Sesquiterpene Coumarins from *Ferula fukanensis* and Their Pro-inflammatory Cytokine Gene Expression Inhibitory Effects

Tsunetake Motai, Akihiro Daikonya, and Susumu Kitanaka*

School of Pharmacy, Nihon University; 7–7–1 Narashinodai, Funabashi, Chiba 274–8555, Japan.

Received November 28, 2012; accepted March 8, 2013

Six new sesquiterpene coumarin derivatives, fukanefuomarin H–M (1–6), were isolated from an 80% aqueous methanol extract of the roots of *Ferula fukanensis*. The structures were elucidated on the basis of spectroscopic evidence, particularly heteronuclear multiple-bond connectivity (HMBC) and high-resolution MS. The sesquiterpene coumarin derivatives inhibited nitric oxide (NO) and inducible NO synthase (iNOS), interleukin-6 (IL-6), and tumor necrosis factor- α (TNF- α) gene expression in a murine macrophage-like cell line (RAW264.7), which was activated by lipopolysaccharide (LPS) and recombinant mouse interferon- γ (IFN- γ).

Key words *Ferula fukanensis*; macrophage; nitric oxide; sesquiterpene coumarin

Ferula fukanensis grows on arid land in Central Asia and has been used as a traditional medicine for the treatment of rheumatoid arthritis and bronchitis. Previous studies have analyzed the polysulfanes found in this plant by GC-MS (chemical ionization/electron ionization (CI/EI)),¹⁾ while the chemical constituents of other plants in the genus *Ferula* (Umbelliferae) have been studied by many groups. In previous papers,^{2–6)} we reported the isolation of sesquiterpene coumarin derivatives, sesquiterpene phenylpropanoids, and sesquiterpene chromone derivatives from *F. fukanensis*. Compounds commonly found in this genus are sesquiterpene coumarins and sesquiterpene chromones.^{7–13)} In our continuing investigations of the *in vitro* anti-inflammatory effects of medicinal herbal extracts, an 80% aqueous methanol (MeOH) extract of the roots of *F. fukanensis* (FFE) was observed to inhibit nitric oxide (NO) production in a lipopolysaccharide (LPS)-activated murine macrophage-like cell line.²⁾

Macrophages play major roles in immunity and the inflammatory responses involved with host defense. After activation, they initiate the production of cytokines, oxygen and nitrogen species, and eicosanoids. Bacterial LPS, either alone or in combination with recombinant mouse interferon- γ (IFN- γ), is the best known stimulus that induces the transcription of genes encoding pro-inflammatory proteins in macrophages. This stimulation results in cytokine release and the synthesis of enzymes such as inducible NO synthase (iNOS), interleukin-6 (IL-6), and tumor necrosis factor- α (TNF- α). The NO radical is known to play a central role in inflammation and immune reactions. However, the excessive production of NO may cause tissue damage. Excessive NO production by activated macrophages has been observed in inflammatory diseases such as rheumatoid arthritis.

We discovered that FFE inhibited activated macrophage NO production (IC₅₀ = 21.9 μ g/mL).²⁾ Therefore, FFE was analyzed to identify the active compounds in the extract. Bioactivity-guided fractionation was used to isolate active compounds that inhibited NO production from the CHCl₃ fraction. These compounds also inhibited iNOS mRNA, IL-6 mRNA, and TNF- α mRNA expression in RAW264.7 cells treated with LPS and IFN- γ .

Results and Discussion

FFE was partitioned by successive extractions using CHCl₃, EtOAc, and H₂O. The CHCl₃-soluble fraction inhibited 60% of the NO production at 30 μ g/mL. Thus, the CHCl₃-soluble fraction was chromatographed on a silica gel column to yield 11 fractions. Fraction 6 inhibited 96.2% of NO production at 30 μ g/mL, while fraction 8 inhibited 84.7% of NO production at 30 μ g/mL. Compounds 1–6 were isolated from fractions 6 and 8.

Fukanefuomarin H (1) was obtained as a colorless oil, with a molecular weight of 398 based on fast atom bombardment mass spectrometry (FAB-MS) data, and with a protonated molecular ion peak at m/z 399 [M+H]⁺ in the positive mode. These results and the ¹H- and ¹³C-NMR data (Tables 1, 2) suggested a molecular formula of C₂₄H₃₀O₅, which was supported by high resolution (HR)-FAB-MS in the positive mode (C₂₄H₃₁O₅, m/z 399.2166). The MS data for compounds 1 and 2 indicated the same molecular ions (see Experimental), which suggested that they were isomeric compounds.

