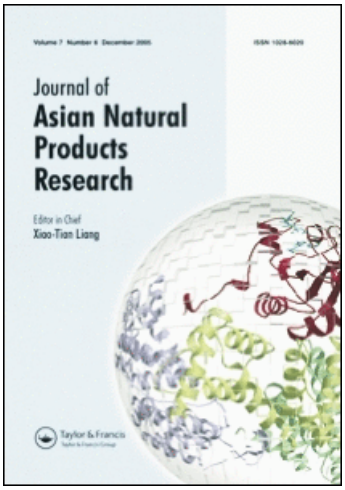

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

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Online publication date: 11 August 2010

To cite this Article Hou, Wen-Rui , Su, Zhi-Qiang , Pi, Hui-Fang , Yao, Guang-Min , Zhang, Peng , Luo, Xiang , Xie, Sheng-Nan and Xiang, Ming(2010) 'Immunosuppressive constituents from *Urtica dentata* Hand', Journal of Asian Natural Products Research, 12: 8, 707 — 713
To link to this Article: DOI: 10.1080/10286020.2010.490779
URL: http://dx.doi.org/10.1080/10286020.2010.490779

Journal of Asian Natural Products Research
Vol. 12, No. 8, August 2010, 707–713

NOTE

Immunosuppressive constituents from *Urtica dentata* Hand

Wen-Rui Hou[a†], Zhi-Qiang Su[c†], Hui-Fang Pi[b], Guang-Min Yao[b], Peng Zhang[b], Xiang Luo[a], Sheng-Nan Xie[a] and Ming Xiang[ab]*

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(*Received 27 January 2010; final version received 30 April 2010*)

A novel biscoumarin, 6,6′,7,7′-tetramethoxyl-8,8′-biscoumarin (**1**), was isolated from the ethyl acetate extract of *Urtica dentata* Hand, together with five known compounds named as 7,7′-dihydroxy-6,6′-dimethoxy-8,8′-biscoumarin (**2**), 7,7′-dimethoxy-6,6′-biscoumarin (**3**), scoparone (**4**), vanillic acid (**5**), and daucosterol (**6**). Structures of the isolated compounds were elucidated on the basis of spectroscopic analysis including 2D NMR experiments. Compounds **1** and **2** were confirmed to be a rare carbon–carbon linked symmetrical biscoumarin. Compounds **1–4**, especially **1** ($IC_{50} = 8.18 \times 10^{-5}$ mol/l), showed potent immunosuppressive activities as determined by the Cell Counting Kit-8 assay for lymphocyte proliferation. Also, in the FACS analysis, **1** ($IC_{50} = 5.19 \times 10^{-4}$ mol/l) promoted the differentiation of $CD4^+CD25^+Foxp3^+$ T regulatory cells distinctly compared to the normal control. Thus, **1** possessed specific immunosuppressive property by eliciting T regulatory cells, which may provide a potential treatment strategy for autoimmune diseases.

Keywords: *Urtica dentata* Hand; biscoumarin; immunosuppression

1. Introduction

Natural products derived from medicinal plants are an abundant source of pharmacologically active compounds, many of which have important allies in the treatment of human diseases [1]. *Urtica dentata* Hand, commonly known as Honghuoma [2], is a perennial herb belonging to Urticaceae and grows indigenously in the southwest of Hubei Province, China. It has been historically used in those places as a remedy for rheumatoid arthritis and some other autoimmune diseases [3].

Our previous research found that the ethyl acetate (EtOAc) extract of *U. dentata* possessed powerful immunosuppressive abilities against arthritis [4] and skin allograft transplantation [5]. These medicinal properties prompted us to carry out phytochemical investigation on *U. dentata*. Our current research has led to the isolation of one novel biscoumarin with rare symmetrical structure linked by carbon–carbon bond (**1**), along with five known compounds (**2–6**) (Figure 1), from the EtOAc extract of *U. dentata*. The immunosuppressive abilities of these isolated compounds were studied *in vitro*. In this paper, we elucidate the isolation and characterization of the immunosuppressive constituents from the EtOAc extract of *U. dentata*.

