}H_{18}O_7$ $[M]^+$ 394.1052); EIMS, 70 eV, *m/z* (rel. int.): 394 ($[M]^+$, 100), 349 (42), 286 (10), 271 (45), 244 (95), 229 (25), 215 (25), 203 (50), 135 (15), 84 (25).

3.6. Immunosuppression assay

T lymphocytes were purified from whole blood by negative selection. The PMA + anti-CD28 monoclonal antibody stimuli mimicked CD28 costimulation. The determination of IL-2 concentration of CD28-costimulated T cells was performed by ELISA assay. Drug cytotoxicity was determined by MTT colorimetric assay. (Lai et al., 1999)

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