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Inducible Nitric Oxide Synthase Inhibitors of Chinese Herbs. Part 2: Naturally Occurring Furanocoumarins

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Abstract—Inducible nitric oxide synthase (iNOS)-dependent production of nitric oxide (NO) plays an important role in inflammation. The effects of various naturally occurring furanocoumarins on NO production in lipopolysaccharide (LPS)-activated RAW 264.7 macrophage cells were evaluated in vitro. The results showed that angelicin, pimpinellin, sphondin, byakangelicol, oxypeucedanin, oxypeucedanin hydrate, xanthotoxin, and cnidilin are potential NO production inhibitors, and their IC_{50} values for inhibition of nitrite production were 19.5, 15.6, 9.8, 16.9, 16.8, 15.8, 16.6, and 17.7 μg/mL, respectively. Distinct structure–activity relationships were also revealed for the NO production inhibitory activities of these furanocoumarins. Activities of the angelicin type such as pimpinellin and sphondin were more potent than those of the psoralen type. Presence of a methoxy at the C_6 position in the angelicin type seemed to be essential to augment the activity. Western blot analysis demonstrated that only sphondin dose-dependently inhibited the expression of the iNOS protein at 2.5–20 μg/mL. However, iNOS enzyme activity was stimulated with LPS for 12 h and sphondin was administered (20 μg/mL) for 24 h, which did not reasonably inhibit iNOS enzyme activity. L-NAME (100 μM), a known specific inhibitor of iNOS, was employed as a positive control with the same protocol and showed more than 50% inhibition activity. The results demonstrate that the NO production inhibitory activity of sphondin is due to the effect of iNOS expression, but not by direct inhibition of iNOS enzyme activity. Thus, sphondin may act as a potent inhibitor of NO production under tissue-damaging inflammatory conditions.

Introduction

Nitric oxide (NO) is synthesized from L-arginine by constitutive and inducible nitric oxide synthase (cNOS and iNOS) in numerous mammalian cells and tissues. Continuous release of NO by cNOS maintains the vasculature in an active state of vasodilation, which is of paramount importance for the maintenance of normal blood pressure.[1,2] Inducible NOS is also an important pharmacological target in inflammatory and mutagenesis research.[3,4] Therefore, inhibition of NO production by iNOS may have potential therapeutic value when related to inflammation and septic shock.

NO is known to cause mutagenesis[5,6] and deamination of DNA bases[6,7] and to play an important role in the formation of carcinogenic *N*-nitroso compounds in vivo.[8] NO rapidly and spontaneously reacts with triplet oxygen ($^3O_2$) to form stable anions, nitrite, and nitrate.[9,10] These compounds non-enzymatically *N*-nitrosylate the primary and secondary amines to produce carcinogenic nitrosamines.[8] Moreover, under inflammatory conditions, macrophages can greatly increase their production of both NO and superoxide anion (O^{2-}) simultaneously, which rapidly react with each other to form the peroxynitrite anion ($ONOO^-$), and thus play a role in inflammation and also possibly in the multistage process of carcinogenesis.[11] The peroxynitrite anion activates the constitutive and inducible forms of cyclo-oxygenase (COX-1 and COX-2, respectively), which are rate-determining enzymes for prostaglandin biosynthesis during the inflammatory process.[12,13] Chronic inflammation of the colon increases the risk of colorectal cancer in rats.[14] On the basis of this evidence, iNOS inhibition has become a new approach for cancer chemoprevention.

Endotoxins and a number of cytokines, including interferon and interleukins, bring about the expression of iNOS in macrophages.[14] Therefore, in this paper, NO released from lipopolysaccharide (LPS) stimulated

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murine macrophage RAW 264.7 cells was quantitatively analyzed by Griess reaction.[15] Moreover, the effects of iNOS enzyme expression were detected by Western blot analysis.[16]

There are many synthetic inhibitors of iNOS; natural products inhibiting NO production have also been found, such as flavonoids, coumarins, and anti-oxidative tannins.[17–22] We have recently reported that NO generation-suppressive properties of two furanocoumarins, deltoin and imperatorin, isolated from *Ledebouriella seseloids*, have the potential to affect NO production and iNOS expression.[22] Both coumarins and chromones were isolated from *L. seseloides*. Interestingly, structures of coumarins (benzo-α-pyrone) and chromones (benzo-γ-pyrone) differ in the position of carbonyl substitution, and only coumarins showed significant inhibition of LPS-induced NO generation in RAW 264.7 cells. Therefore, we continued to explore the naturally occurring furanocoumarins as a conspicuous class of NO generation inhibitors. In the present investigation, we evaluate the effects of structurally diverse furanocoumarin-derived activities (Fig. 1) on NO production in LPS-activated RAW 264.7 macrophages. Structure–activity relationships to NO inhibitory potency of the 17 furanocoumarins are discussed.

