Sesquiterpene Coumarins from *Ferula fukanensis* and Nitric Oxide Production Inhibitory Effects[1]

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Received September 9, 2003

Six new sesquiterpene coumarin derivatives, 2,3-dihydro-7-hydroxy-2*R**,3*R**-dimethyl-2-[4,8-dimethyl-3(*E*),7-nonadien-6-onyl]furo[3,2-*c*]coumarin (**4**), fukanefuromarin A (**5**), fukanefuromarin B (**6**), fukanefuromarin C (**7**), fukanefuromarin D (**8**), and fukanemarin A (**9**), were isolated from a 80% aqueous methanol extract of the roots of *Ferula fukanensis*. The structures were elucidated on the basis of spectral evidence, especially heteronuclear multiple-bond connectivity (HMBC), nuclear Overhauser exchange spectroscopy (NOESY), and high-resolution MS. An extract of *F. fukanensis* (FFE) and the sesquiterpene coumarin derivatives inhibited nitric oxide (NO) production and inducible NO synthase (iNOS) gene expression by a murine macrophage-like cell line (RAW 264.7), which was activated by lipopolysaccharide (LPS) and recombinant mouse interferon-γ (IFN-γ).

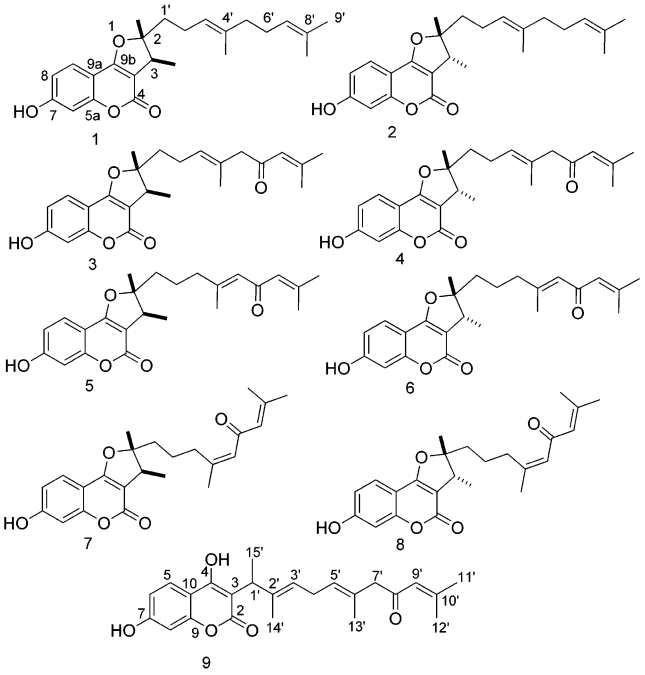
Ferula fukanensis grows on arid land in Central Asia and has been used as a traditional medicine for treatment of rheumatoid arthritis and bronchitis. Previous studies have analyzed the polysulfanes in this plant by GC−MS (CI/EI),[2] and the chemical constituents of plants in the genus *Ferula* (Umbelliferae) have been studied by many groups. Compounds commonly found in this genus are sesquiterpenes[3−9] (especially daucanes, humulanes, and guaianes), sesquiterpene coumarins, and sesquiterpene chromones.[10−14]

Macrophages play major roles in the immunity and inflammatory responses involved in host defense. Once activated, they initiate the production of cytokines, oxygen and nitrogen species, and eicosanoids. In macrophages, bacterial lipopolysaccharide (LPS), alone or in combination with recombinant mouse interferon-γ (IFN-γ), is the stimuli best characterized to induce the transcription of gene encoding proinflammatory proteins. Such stimulation results in cytokine release and the synthesis of enzymes such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). The nitric oxide (NO) radical is known to play a central role in inflammatory and immune reactions. However, excessive production of NO may cause tissue damage. In inflammatory diseases such as rheumatoid arthritis, excessive NO production by activated macrophages has been observed.

We discovered that FFE inhibited activated macrophage NO production (IC_{50} = 21.9 μg/mL). Therefore, the analysis of FFE was undertaken to identify the active compounds in the extract. Through bioactivity-guided fractionation, active compounds that inhibited NO production were isolated from the chloroform fraction. FFE and these compounds also inhibited iNOS mRNA expression in RAW 264.7 cells treated with LPS and IFN-γ.

Results and Discussion

FFE was partitioned by successive extraction with chloroform, ethyl acetate, and water. The chloroform-soluble fraction inhibited 60% of NO production at 30 μg/mL. Therefore, the chloroform-soluble fraction was separated by silica gel column chromatography to yield 11 fractions. Fraction 6 inhibited 96.2% of NO production at 30 μg/mL. Compounds **1**−**9** were isolated from fraction 6.