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ISSN 1028-6020 print/ISSN 1477-2213 online

DOI: 10.1080/10286020.2010.490779
http://www.informaworld.com

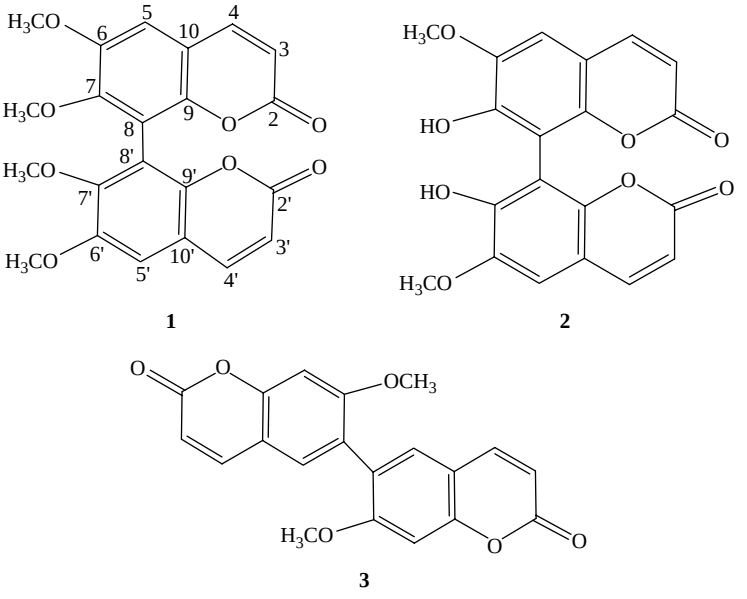

Figure 1. Structures of compounds **1**–**3**.

2. Results and discussion

Compound **1** was obtained as a yellow buff needle crystal. Its molecular formula was determined to be $C_{22}H_{18}O_8$ by the HR-EI-MS at *m/z* 410.1028 $[M]^+$. The IR spectrum showed the presence of carbonyl (1702 cm^{-1}) and aromatic rings (1603, 1573 and 1497 cm^{-1}). The ^1H NMR spectrum (Table 1) indicated the presence of two methoxyls at δ 3.94 (3H, s) and 4.09 (3H, s), an aromatic proton at δ 6.66 (1H, s), and an AB system at δ 6.27 (1H, d, $J = 8.0$ Hz) and 7.60 (1H, d, $J = 8.0$ Hz). ^{13}C NMR spectroscopic data (Table 1) showed 11 carbon signals, which were only half of the number of carbon atoms in the molecular formula of $C_{22}H_{18}O_8$, indicating that **1** might have a symmetrical structure. ^1H and ^{13}C NMR spectroscopic data of **1** were similar to the corresponding data of scoparone [6–7]. The differences were that the signal of H-8 had disappeared, while the chemical shift of C-8 was shifted toward downfield. These observations suggested that **1** was a symmetrical scoparone dimer coupled by a carbon–carbon bond. This was further confirmed by the HMBC spectrum (Figure 2), which showed the vital correlations of H-5 at δ 6.66 with C-4 at δ 144.6, C-6 at δ 143.8 and C-10 at δ 111.2; H-3 at δ 6.27 with C-2 at δ 160.6 and C-10 at δ 111.2; H-4 at δ 7.60 with C-5 at δ 103.2, C-9 at δ 142.5 and C-10 at δ 111.2; 6-OCH_3 at δ 3.94 with C-6 at δ 143.8; and 7-OCH_3 at δ 4.09 with C-8 at δ 134.5. Thus, the structure of **1** was established to be 6,6′,7,7′-tetramethoxyl-8,8′-biscoumarin.

Table 1. ^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectral data of compound **1** in $CDCl_3$ (δ in ppm, J values in Hz).