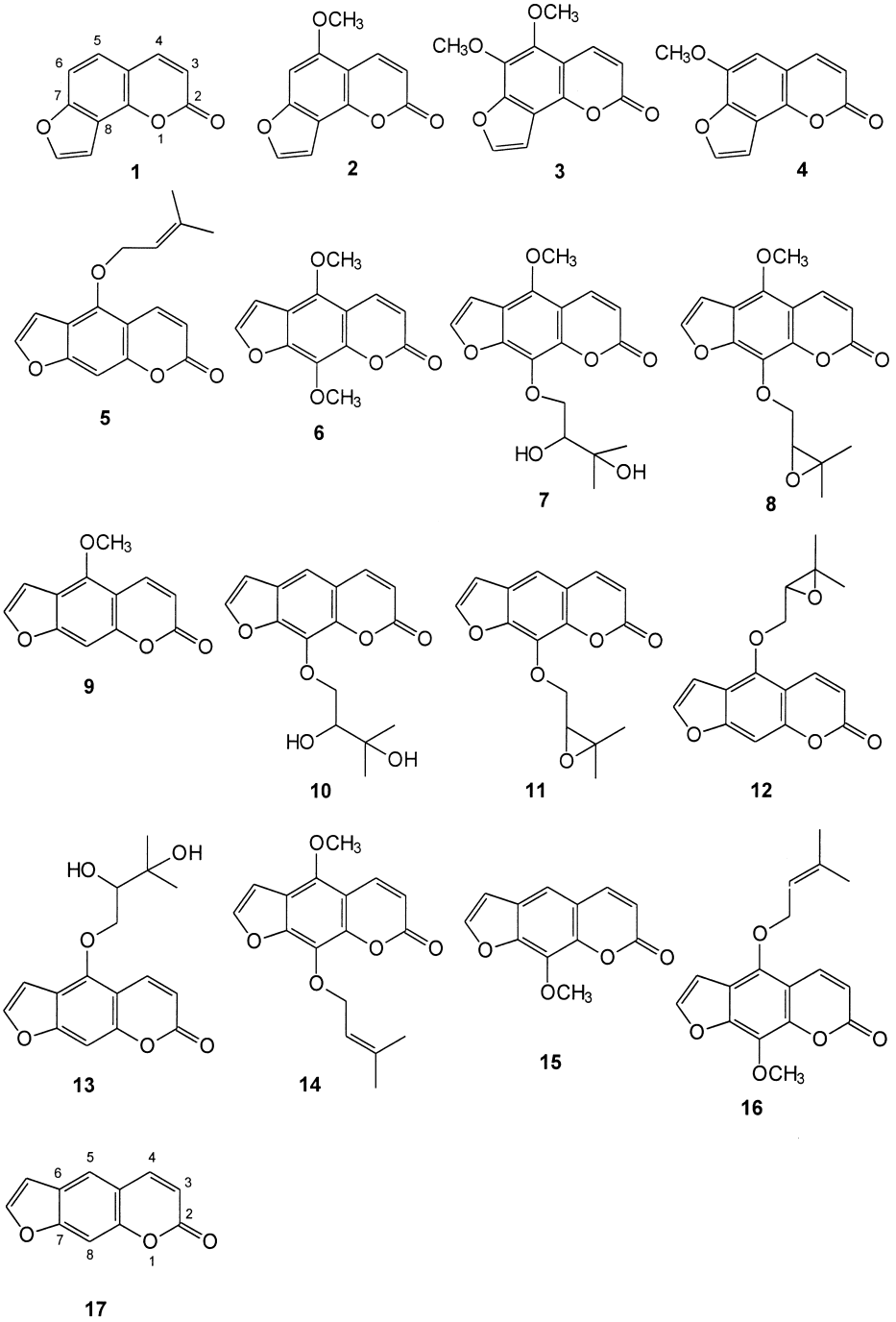


Figure 1. Structures of naturally occurring angelicin-type (**1**–**4**) and psoralen-type furanocoumarins (**5**–**17**).