The ¹H-NMR data for 1 indicated the presence of a 1,2,4-trisubstituted benzene ring at δ_H 7.49 (1H, d, J = 8.4 Hz, H-9), δ_H 6.83 (1H, dd, J = 2.2, 8.5 Hz, H-8), δ_H 7.08 (1H, d, J = 2.2 Hz, H-6), while the other signals were similar to those for the sesquiterpene unit of 2,3-dihydro-7-hydroxy-2S*,3R*-dimethyl-2-[4,8-dimethyl-3(*E*),7-nonadienyl]-furo[3,2-*c*]coumarin.^{2,10)} The remaining ¹H- and ¹³C-NMR data for 1, except those related to the sesquiterpene unit, indicated a 7-hydroxy-substituted coumarin compound.^{12,13)}

In the heteronuclear multiple bond connectivity (HMBC) spectrum of 1, the correlations of Me-2 with C-2, C-3 and C-1' and Me-3 with C-2, C-3, and C-3a, confirmed that C-1' in the sesquiterpene unit was attached to C-2 in coumarine. On the basis of the above analysis, the ¹³C-NMR results (δ_{C-2} 96.8, δ_{C-9b} 165.6), and the molecular formula of 1, it was deduced that C-2 and C-9b were linked by an oxygen atom. Furthermore, the HMBC correlations of H-9' with C-7', C-8', and Me-8', and Me-8' with C-7', C-8', and C-9' confirmed that 1 had a 2-butenyl system. The relative configuration of the dimethyldihydrofuran moiety at C-2 and C-3 was determined on the basis of difference nuclear Overhauser effect (NOE) experiments. Compound 1 had significant NOE correlations between Me-2 and Me-3, and between H-2 and H-1',

The authors declare no conflict of interest.

* To whom correspondence should be addressed. e-mail: kitanaka.susumu@nihon-u.ac.jp

Fig. 1. Sesquiterpene Coumarins from *F. fukanensis*Table 1. ^1H -NMR Data for Compounds **1–4** (300 MHz) (CDCl_3 , TMS, δ (ppm), $J=\text{Hz}$)^{a)}

H	1	2	3	4
3	3.28 q (7.0)	3.19 q (7.0)	3.27 q (7.3)	3.21 q (7.0)
6	7.08 d (2.2)	7.07 d (2.3)	7.07 d (2.2)	7.05 d (2.3)
8	6.83 dd (2.2, 8.5)	6.82 dd (2.3, 8.5)	6.85 dd (2.2, 8.4)	6.84 dd (2.3, 8.5)
9	7.49 d (8.4)	7.50 d (8.8)	7.50 d (8.8)	7.50 d (8.8)
1'	1.79 m	1.45 m	1.79 m	1.74 m
2'	2.14 m	2.25 m	2.15 m	2.27 m
3'	5.08 t (6.2)	5.15 t (6.5)	5.23 t (7.0)	5.30 t (6.5)
5'	1.94 m	2.00 m	2.28 m	2.33 m
6'	2.09 m	2.14 m	4.93 m	4.99 m
7'	5.23 t (7.0)	5.27 t (7.0)	6.99 brs	7.02 brs
9'	4.11 s	4.12 s		
2-Me	1.45 s	1.48 s	1.45 s	1.49 s
3-Me	1.29 d (7.0)	1.28 d (7.0)	1.30 d (7.0)	1.29 d (7.0)
4'-Me	1.55 s	1.62 s	1.65 s	1.71 s
8'-Me	1.77 s	1.79 s	1.89 s	1.91 s

a) Assignments confirmed by decoupling, ^1H - ^1H COSY, HMQC, HMBC and difference NOE spectra.

which indicated a *cis* relationship between Me-2 and Me-3. Thus, **1** was 2,3-dihydro-7-hydroxy-2*S**,3*R**-dimethyl-2-[9-hydroxy-4,8-dimethyl-3(*E*),7-nonadienyl]-furo[3,2-*c*]coumarin.