Fukanemarin A (**9**) was obtained as a yellow oil, with a molecular weight of 396 based on fast atom bombardment mass spectrometry (FABMS) data that showed a protonated molecular ion peak at *m*/*z* 397 $(M + H)^+$ and a deprotonated molecular ion at *m*/*z* 395 $(M - H)^-$ in the negative mode. These data, together with the ^1H and ^{13}C NMR spectral data (Table 3), suggest a molecular formula of $C_{24}H_{28}O_5$, which was supported by HRFABMS in the positive mode ($C_{24}H_{29}O_5$, *m*/*z* 397.20147). The mass spectral data for compounds **3**−**9** showed the same molecular ions (see Experimental Section), which indicated the presence of isomeric compounds.

The ^1H NMR spectral data of **9** showed the presence of a 1,2,4-trisubstituted benzene ring at δ_H 7.59 (1H, d, J = 8.8 Hz, H-5), δ_H 6.81 (1H, dd, J = 8.8, 2.3 Hz, H-6), and δ_H 6.92 (1H, d, J = 2.3 Hz, H-8) and other signals characteristic of a sesquiterpene unit, determined on the basis of

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10.1021/np030408k CCC: $27.50
Published on Web 02/24/2004

Table 1. ^{1}H NMR Data for Compounds **3**−**8** (300 MHz) ($CDCl_3$, TMS, δ (ppm), J = Hz)[a]

H	**4**	**5**	**6**	**7**	**8**
3	3.29 q (7.0)	3.24 q (7.0)	3.20 q (7.0)	3.26 q (7.0)	3.16 q (7.0)
6	7.07 brd	7.05 d (2.1)	7.07 d (2.3)	7.07 d (2.2)	7.05 d (2.6)
8	6.87 dd (2.2, 8.8)	6.86 dd (2.1,8.5)	6.86 dd (2.3, 8.5)	6.82 dd (2.2, 8.4)	6.80 dd (2.6, 8.7)
9	7.49 d (8.8)	7.50 d (8.5)	7.50 d (8.5)	7.46 d (8.4)	7.44 d (8.7)
1′	1.71 m	1.71 m	1.69 m	1.82 m	1.78 m
2′	2.30 m	1.61 m	1.80 m	1.60 m	1.75 m
3′	5.29 t (7.0)	2.18 m	2.21 m	2.64 m	2.69 m
5′	3.08 s	6.04 s	6.06 s[b]	6.03 s	6.06 s
7′	6.12 brs	6.05 s	6.06 s[b]	6.05 s	6.08 s
9′	1.87 s	1.88 s	1.89 s	1.87 s	1.89 s
2Me	1.48 s	1.44 s	1.47 s	1.41 s	1.44 s
3Me	1.28 d (7.0)	1.30 d (7.0)	1.27 d (7.0)	1.28 d (7.0)	1.27 d (7.0)
4′Me	1.66 s	2.13 s	2.16 s	1.86 s	1.92 s
8′Me	2.15 s	2.15 s	2.17 s	2.13 s	2.14 s

[a] Assignments confirmed by decoupling, ^{1}H−^{1}H COSY, HMQC, HMBC, NOESY, and different NOE spectra. [b] Overlapped signal.

Table 2. ^{13}C NMR Data for Compounds **3**−**8** (75 MHz, $CDCl_3$, TMS, δ (ppm))[a]

C	**4**	**5**	**6**	**7**	**8**
2	96.9	97.1	96.6	97.4	96.1
3	44.9	42.5	44.7	42.4	44.0
3a	103.8	103.3	103.6	103.2	102.8
4	162.8	162.4	162.4	162.3	161.7
5a	157.3	156.8	156.8	156.7	156.0
6	103.8	103.5	103.5	103.4	103.1
7	161.6	161.3	161.3	161.1	160.3
8	113.9	113.7	113.7	113.5	112.8
9	124.5	124.3	124.3	124.3	123.7
9a	106.1	105.7	105.9	105.9	105.4
9b	166.2	166.0	165.9	166.0	165.1
1′	35.6	41.5	35.1	41.9	34.8
2′	23.8	21.9	22.6	22.6	22.7
3′	129.1	41.6	41.9	33.9	33.5
4′	130.7	157.2	157.3	157.0	157.4
5′	55.8	126.3[b]	126.2	126.9	126.2
6′	200.1	191.9	192.0	191.4	190.8
7′	123.3	126.3[b]	126.3	126.4	125.8
8′	157.0	155.5	155.5	155.2	154.6
9′	28.6	28.4	28.4	28.4	27.7
2Me	26.1	21.0	26.0	21.0	25.3
3Me	14.4	14.7	14.2	14.6	13.5
4′Me	17.3	19.6	19.8	26.0	25.4
8′Me	21.7	21.3	21.3	21.3	20.6

[a] Assignments confirmed by decoupling, ^{1}H−^{1}H COSY, HMQC, HMBC, NOESY, and different NOE spectra. [b] Overlapped signal.