Results

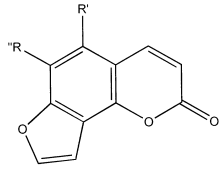
Effects of furanocoumarins on NO produced from RAW 264.7 cells

Seventeen furanocoumarins were isolated from *Angelica dahurica* var. *pai-chi*, *A. dahurica* var. *dahurica*, *A. formosana, A. hirsutiflora*, and other Chinese herbs.[23–25] The inhibition effects of the 17 compounds on the generation of NO were examined in lipopolysaccharide (LPS, 1 μg/mL)-stimulated RAW 264.7 macrophages. The total activity showing the level of nitrite production was measured by the Griess method.[15] Primary screening tests were done at a sample concentration of 20 μg/mL, and no cytotoxicity was observed at this concentration (data not shown). The cytotoxic effects of the furanocoumarins were measured using the MTT assay, and the viability effects of treated cells were all greater than 95%. As shown in Tables 1 and 2, pimpinellin, sphondin, byakangelicol, oxypeucedanin, oxypeucedanin hydrate, xanthotoxin and cnidilin showed more than 50% inhibition of NO production at 20 μg/mL. The amount of NO production at 10 and 20 μg/mL (6×10^4 cells/well in 96-well plates) was continuously measured and the IC_{50} values for the above compounds were counted. Sphondin showed stronger inhibition of nitrite accumulation in the cell culture supernatants of LPS-stimulated RAW 264.7 cells at 10 μg/mL than did the other compounds, as shown in Figure 2(A). Sphondin dose-dependently reduced the induction of NO products at 2.5–20 μg/mL (6×10^5 cells/well in 6-well plates) as shown in Figure 3(A) and the IC_{50} value was 9.8 μg/mL as described in Table 1.

Effects of furanocoumarins on iNOS enzyme activity

It is not known whether the reduction in nitrite accumulation by sphondin is a result of inhibition of iNOS expression or inhibition of its enzymatic activity. The effect of sphondin was compared with that of L-NAME, a specific inhibitor of NO synthase enzyme activity. RAW 264.7 cells were activated by LPS (1 μg/mL) for 12 h, and the medium was replaced with fresh medium with tested samples. Sphondin (20 μg/mL) or the control solvent (0.5% DMSO) weakly inhibited iNOS activity

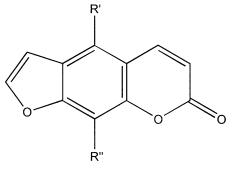
Table 1. Inhibition effects of angelicin-type furanocoumarins on NO production in RAW 264.7 cells[a]



Furanocoumarin	Formula	R′	R″	%I[b] (20 μg/mL)	IC_{50} (μg/mL)
Angelicin (**1**)	$C_{11}H_6O_3$	H	H	53.6±7.6	19.5
Isobergapten (**2**)	$C_{12}H_8O_4$	OCH_3	H	8.8±12.4	>20.0
Pimpinellin (**3**)	$C_{13}H_{10}O_5$	OCH_3	OCH_3	64.7±10.9	15.6
Sphondin (**4**)	$C_{12}H_8O_4$	H	OCH_3	85.3± 8.7	9.8

[a]Results are expressed as the mean ± S.D. of three experiments.
[b]Inhibition of expression in percent compared with the DMSO solvent control in LPS-activated RAW 264.7 cells.

Table 2. Inhibition effects of psoralen-type furanocoumarins on NO production in RAW 264.7 cells[a]