Fukanefuomarin I (**2**) was obtained as a colorless oil. The HMBC experiments suggested that the structure of **2** was similar to that of **1**. However, the NMR spectra of **2** differed slightly from those of **1**, especially at C-1' (δ_{C} 35.0 for **2**, δ_{C} 41.6 for **1**) and Me-2 (δ_{C} 25.3 for **2**, δ_{C} 20.6 for **1**), which suggested that **2** was a diastereomer of **1** at the chiral centers C-1' and C-2'. The relative configuration of the dimethyldihydrofuran moiety at C-1' and C-2' was determined on the basis of difference NOE experiments. Compound **2** had significant NOE correlations between Me-2 and H-3', and between Me-3 and H-1', which indicated a *trans* relationship between Me-2 and Me-3. Thus, **2** was 2,3-dihydro-7-hydroxy-2*R**,3*R**-dimethyl-2-[9-hydroxy-4,8-dimethyl-3(*E*),7-nonadienyl]-furo[3,2-*c*]coumarin.

Fukanefuomarin J (**3**) was obtained as a colorless oil. Its

molecular formula, $\text{C}_{24}\text{H}_{27}\text{O}_6$, was determined by HR-FAB-MS $\{m/z\}$ 411.1806 $[\text{M}+\text{H}]^+$ with 12 degrees of unsaturation. The ^1H - and ^{13}C -NMR spectra of **3** were similar to those of **1**, except for those of the sesquiterpene unit.

In the HMBC spectrum of **3**, the correlations between Me-8' and C-7', C-8', and C-9'; H-7' and C-6', C-8', and C-9'; H-6' and C-5', C-7', and C-8', and the characteristic carbonyl absorption (ν 1757 cm^{-1}) in the IR spectrum of **3** supported the presence of a butenolide in this compound. The relative configuration of the dimethyldihydrofuran moiety at C-2 and C-3 was determined on the basis of difference NOE experiments. Compound **3** had significant NOE correlations between Me-2 and H-3', and between Me-3 and H-1', which indicated a *cis* relationship between Me-2 and Me-3. Thus, **3** was 2,3-dihydro-7-hydroxy-2*S**,3*R**-dimethyl-2-[4-methyl-5-(4-methyl-5-(4-methyl-5-oxo-2,5-dihydro-2-furyl)-3(*E*)-pentenyl]-furo[3,2-*c*]coumarin.

Fukanefuomarin K (**4**) was obtained as a colorless oil. The

Table 2. ^{13}C -NMR Data for Compounds **1–4** (75 MHz, CDCl_3 , TMS, δ (ppm))^{a)}

C	1	2	3	4
2	96.8	96.1	96.5	95.9
3	41.4	44.1	41.7	44.1
3a	102.8 ^{b)}	103.0	102.8	103.0
4	161.9	161.8	161.7	161.7
5a	156.1	156.1	156.1	156.2
6	102.7 ^{b)}	102.8	102.9	103.1
7	160.7	160.6	160.5	160.4
8	113.1	113.0	113.0	112.9
9	123.7	123.7 ^{b)}	123.7	123.7
9a	105.2	105.4	105.2	105.6
9b	165.6	165.3	165.4	165.2
1'	41.6	35.0	41.2	34.8
2'	22.1	22.7	22.2	22.8
3'	123.3	123.6 ^{b)}	127.5	123.7
4'	135.0	134.9	129.7	129.7
5'	39.6	39.6	43.2	43.2
6'	26.0	26.0	79.8	79.8
7'	127.8	127.8	148.3	148.3
8'	133.7	133.8	129.5	129.6
9'	61.3	61.4	173.8	173.9
2-Me	20.6	25.3	20.5	25.3
3-Me	14.1	13.5	14.1	13.5
4'-Me	16.0	16.0	16.6	16.7
8'-Me	21.2	21.2	10.6	10.6

a) Assignments confirmed by decoupling, ^1H - ^1H COSY, HMQC, HMBC and difference NOE spectra. b) Assignments may be interchanged.