Position	δ_H	δ_C
2,2′	–	160.6
3,3′	6.27 d ($J = 8.0$)	113.5
4,4′	7.60 d ($J = 8.0$)	144.6
5,5′	6.66 s	103.2
6,6′	–	143.8
7,7′	–	143.1
8,8′	–	134.5
9,9′	–	142.5
10,10′	–	111.2
6,6′-OCH_3	3.94 s	59.5
7,7′-OCH_3	4.09 s	61.6

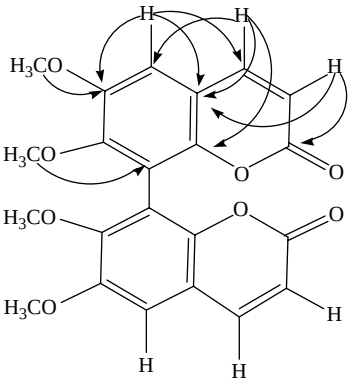

Figure 2. Key HMBC correlations of compound **1**.

Compound **1** was found to be a rare carbon–carbon linked symmetrical biscoumarin and presented as the main constituent in the EtOAc extract of *U. dentata.* It might be formed as an artifact in our isolation process. In order to further confirm whether compound **1** is an artifact or a novel natural product, scoparone (150 mg) was dissolved in EtOH (25 ml) and heated for 8 h at reflux temperature. However, **1** could not be found in this reaction evidenced by HPLC analysis. From the chemical synthetic pathway, biscoumarin with the carbon–carbon bond could be coupled by two coumarin monomers in a process rationalized by means of free radical reactions. These reactions can be catalyzed by oxidases, including peroxidase and laccase systems, known to be radical generators in plants [8]. The conditions of these reactions are rigorous, so it is difficult to produce compound **1** in our isolation process. On the basis of the above information, compound **1** should be a novel natural product, not an artifact formed in the isolation process.

Additionally, five known compounds, namely, 7,7′-dihydroxy-6,6′-dimethoxy-8,8′-biscoumarin (**2**), 7,7′-dimethoxy-6,6′-biscoumarin (**3**) (Figure 1), scoparone (**4**), vanillic acid (**5**), and daucosterol (**6**), were isolated from the EtOAc extract of *U. dentata*. Their structures were identified by spectroscopic methods and determined by comparing with the literature data [9–11].

The immunosuppressive abilities of these isolated compounds were tested *in vitro* by the Cell Counting Kit-8 (CCK-8) assay. As shown in Figure 3, **1** conspicuously suppressed the proliferation of splenic lymphocytes induced by concanavalin A (ConA) in a dose-dependent manner ($P < 0.05$) with an IC_{50} value of 8.18×10^{-5} mol/l; while IC_{50} values of compounds **2–4** were 4.22×10^{-4}, 3.72×10^{-4}, and 3.46×10^{-4} mol/l, respectively. Nevertheless, **5** and **6** showed no anti-proliferative capability against splenic lymphocytes, and **6** even possessed significant pro-proliferative abilities ($P < 0.01$) at concentrations of 2.4×10^{-5} and 2.4×10^{-6} mol/l.

T regulatory (Treg) cells, especially $CD4^+CD25^+$ Treg cells, play a very important role in the development process of autoimmune diseases [12–15]. Considering that $CD4^+CD25^+Foxp3^+$ Treg cells functioned as a critical mediator of self-tolerance and immune homeostasis [16–18], we further studied the effects of compounds **1–6** on the differentiation of $CD4^+CD25^+Foxp3^+$ Treg cells by FACS analysis. As shown in Figure 4, compound **1** ($IC_{50} = 5.19 \times 10^{-4}$ mol/l) promoted the differentiation of $CD4^+CD25^+Foxp3^+$ Treg cells distinctly in a dose-dependent way compared to the normal control. In contrast, no significant changes were observed in the cells treated with compounds **2–6** compared to the normal control (data not shown).