Furanocoumarins	Formula	R′	R″	%I[b] (20 μg/mL)	IC_{50} (μg/mL)
Isoimperatorin (**5**)	$C_{16}H_{14}O_4$	$OCH_2CH{=}C(CH_3)_2$	H	28.1±39.7	>20.0
Isopimpinellin (**6**)	$C_{13}H_{10}O_5$	OCH_3	OCH_3	33.0±46.7	>20.0
Byakangelicin (**7**)	$C_{17}H_{18}O_7$	OCH_3	$OCH_2CH(OH)C(OH)(CH_3)_2$	35.2±1.8	>20.0
Byakangelicol (**8**)	$C_{17}H_{16}O_6$	OCH_3	$OCH_2CHOC(CH_3)_2$	61.1±8.6	16.9
Bergapten (**9**)	$C_{12}H_8O_4$	OCH_3	H	0.0±0.0	>20.0
Heraclenol (**10**)	$C_{16}H_{16}O_6$	H	$OCH_2CH(OH)CH(OH)(CH_3)_2$	0.0±0.0	>20.0
Heraclenin (**11**)	$C_{16}H_{14}O_5$	H	$OCH_2CHOC(CH_3)_2$	0.0±0.0	>20.0
Oxypeucedanin (**12**)	$C_{16}H_{14}O_5$	$OCH_2CHOC(CH_3)_2$	H	53.7±11.2	16.8
Oxypeucedanin hydrate (**13**)	$C_{16}H_{16}O_6$	$OCH_2CH(OH)CH(OH)(CH_3)_2$	H	83.2±17.1	15.8
Phellopterin (**14**)	$C_{17}H_{16}O_5$	OCH_3	$OCH_2CH{=}C(CH_3)_2$	37.6±2.7	>20.0
Xanthotoxin (**15**)	$C_{12}H_8O_4$	H	OCH_3	57.8±8.9	16.6
Cnidilin (**16**)	$C_{17}H_{16}O_5$	$OCH_2CH{=}C(CH_3)_2$	OCH_3	53.7±11.3	17.7
Psoralen (**17**)	$C_{11}H_6O_3$	H	H	27.1±11.1	>20.0

[a]Results are expressed as the mean ± S.D. of three experiments.
[b]Inhibition of expression in percent compared with the DMSO solvent control in LPS-activated RAW 264.7 cells.

in activated RAW 264.7 macrophages. In contrast, L-NAME significantly inhibited nitrite accumulation by more than 50% at 100 μM (Table 3). According to the above results, we suggest that sphondin does not exhibit a direct effect on the enzymatic activity of inducible NO synthase.

Effects of furanocoumarins on iNOS enzyme expression

The effects of tested compounds on the induction of iNOS enzyme expression (6×10^5 cells/well in 6-well plates) were checked using a Western blotting technique. As shown in Figure 2(B), pimpinellin and sphondin significantly reduced the induction of iNOS expression at 20 μg/mL. Sphondin concentration-dependently reduced the induction of iNOS enzyme expression at 2.5–20 μg/mL by Western blot assay in Figure 3(B). However, pimpinellin did not show inhibition effects of iNOS enzyme expression at 10 μg/mL (data not shown). Moreover, byakangelicol, oxypencedanin, oxypeucedanin hydrate, xanthotoxin, and cnidilin showed inhibition of NO production, but none significantly inhibited iNOS expression at 20 μg/mL. The results showed that sphondin possessed the greater effect on iNOS expression of the 17 furancoumarins tested.

Discussion

Coumarins have been reported to have several pharmacological activities, such as antitumor activities,[26–28] anticarcinogenesis, and DNA-repair deficiencies.[29–31] Recently, some papers reported the inhibitory activity of various coumarins on NO production.[20,21,32–35] Otherwise, in previous studies, furanocoumarins showed more inhibition effects of iNOS expression than both simple coumarins and chromones.[22] Therefore, in the present study, 17 kinds of furanocoumarins were screened for their effects on NO production. Furanocoumarins, based on the position of the furan-ring, are divided into the angelicin type (furan-ring at position 6,7 and also called angular-type) and the psoralen type (furan-ring at position 7,8 and also called linear-type). As shown above, it is interesting to note that the characteristics of the side chain affect NO production inhibition by angelicin- and psoralen-type furanocoumarins.