HMBC experiments suggested that the structure of **4** was similar to that of **3**. However, the NMR spectra of **4** differed slightly from those of **3**, especially at C-1' (δ_{C} 34.8 for **4**, δ_{C} 41.2 for **3**) and 2-Me (δ_{C} 25.3 for **4**, δ_{C} 20.5 for **3**), which suggested that **4** was a diastereomer of **3** at the chiral centers C-1' and C-2'. The relative configuration of the dimethyldihydrofuran moiety at C-1' and C-2' was determined on the basis of difference NOE experiments. Compound **4** had significant NOE correlations between Me-2 and H-1', and between H-2 and Me-3, which indicated a *trans* relationship between Me-2 and Me-3. Thus, **4** was 2,3-dihydro-7-hydroxy-2*R**,3*R**-dimethyl-2-[4-methyl-5-(4-methyl-5-(4-methyl-5-oxo-2,5-dihydro-2-furyl)-3(*E*)-pentenyl]-furo[3,2-*c*]coumarin.

Fukanefuromarin L (**5**) was obtained as a yellow oil, with a molecular weight of 398 based on FAB-MS data, and with a protonated molecular ion peak at m/z 399 $[\text{M}+\text{H}]^+$. These results and the ^1H - and ^{13}C -NMR data (Table 3) suggested a molecular formula of $\text{C}_{24}\text{H}_{31}\text{O}_5$, which was supported by HR-FAB-MS in the positive mode ($\text{C}_{24}\text{H}_{31}\text{O}_5$, m/z 399.2171). The MS data for compounds **5** and **6** showed the same molecular ions (see Experimental), which indicated that they were structural isomers.

In the HMBC spectrum of **5**, the correlations of H-15' and C-2', C-3', and C-4'; and H-2' and C-3, C-1', and C-3', confirmed that C-1' in the sesquiterpene unit was attached to C-2' in coumarin. On the basis of this analysis, the ^{13}C -NMR data ($\delta_{\text{C-2'}}$ 92.9, $\delta_{\text{C-4'}}$ 166.8), and the molecular formula of **5**, it was deduced that C-4 and C-2' were linked by an oxygen atom. Thus, **5** was 2,3-dihydro-7-hydroxy-2-(1-hydroxy-1,5,9-trimethyl-deca-4,8-dienyl)-furo[3,2-*c*]coumarin.

Fukanefuromarin M (**6**) was obtained as a colorless powder. The HMBC experiments suggested that the structure of **6** was similar to that of **5**. However, the NMR spectra of **6**

differed slightly from those of **5**, especially at C-2' (δ_{C} 67.0 for **6**, δ_{C} 92.9 for **5**) and C-3' (δ_{C} 82.7 for **6**, δ_{C} 73.1 for **5**), which suggested that C-4 and C-3' were linked by an oxygen atom. The relative configuration at C-1', C-2', and C-3' was determined on the basis of difference NOE experiments. Compound **6** had significant NOE correlations between H-2' and H-1' (δ_{H} 2.75), and between H-15' and H-1' (δ_{H} 2.44), which indicated a *cis* relationship between H-2' and H-15'. Thus, **6** was 3,4-dihydro-3,8-hydroxy-2-(4,8-dimethyl-nona-3(*E*),7-dienyl)-2-methyl-2*H*-pyrano[3,2-*c*]coumarin.

The inhibitory effects of these compounds on NO production were examined after its induction by LPS/IFN- γ . Compounds **1–4** had inhibitory activities [**1**: IC_{50} =9.6 $\mu\text{g/mL}$ (24.3 μM); **2**: IC_{50} =22.0 $\mu\text{g/mL}$ (55.6 μM); **3**: IC_{50} =4.5 $\mu\text{g/mL}$ (11.1 μM); **4**: IC_{50} =14.6 $\mu\text{g/mL}$ (35.7 μM)] (Fig. 2). The cytotoxic effects of these compounds were measured using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Compounds **1**, **2**, **4** (3–30 $\mu\text{g/mL}$), and **3** (1–10 $\mu\text{g/mL}$) had no significant cytotoxicity after LPS/IFN- γ treatment for 24 h. In contrast, **5** and **6** (10 $\mu\text{g/mL}$) exhibited significant cytotoxicity. By comparing the inhibitory activity of these compounds, we identified interesting features that may affect the activity level. Inhibitory activities of **1** were stronger than **2**, and **3** were stronger than **4**, suggesting that the configuration of the dimethyldihydrofuran moiety enhances sesquiterpene coumarine inhibitory activity. Furthermore, the reverse transcription-polymerase chain reaction (RT-PCR) analysis in the present study indicated that LPS/IFN- γ treatment increased the expression level of iNOS mRNA, IL-6 mRNA, and TNF- α mRNA, whereas **1** and **4** inhibited this increase in a dose-dependent manner (Fig. 3).