Additionally, the acute cytotoxicity of compound **1** was evaluated against HepG2 cells by CCK-8 analysis. No obvious cell death was observed in the cells treated with **1** even at the concentration of 2.4×10^{-4} mol/l for 2 days, which indicated that

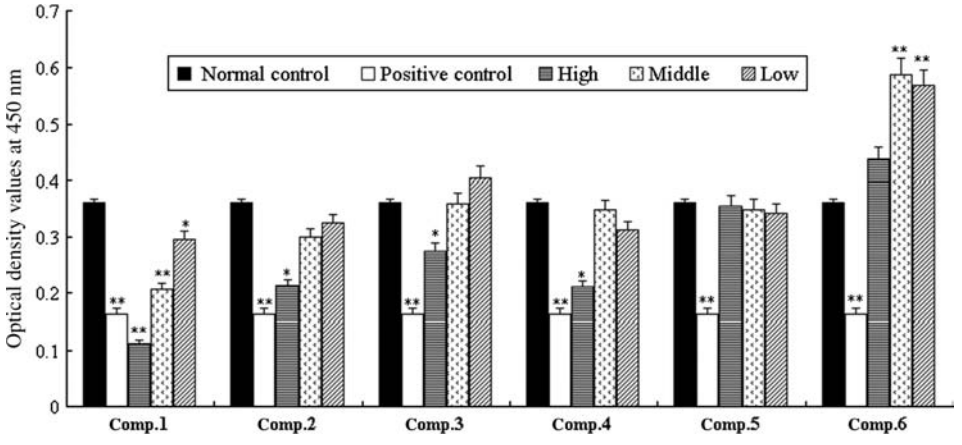

Figure 3. Effects of the isolated compounds on the proliferation of splenic lymphocytes determined by CCK-8 assay. High, middle and low: cells treated with the isolated compounds **1**–**6** at concentrations of 2.4×10^{-4}, 2.4×10^{-5}, and 2.4×10^{-6} mol/l, respectively. Cells treated with CsA served as the positive control, while the cells which were not treated with any drugs were used as the normal control. All tests were run in triplicate and were averaged. Data were presented as mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. normal control.