All coumarins in this experiment were screened for NO production inhibition activity at a concentration of 20 μg/mL. Then, a series of concentrations were used for

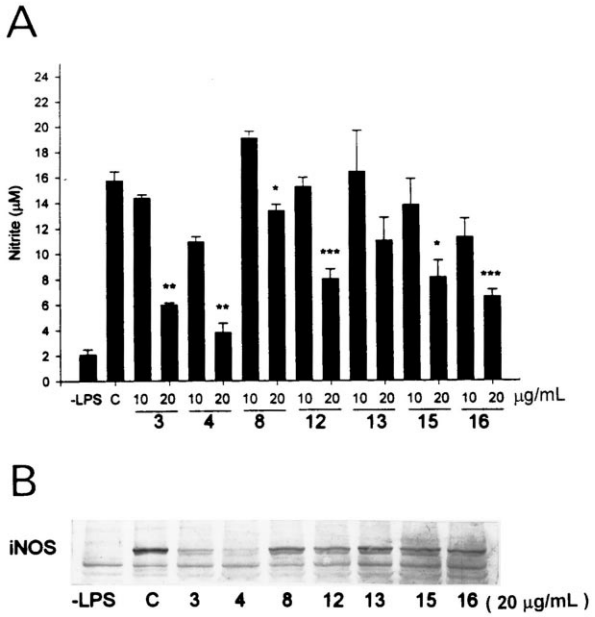


Figure 2. Effects of nitrite production and iNOS expression on LPS-activated RAW 264.7 cells by furanocoumarins at 10 and 20 μg/mL. Inhibitions of nitrite production and iNOS expression were measured by the Griess reaction (A) and Western blot analysis (B), respectively. Statistical analysis was done by Student's *t*-test. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$, significantly different from the LPS-activated group.

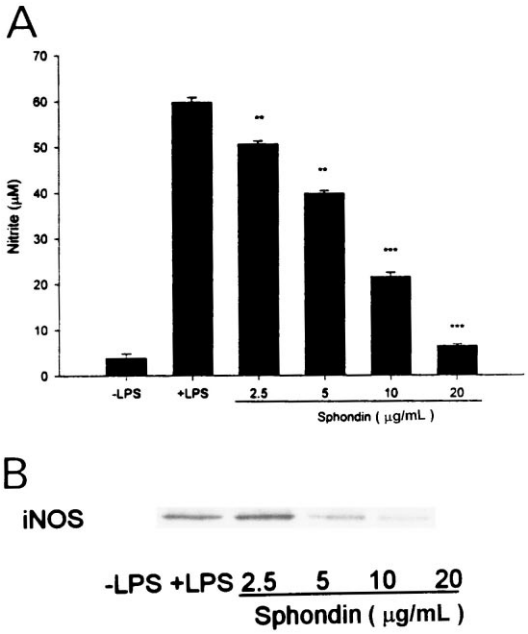


Figure 3. Dose-dependent inhibition of nitrite production and iNOS expression on LPS-activated RAW 264.7 cells by sphondin. Inhibitions of nitrite production and iNOS expression were measured by the Griess reaction (A) and Western blot analysis (B), respectively. Statistical analysis was done by Student's *t*-test. $^{**}P<0.01$; $^{***}P<0.001$, significantly different from the LPS-activated group.

Table 3. Effect of indicated compounds after LPS induction of iNOS enzyme in RAW 264.7 cells[a,b]

LPS pretreatment of cells	Addition to LPS-treated RAW 264.7 cells	NO in medium (μM/5×10^5cells)	Inhibition (%)
None	DMSO[c], 0.5%	0.0±0.0	—
LPS (1 μg/mL), 12 h	Control	6.73±0.44	—
	DMSO, 0.5%	5.59±0.38	16.9
	Sphondin, 20 μg/mL	5.58±0.09	15.3
	L-NAME, 100 μM	3.37±0.54[d]	50.8

[a]RAW 264.7 cells were stimulated with LPS (1 μg/mL) for 12 h, and cells were washed twice with PBS to remove LPS. RAW 264.7 cells were then scraped and placed in a 96-well plate, and indicated compounds were added and incubated at 37 °C for a further 12 h.
[b]Results are expressed as the mean ± S.D. of three experiments.
[c]DMSO (0.5%) was used as a solvent in this experiment.
[d]$P < 0.05$; significantly different from the control.

IC_{50} determination. Some structure–activity relationships of linear- and angular-type furanocoumarins can be deduced.

Of the angelicin-type furanocoumarins in Table 1, angelicin (**1**), which is the fundamental skeleton lacking substitutions on the benzene ring, showed a moderate inhibitory effect (53.6%) on LPS-induced NO generation in RAW 264.7 cells. The activity of isobergapten (**2**), with a methoxy group at the C_5 position, exhibited a considerably lower inhibitory effect (8.8%), whereas sphondin (**4**), with a methoxy group at the C_6 position, showed a strong inhibitory effect. The activity of pimpinellin (**3**), having two methoxy groups at the C_5 and C_6 positions, was slightly higher than that of angelicin (**1**). On the basis of this evidence, a methoxy group at the C_6 position of the angular furanocoumarin skeleton seems to be very important for activity, and the addition of a methoxy group at C_5 decreases activity.