Inhibitors of NO production by macrophages act mainly through two mechanisms; *i.e.*, the inhibition of iNOS ex-

Table 3. NMR Spectral Data for Compounds **5** and **6** (300 MHz for ^1H -NMR, 75 MHz for ^{13}C -NMR, Acetone- d_6 , TMS, δ (ppm), $J=\text{Hz}$)^{a)}

Position	5		6	
	H	C	H	C
2		160.0		162.5
3		99.4		95.6
4		166.8		158.7
5	7.53 d (8.8)	124.4	7.64 brd (8.9)	124.1
6	6.85 dd (2.3, 8.5)	113.0	6.82 brdd (2.2, 8.9)	112.8
7		161.7		161.0
8	6.77 d (2.1)	103.0	6.71 brd (2.0)	102.5
9		157.2		154.6
10		105.6		108.8
1'	3.07 m	27.7	2.44 brdd (7.0, 9.9) 2.75 brdd (7.0, 9.9)	26.9
2'	5.04 m	92.9	3.97 brt (7.0)	67.0
3'		73.1		82.7
4'	1.62 m	38.8	1.81 m	38.1
5'	2.19 m	22.4	2.24 m	22.2
6'	5.19 t (7.0)	124.9	5.19 brt (6.6)	124.4
7'		135.2		135.4
8'	2.02 m ^{b)}	40.3	1.97 m	40.2
9'	2.05 m ^{b)}	27.3	2.05 m	27.3
10'	5.10 t	124.7	5.07 brt (7.0)	124.6
11'		131.2		131.2
12'	1.64 s ^{b)}	25.7	1.64 s	25.7
13'	1.59 s	17.7	1.57 s	17.7
14'	1.64 s ^{b)}	16.0	1.62 s	16.0
15'	1.35 s	22.3	1.40 s	18.6

a) Assignments confirmed by decoupling, ^1H - ^1H COSY, HMQC, HMBC and different NOE spectra. b) Overlapped signal.

Fig. 2. Inhibitory Effect of Compounds **1**–**4** on NO Production Stimulated by LPS and IFN- γ

RAW264.7 cells were treated with LPS/IFN- γ alone or together with each compounds at concentrations indicated. After 16h incubation, the supernatants were tested by Griess assay and the inhibitory rates were calculated. The experiment was performed three times and the data are expressed as mean $\pm$ S.D. values.

pression and inhibition of enzyme activity. For **1** and **4**, the mechanism appears to be the inhibition of iNOS expression.

Experimental

General Experiment Procedures UV spectra were obtained in MeOH on a Shimadzu UV-160 spectrophotometer, and IR spectra were recorded on a JASCO IR A-2 spectrophotometer. The NMR spectra were taken on a Mercury-300BB Varian spectrometer, with TMS as an internal standard. MS were obtained on a JEOL GCmate spectrometer. Column chromatography was carried out on silica gel (Wako gel C-300, Wako Pure Chemical Industries, Ltd., Osaka, Japan).

TLC was performed on Merck TLC plates (0.25 mm thickness), with compounds visualized by spraying with 5% (v/v) H_2SO_4 in EtOH and then heating on a hot plate. HPLC was performed on a JASCO PU-2089 apparatus equipped with a JASCO UV-2075 detector. YMC-Pak SIL-06 (10 $\times$ 150 mm i.d.) columns and YMC-PAK Pro-C₁₈ (10 $\times$ 150 mm i.d.) were used for preparative purposes.

Plant Material Dried roots of *F. fukanensis* were collected in the Urumqi, Xinjiang, People's Republic of China, in October 2002. Voucher specimens (NK03044) have been deposited at College of Pharmacy, Nihon University.

Extraction and Isolation Dried roots of *F. fukanensis*

Fig. 3. Inhibitory Effect of Compounds **1** and **4** on Gene Expression on Stimulated by LPS and IFN- γ

RAW264.7 cells were treated with LPS/IFN- γ alone or together with each compounds at concentrations indicated. The untreated and treated cells were collected and examined by RT-PCR.