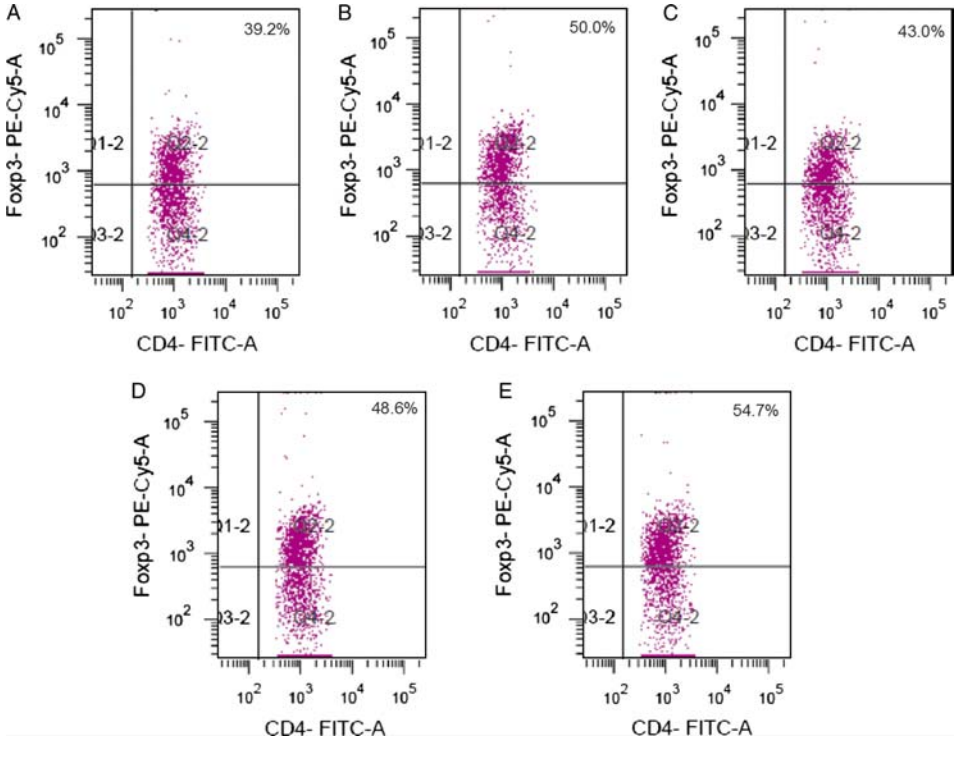

Figure 4. Effects of compound **1** on the differentiation of $CD4^{+}CD25^{+}Foxp3^{+}$ Treg cells. (A) Normal control: cells treated with the medium. (B) Positive control: cells treated with TGF-β. (C) Cells treated with **1** at a concentration of 2.4×10^{-6} mol/l. (D) Cells treated with **1** at a concentration of 2.4×10^{-5} mol/l. (E) Cells treated with **1** at a concentration of 2.4×10^{-4} mol/l.

1 had very low cytotoxicity to HepG2 cells (data not shown).

In conclusion, **1** was found to be the most abundant and active component, likely responsible for most of the immunosuppressive effects of the EtOAc extract of *U. dentata*. It possessed distinct immunosuppressive abilities by inhibiting the proliferation of lymphocytes and enhancing the differentiation of Treg cells, which may provide a new therapy approach for autoimmune diseases.

3. Experimental

3.1 General experimental procedures

All melting points were determined on an XT-4100Xb melting point apparatus and are uncorrected. Optical rotations were measured on a Perkin-Elmer 241MC polarimeter. UV and IR spectra were recorded on UV detector 756 PC and Perkin-Elmer FT-IR spectrometer, respectively. ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz) and HMBC spectra were run on a Bruker AM-400 spectrometer with TMS as the internal standard and values were given in ppm (δ). ESI-MS was recorded on a Finnigan LTQ FTMS. Silica gel (100–200 mesh; Qingdao Marine Chemical Ltd, Qingdao, China) and Sephadex LH-20 (Fuji Silysia Chemical Ltd, Aichi-ken, Japan) were used for column chromatography (CC). TLC was carried out with silica gel GF-254 (Qingdao Marine Chemical Ltd). All solvents used in this experiment were of analytical grade.

3.2 Plant material

The whole plants of *U. dentata* were collected in September 2007 from mountain areas of Enshi, an autonomous region in Hubei Province of China. The plant materials were identified by Jia-Chun Chen, a botanical expert in Tongji Medical College. A voucher specimen (20070913) has been deposited in the herbarium of our department.

3.3 Extraction and isolation

Air-dried and powdered roots of *U. dentata* (5 kg) were exhaustively extracted three times with 95% EtOH (30 liters) for 2 h under reflux. Then, the 95% EtOH extract was concentrated under reduced pressure to give a residue (2230 g), which was suspended in distilled water (3.5 liters), partitioned successively with petroleum ether (3 liters), EtOAc (4 liters), and *n*-BuOH (2.5 liters). The combined EtOAc parts were evaporated *in vacuo* to give a condensed residue (90 g), with the latter subjected to CC on silica gel using petroleum ether–EtOAc (in gradient) as the eluting solvent, followed by MeOH to yield seven fractions. Compound **1** (178 mg) was crystallized from fraction 4 [petroleum ether–EtOAc (2:1)] as buff needle crystals. Fraction 5 was subjected to silica gel CC using EtOAc–MeOH (gradient) as the eluting solvent to yield three subfractions. Subfraction 3 was subjected to Sephadex LH-20 using EtOAc–MeOH (20:1) to yield **2** (73 mg) and **3** (24 mg). Fraction 2 was again subjected to silica gel using $CHCl_3$–MeOH (gradient) as the eluting solvent to yield **4** (35 mg) and **5** (20 mg). Compound **6** (18 mg) was crystallized from fraction 6 as a white amorphous powder.