In the series of psoralen-type furanocoumarins in Table 2, psoralen (**17**), having no additional substitutions on the benzene ring, was found to have significantly lower activity than did angelicin (**1**). The activities of xanthotoxin (**15**) and cnidilin (**16**), with a methoxy group at the C_8 position, showed increased inhibitory effects of 57.8 and 53.7%, respectively, whereas in the latter, an additional dimethylallyoxy group at the C_5 position seems to have no significant influence. This evidence was confirmed by comparing the structure and activity of psoralen (**17**, 27.1%) and isoimperatorin (**5**, 28.1%). On the other hand, bergapten (**9**), with a methoxy group at the C_5 position, showed no activity. Isopimpinellin (**6**, 33.0%), with two methoxy groups at the C_5 and C_8 positions, had comparable activity with that of xanthotoxin (**15**, 57.8%) to support this inference.

Oxypeucedanin (**12**, 53.7%) and oxypeucedanin hydrate (**13**, 83.2%), with an oxidized prenyoxy group at the C_5 position, in which the double bond of the dimethylallyoxy group is replaced by an epoxyl or two hydroxyl groups, were found to be significantly more active than was a non-oxidative one (isoimperatorin, **5**). However, of byakangelicin (**7**), byakangelicol (**8**), and phellopterin (**14**), only byakangelicol, with a dimethylallyoxy group saturated by an epoxyl at the C_8 position, showed high activity. On the other hand, heraclenin (**11**), compared with byakangelicol, having the same moiety at the C_8 position but lacking a substitution at the C_5 position, showed no activity. Based on the results mentioned above, functional-group substitutions on the benzene ring selectively enhance or decrease NO production activity, and this cannot be simply explained as hydrophilic or hydrophobic group substitution.

In conclusion, our studies clearly demonstrate that the angelicin type is more potent than the psoralen type. For the angelicin type, the C_5-methoxy group is important for activity. For the psoralen type, the C_8-methoxy or the double bond of the dimethylallyoxy group saturated by two hydroxyl groups at the C_5 position enhances the activity. In this investigation, sphondin most strongly inhibited NO production and iNOS expression in LPS-induced RAW 264.7 macrophages as compared to the other furancoumarins tested.

The present study suggests that sphondin is able to inhibit the induction of iNOS in LPS-activated murine macrophages. This notion is based on the following lines of evidence: (1) The least nitrite accumulated in cell supernatants when sphondin was added simultaneously with LPS; a delayed addition resulted in a decreased effect. (2) Sphondin did not inhibit NO accumulation after LPS stimulation of RAW 264.7 cells, and did not show a direct effect on enzymatic activity of iNOS. (3) Western blot analyses demonstrated markedly reduced levels of iNOS protein in LPS-activated cells treated with sphondin as compared to untreated cells.

Macrophages play a major role in host defences against infection and tumor development, and this activity is regulated through the production of several mediators.[36–38] In particular, the production of NO by macrophages mediates killing or growth inhibition of tumor cells, bacteria, fungi and parasites.[36–38] However, over-expression of iNOS by various stimuli, resulting in over-production of NO, contributes to the pathogenesis of septic shock and some inflammatory and autoimmune diseases.[39] It has been suggested that the interaction between NO and superoxide ($O_2^{-}\cdot$) to yield peroxynitrite ($ONOO^-$) and its conjugated acid, peroxynitrous acid (ONOOH), dramatically enhances the toxicity of either NO or $O_2^{-}\cdot$ alone. Therefore, it would be valuable to develop potent and selective inhibitors of iNOS for potential therapeutic use. Thus sphondin, which inhibits the expression of iNOS and is easily synthesized, might be useful for the prevention of various diseases. Moreover sphondin is contained in plants of the Umbelliferae family such as *Heracleum sphondylium, H. panamces, and H. villosa,* etc.[23,24] The findings presented here might therefore provide a scientific basis for the use of plant extracts containing sphondin and related compounds against inflammatory diseases in phytotherapy.