(5.9 kg) were chopped and extracted twice with 80% MeOH (18 L). Solvent was evaporated under reduced pressure and the extract (448 g) was suspended in H₂O (3.0 L), and partitioned with CHCl₃ (3×3 L) and EtOAc (3×3 L), successively. Evaporation of the solvent yielded a CHCl₃ fraction (272 g), EtOAc fraction (142 g), and the aqueous fraction (96 g). The CHCl₃ fraction was subjected to silica gel column chromatography (12×17 cm, eluted with hexane and EtOAc in increased polarity). The fractions (200 mL each) were combined according to TLC monitoring into eleven portions. Fraction 8, eluted with hexane–EtOAc (1:1), was isolated and further purified by coeum chromatography and reverse phase HPLC (CH₃CN–H₂O, 52:48) to give **1** (12.0 mg), and **2** (8.3 mg), and reverse phase HPLC (CH₃CN–H₂O, 43:57) to give **3** (13.1 mg), **4** (5.3 mg). Fraction 6, eluted with hexane–EtOAc (4:1), was isolated and further purified by column chromatography, HPLC (hexane–EtOAc, 60:40) and reverse phase HPLC (CH₃CN–H₂O, 53:47) to give **5** (10.0 mg) and **6** (18.7 mg).

Fukaneufuromarin H (1): Colorless oil. $[\alpha]_D^{23} +1.8^\circ$ ($c=0.99$, MeOH). UV (MeOH) $\lambda_{\max}$ (log ϵ) nm: 328 (3.87), 317 (4.12), 289 (3.83), 226 (4.10), 211 (4.20). IR (LF) $\nu_{\max}$ cm⁻¹: 3333, 2973, 2936, 1694, 1633, 1574, 1518, 1452, 1416, 1383, 1267, 1234, 1151, 1094, 1000, 958, 851, 762. ¹H- and ¹³C-NMR data, see Tables 1 and 2. FAB-MS m/z : 399 [M+H]⁺. HR-FAB-MS m/z : 399.21665 [M+H]⁺ (Calcd for C₂₄H₃₁O₅ 399.2171).

Fukaneufuromarin I (2): Colorless oil. $[\alpha]_D^{23} -4.3^\circ$ ($c=0.56$, MeOH). UV (MeOH) $\lambda_{\max}$ (log ϵ) nm: 328 (3.76), 317 (4.15), 289 (3.87), 226 (4.15), 209 (4.37). IR (KBr) $\nu_{\max}$ cm⁻¹: 3271, 2973, 2935, 1694, 1634, 1574, 1519, 1452, 1416, 1379, 1267, 1234, 1153, 1111, 1001, 958, 850, 762. ¹H- and ¹³C-NMR data, see Tables 1 and 2. FAB-MS m/z : 399 [M+H]⁺. HR-FAB-MS m/z : 399.2173 [M+H]⁺ (Calcd for C₂₄H₃₁O₅ 399.2171).

Fukaneufuromarin J (3): Colorless oil. $[\alpha]_D^{23} -4.3^\circ$ ($c=0.40$, MeOH). UV (MeOH) $\lambda_{\max}$ (log ϵ) nm: 331 (4.13), 317 (4.20), 289 (3.80), 238 (3.98), 225 (4.24), 208 (4.51). IR (KBr) $\nu_{\max}$ cm⁻¹: 3409, 2978, 2927, 1757, 1722, 1689, 1635, 1611, 1573, 1519, 1449, 1415, 1383, 1267, 1231, 1150, 1115, 1065, 995, 957, 852, 767. ¹H- and ¹³C-NMR data, see Tables 1 and 2. FAB-MS m/z : 411 [M+H]⁺. HR-FAB-MS m/z : 411.1806 [M+H]⁺ (Calcd for C₂₄H₂₇O₆ 411.1807).

Fukaneufuromarin K (4): Colorless oil. $[\alpha]_D^{23} -10.0^\circ$ ($c=0.1$, MeOH). UV (MeOH) $\lambda_{\max}$ (log ϵ) nm: 331 (4.02), 318 (4.10), 289 (3.76), 238 (3.84), 225 (4.15), 207 (4.51). IR (LF)

$\nu_{\max}$ cm⁻¹: 3235, 2976, 2935, 1756, 1694, 1635, 1573, 1519, 1453, 1417, 1378, 1265, 1230, 1145, 1109, 1067, 999, 959, 852, 758. ¹H- and ¹³C-NMR data, see Tables 1 and 2. FAB-MS m/z : 411 [M+H]⁺. HR-FAB-MS m/z : 411.1806 [M+H]⁺ (Calcd for C₂₄H₂₇O₆ 411.1807).