3.3.1 6,6′,7,7′-Tetramethoxyl-8,8′-bisco umarin (*1*)

Yellow buff needle crystal; mp 149–151°C; $[\alpha]_D^{25}$ 0 ($c = 0.05$, CH_3OH). UV (CH_3OH) λ_{max} (log ε): 346 (4.5), 210 (5.8), 227 (4.7) nm. IR (KBr, ν_{max}, cm^{-1}): 2957, 1702, 1604, 1573, 1497, 1459, 1417, 1368, 1300, 1238, 1122, 1071, 967, 918, 866, 833, 594, 540, 516. ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) spectral data: see Table 1. ESI-MS *m/z*: 410.2 (M^+),

223.2, 189.8. HR-ESI-MS: 410.1028 $[M]^+$(calcd for $C_{22}H_{18}O_8$, 410.1002).

3.4 Preparation of splenic lymphocytes

Splenic lymphocytes were prepared as previously described by Kawaguchi *et al.* [19] with a slight modification. The spleens of healthy Balb/c mice were obtained under sterile conditions and grinded through nylon meshes. Splenic lymphocytes were isolated by gradient centrifugation with Ficoll solution. The viability of splenic lymphocytes was examined under the inverted microscope with trypan blue and the concentration of splenic lymphocytes was adjusted to 1×10^6 cells/ml.

3.5 Assessment of the proliferation of splenic lymphocytes

CCK-8 assay was used to determine the effects of the isolated compounds on the proliferation of splenic lymphocytes. To each well of a 96-well plate, 2×10^5 splenic lymphocyte cells supplemented in 200 μl RPMI-1640 medium with 10% heat-inactivated fetal bovine serum were added. ConA (5 μg/ml) was used as the mitogen of splenic T lymphocytes. Isolated compounds at concentrations of 2.4×10^{-4}, 2.4×10^{-5}, and 2.4×10^{-6} mol/l were added to corresponding wells, respectively. Three wells were set as parallel contrast for each concentration. Cyclosporin A (CsA) served as the positive control. After incubating for 48 h at 37°C with 5% CO_2, 10 μl CCK-8 was added to each well 2 h before terminating the incubation. Then, the optical density was measured at the wavelength of 450 nm. The average of the triplicate sets was taken as the final value. The IC_{50} values were calculated based on these data.

3.6 Analysis of the differentiation of $CD4^+CD25^+Foxp3^+$ Treg cells

Splenic lymphocytes (2×10^5 cells/well) were planted in each well of a 24-well plate. Compounds **1**–**6** at concentrations of 2.4×10^{-6}, 2.4×10^{-5}, and 2.4×10^{-4} mol/l, and TGF-β (8×10^{-7} mol/l) were added to corresponding wells, respectively. The splenic lymphocytes were cultured at 37°C, 5% CO_2 and stimulated with anti-CD3 antibody (5 μg/ml) for 3 days. Then, the lymphocytes were incubated with appropriately diluted fluorescein isothiocyanate (FITC)-labeled anti-CD4 and phycoerythrin (PE)-labeled anti-CD25 antibodies for half-an-hour. After blocking the Fc receptors, the cells were then stained with PE-Cy5-labeled Foxp3 and homotype antibodies for 30 min, respectively. Samples were acquired on a FACSort (BD Biosciences, Mountain View, CA, USA) and data analysis was conducted by using CellQuest software.

3.7 Cytotoxicity assay

The cytotoxic activities *in vitro* of compound **1** were determined against a human cancer cell line, HepG2. This assay was executed in 96-well plates. In each well of the plate, 2×10^5 HepG2 cells were seeded and cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 24 h. Different doses of **1** were added to the plates and the cells were continued to incubate at the same conditions for another 24 h. Then, the viability of HepG2 cells was determined by CCK-8 analysis according to the modified procedure of Tully *et al.* [20].

Acknowledgement

This work was financially supported by the National Science Foundation of China (No. 30672655).

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