Materials and Methods

Chemicals and cells

Dimethyl sulfoxide (DMSO), *N*-nitro-L-arginine-methyl ester (L-NAME), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide], trypan blue, LPS (*E. coli* serotype 0127-8B), and other chemicals were purchased from Sigma Chemical (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), antibiotics, glutamine, and trypsin-EDTA were purchased from GIBCO BRL (Grand Island, NY, USA). The murine macrophage cell line, RAW 264.7, was obtained from American Type Cell Culture (ATCC; Rockville, MD, USA).

Sample preparation

Isoimperatorin (**5**), byakangelicin (**7**), oxypeucedanin (**12**) and phellopterin (**14**) were isolated from the dried

root of *Angelica dahurica* Benth. et Hook. var. *pai-chi* Kimura, Hata et Yen (Umbelliferae). Byakangelicol (**8**) was isolated from the dried root of *A. dahurica* Benth. et Hook. var. *dahurica* (Umbelliferae).[23] Bergapten (**9**) and oxypeucedanin hydrate (**13**) were isolated from the dried roots of *A. formosana* Borss. (Umbelliferae).[24] Xanthotoxin (**15**) and psoralen (**17**) were isolated from the dried roots of *A. hirsutiflora* Liu, Chao et Chuang.[25] All other coumarins used in this study were isolated from Umbelleferae or Rutaceae medicinal herbs in our laboratory. Each tested solution (4 mg/mL) was prepared by dissolving each compound in DMSO, after which they were stored at 4 °C until use. Serial dilutions of the tested solutions with culture medium were prepared before in vitro assays.

NO production from RAW 264.7 cells

The murine macrophage cell line, RAW 264.7, was cultivated in DMEM medium supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2.[40] The cells (0.2 mL, 3×10^5 cells/mL) were placed in 96-well plates and treated with LPS (1 μg/mL) and tested compounds. After 24 h, the level of nitrite was measured as described below. Furanocoumarins were dissolved in DMSO diluted with culture medium into appropriate concentrations. The final concentration of DMSO was adjusted to 0.5% (v/v). At this concentration of DMSO, no significant changes in cytotoxicity and NO productivity were observed in preliminary experiments.

iNOS activity assay

The enzyme preparation was obtained from RAW 264.7 cells cultured in a 100-mm plate after activation with LPS (1 μg/mL) for 12 h. The cells were collected and washed twice with PBS to remove LPS. RAW 264.7 cell suspensions (0.2 mL, 3×10^5 cells/mL) were placed in 96-well plates and indicated compounds were added. L-NAME was a specific inhibitor of NO synthase enzyme activity as a positive control and 0.5% DMSO as a solvent control. After 12 h, the amount of nitrite was measured by Griess reaction as described below.

Cell viability

Mitochondrial respiration, as an indicator of cell viability, was assayed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to formazan. Cells in 96-well plates were incubated with MTT (0.25 mg/mL) for 4 h. The cells were solubilized in 0.04 N HCl in isopropanol. The extent of the reduction was measured by absorbance at 600 nm.[39]

Measurement of nitrite formation

Nitrite, as an indicator of NO synthesis, was determined in the supernatant of media by Griess reaction.[15] After incubation of the cells for 24 h, the supernatants (0.1 mL) were added to a solution of 0.1 mL Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylene diamine dihydrochloride in 5% H_3PO_4) to form a purple azodye. Using $NaNO_2$ to generate a standard curve, nitrite production was measured by an absorption reading at 530 nm.

Western blot analysis

Macrophages (2 mL, 3×10^5 cells/mL), grown in 6-well plates to confluence, were incubated with or without LPS in the absence or presence of tested samples for 24 h, respectively. Cells were washed with ice-cold phosphate-buffered saline and stored at −70 °C until further analysis. Western blot analysis was performed according to Rothe et al.[16,41] using a polyclonal rabbit IgG antibody against inducible NO synthase (Santa Cruz, sc-651) and alkaline phosphatase detection system (BCIP/NBT, GIBCO BRL).

Statistical analysis

Each experiment was performed at least in triplicate. Results are expressed as the mean value ± standard deviation (S.D.). Statistical analysis was performed using an unpaired Student's *t*-test. P values < 0.05 were considered significant.

Acknowledgements

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