Fukaneufuromarin L (5): Yellow oil. $[\alpha]_D^{23} +3.3^\circ$ ($c=0.45$, MeOH). UV (MeOH) $\lambda_{\max}$ (log ϵ) nm: 318 (4.23), 290 (3.78), 226 (4.17), 209 (4.33). IR (KBr) $\nu_{\max}$ cm⁻¹: 3281, 2970, 2921, 2855, 1685, 1626, 1577, 1456, 1413, 1379, 1273, 1216, 1165, 1108, 1043, 872, 757. ¹H- and ¹³C-NMR data, see Table 3. FAB-MS m/z : 399 [M+H]⁺. HR-FAB-MS m/z : 399.2171 [M+H]⁺ (Calcd for C₂₄H₃₁O₅ 399.2171).

Fukaneufuromarin M (6): Colorless powder. $[\alpha]_D^{23} +2.7^\circ$ ($c=0.23$, MeOH). UV (MeOH) $\lambda_{\max}$ (log ϵ) nm: 311 (3.82), 285 (3.49), 224 (3.84), 207 (4.06). IR (KBr) $\nu_{\max}$ cm⁻¹: 3268, 2963, 2923, 2856, 1704, 1641, 1607, 1440, 1381, 1262, 1236, 1157, 1098, 1041, 921, 844, 755. ¹H- and ¹³C-NMR data, see Table 3. FAB-MS m/z : 399 [M+H]⁺. HR-FAB-MS m/z : 399.2174 [M+H]⁺ (Calcd for C₂₄H₃₁O₅ 399.2171).

Nitrite Assay^{14–16} RAW264.7 cells, a mouse macrophage-like cell line transformed with the Abelson leukemia virus, were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). The cells were incubated in Ham's F12 medium containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 50 μ g/mL kanamycin. Cell concentration was adjusted to 1.2×10^6 cells/mL, and 200 μ L of cell suspension was seeded in each well of a 96-well flat-bottomed plate. After a 2 h incubation, cells were treated with *Escherichia coli* LPS (100 ng/mL), recombinant mouse IFN- γ (0.33 ng/mL) and test samples dissolved in DMSO (final DMSO concentration 0.2%, v/v) for 16 h at 37°C. The culture supernatant (100 μ L) was placed in each well in duplicate 96-well flat-bottomed plate. A standard solution of NaNO₂ was also placed in other wells on the same plate. Griess reagent (50 μ L of 1% sulfanilamide in 5% H₃PO₄, and 50 μ L 0.1% *N*-1-naphthylethylenediamide dihydrochloride) was added to each well. After 10 min, the reaction products were quantified at 520 nm, with subtraction of the background absorbance at 655 nm, using a microplate reader. Cytotoxicity was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. An MTT solution (200 μ g/mL) was added after 16 h treatment and then incubated for another 8 h at 37°C. The reduced MTT-formazan was dissolved in 150 μ L

of DMSO, and the absorbance of the MTT-formazan solution at 520nm was measured by a microplate reader. The percentage of suppression was calculated by comparing the absorbance of sample-treated cells with that of nontreated cells.

Reverse Transcription-Polymerase Chain Reaction Analysis of iNOS mRNA, IL-6 mRNA and TNF- α mRNA^{14,15,17)}

Cell concentration was adjusted to 1.2×10^6 cells/mL, and 800 μ L (200 μ L $\times$ 4) of cell suspension was seeded in each well of a 96-well flat-bottomed plate. After a 2 h incubation, cells were treated with LPS (100 ng/mL), IFN- γ (0.33 ng/L) and test samples dissolved in DMSO (final DMSO concentration 0.2%, v/v) for 8 h at 37°C. After incubation, the cells were collected in a microfuge tube, chilled on ice and then centrifuged at 2800 $\times g$ for 1 min. Total RNA was isolated from the cell pellet using a RNA isolation kit (QIAGEN, Hilden, Germany). Total RNA (250 ng) was reverse-transcribed into cDNA by oligo (dT)₁₂₋₁₈ primer. The PCR sample contained 30 μ L of the reaction mixture, comprised of 50 mM KCl, 5 mM MgCl₂, 0.2 mM dNTP, 0.6 units of Ampli Taq GOLD (Applied Biosystems, CA, U.S.A.), and 0.4 μ mol of sense and antisense primers. The sense primer for iNOS was 5'-ACCTACTTCTGAC ATTACGACCC-3' and the antisense primer was 5'-AAG GGAGCAATGCCGTACCAAGCC-3'. The sense primer for IL-6 was 5'-TGG AGT CAC AGA AGG AGT GGCTAA G-3', and the antisense primer was 5'-TCTGACCACAGT GAGGAATGTCCAC-3. The sense primer for TNF- α was 5'-CAGCCTCTTCTCATTCCTGCTTG-3', and the antisense primer was 5'-GTCTTTGAGATCCATGCCGTTG-3. The sense primer for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was 5'-ACCACAGTCCATGCCATCAC-3', and the antisense primer was 5'-TCCACCACCCTGTTGCTG TA-3'. The PCR reaction was performed under the following conditions: 25 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1.5 min, using a thermal cycler (GeneAmp PCR Systems 9770; PE Applied Biosystems, U.S.A.). The PCR products were run on a 2% agarose gel and visualized by ethidium bromide staining. The bands in the gel were then photographed.

Acknowledgments This investigation was supported in part by Grants-in-Aid for Scientific Research (C) (No. 24590028) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan, and by the Nihon University Multidisciplinary Research Grant (No. 12-021).

References

- 1) Zhi-da M., Qi-Fi M., Mizuno M., Tanaka T., Iinuma M., *Planta Med.*, **53**, 300–302 (1987).
- 2) Motai T., Daikonya A., Kitanaka S., *J. Nat. Prod.*, **67**, 432–436 (2004).
- 3) Motai T., Kitanaka S., *Chem. Pharm. Bull.*, **52**, 1215–1218 (2004).
- 4) Motai T., Kitanaka S., *J. Nat. Prod.*, **68**, 365–368 (2005).
- 5) Motai T., Kitanaka S., *J. Nat. Prod.*, **68**, 1732–1735 (2005).
- 6) Motai T., Kitanaka S., *J. Nat. Med.*, **60**, 54–57 (2006).
- 7) Kojima K., Isaka K., Ondognii P., Jargalsaikhan G., Suran D., Mizukami H., Ogihara Y., *Chem. Pharm. Bull.*, **46**, 1781–1784 (1998).
- 8) Kojima K., Isaka K., Ondognii P., Jargalsaikhan G., Suran D., Mizukami H., Ogihara Y., *Chem. Pharm. Bull.*, **47**, 690–691 (1999).
- 9) Kojima K., Isaka K., Ondognii P., Zevgeegiin O., Davgiin K., Mizukami H., Ogihara Y., *Chem. Pharm. Bull.*, **47**, 1145–1147 (1999).
- 10) Kojima K., Isaka K., Ondognii P., Zevgeegiin O., Gombosurengyin P., Davgiin K., Mizukami H., Ogihara Y., *Chem. Pharm. Bull.*, **48**, 353–356 (2000).
- 11) Isaka K., Nagatsu A., Ondognii P., Zevgeegiin O., Gombosurengyin P., Davgiin K., Kojima K., Ogihara Y., *Chem. Pharm. Bull.*, **49**, 1072–1076 (2001).
- 12) Nagatsu A., Isaka K., Kojima K., Ondognii P., Zevgeegiin O., Gombosurengyin P., Davgiin K., Irfan B., Iqbal C. M., Ogihara Y., *Chem. Pharm. Bull.*, **50**, 657–677 (2002).
- 13) Su B.N., Takaishi Y., Honda G., Itoh M., Takeda Y., Kodzhimatov O. K., Ashurmetov O., *J. Nat. Prod.*, **63**, 436–440 (2000).
- 14) Ishii R., Saito K., Horie M., Shibano T., Kitanaka S., Amano F., *Biol. Pharm. Bull.*, **22**, 647–653 (1999).
- 15) Daikonya A., Kastuki S., Wu J.-B., Kitanaka S., *Chem. Pharm. Bull.*, **50**, 1566–1569 (2002).
- 16) Green L. C., Wagner D. A., Glogowski J., Skipper P. L., Wishnok J. S., Tannenbaum S. R., *Anal. Biochem.*, **126**, 131–138 (1982).
- 17) Zhao F., Nozawa H., Daikonnya A., Kondo K., Kitanaka S., *Biol. Pharm. Bull.*, **26**, 61–65 (2003).