Table 3. NMR Spectral Data for Compound **9** (300 MHz for ^{1}H NMR, 75 MHz for ^{13}C NMR, $CDCl_3$, TMS, δ (ppm), J = Hz)[a]

position	H	C
2		164.2
3		103.8
4		161.7
5	7.59 d (8.8)	123.9
6	6.81 dd (2.3,8.8)	112.7
7		159.6
8	6.92 d (2.3)	102.2
9		153.5
10		108.5
1′	3.78 q (7.0)	37.8
2′		140.0
3′	5.74 t (7.0)	124.6
4′	2.93 m	27.0
5′	5.29 t (7.3)	125.7
6′		130.8
7′	3.12 s	54.9
8′		198.8
9′	6.14 brs	122.4
10′		156.3
11′	1.89 s	27.7
12′	1.35 d (7.0)	16.2
13′	1.75 s	17.0
14′	1.70 s	16.6
15′	2.17 s	20.8
4OH	7.76 s	

[a] Assignments confirmed by decoupling, ^{1}H−^{1}H COSY, HMQC, HMBC, and NOESY spectra.

correlations of ^{1}H−^{1}H COSY, HMQC, HMBC, and NOESY spectra. The remaining ^{1}H and ^{13}C NMR data of **9**, except for those of the sesquiterpene unit, indicated a 7-hydroxy-substituted coumarin compound.

In the HMBC spectrum of **9**, the correlations of δ_H 3.78 (H-1′) with δ_C 164.2 (C-2), δ_C 103.8 (C-3), δ_C 161.7 (C-4), δ_C 140.0 (C-2′), and δ_C 124.6 (C-3′); δ_H 1.35 (H-15′) with δ_C 103.8 (C-3), δ_C 140.0 (C-2′), and δ_C 37.8 (C-1′); and δ_H 7.76 (OH) with δ_C 103.8 (C-3), δ_C 161.7 (C-4), and δ_C 108.5 (C-10) confirmed that the sesquiterpene unit is connected to C-3 of the coumarin unit and a hydroxyl group is attached to C-4. The chemical shifts of C-3 and C-4 are very close to those reported for similar compounds from *F. pallida*.[15] The configurations of the two double bonds of the sesquiterpene unit were determined as 2′*E* and 5′*E* on the basis of the ^{13}C NMR spectral data (δ_C 16.6, C-13′; δ_C 17.0, C-14′)[16] and NOESY experiments, in which cross-peaks were observed from H-4′/H-13′, H-4′/H-14′, and the H-5′/H-7′ pairs. Thus, compound **9** is 4,7-dihydroxy-3-(1,2,6,10-tetramethyl-8-oxo-undeca-2(*E*),5(*E*),9-trienyl)coumarin.

Fukanefuromarin A (**5**) was obtained as a colorless oil. The ^{1}H NMR spectral data of **5** showed the presence of a 1,2,4-trisubstitued benzene ring at δ_H 7.50 (1H, d, J = 8.5 Hz, H-9), δ_H 7.05 (1H, d, J = 2.1 Hz, H-6), and δ_H 6.86 (1H, dd, J = 8.5, 2.1 Hz, H-8) and other characteristic signals based on correlations of ^{1}H−^{1}H COSY, HMQC, and HMBC spectra. The remaining ^{1}H and ^{13}C data of **5**, except for those of the sesquiterpene unit, indicated a 7-oxygen-substituted coumarin compound.

In the HMBC spectrum of **5**, the correlations of δ_H 1.44 (2-Me) with δ_C 97.1 (C-2), δ_C 42.5 (C-3), and δ_C 41.5 (C-1′) and δ_H 1.30 (3-Me) with δ_C 97.1 (C-2), δ_C 42.5 (C-3), and δ_C 103.3 (C-3a) suggested that C-1′ is connected to C-2. That C-2 is connected to C-9b by an ether bond was deduced according to the unsaturation value and the chemical shifts of C-2 (δ_C 97.1) and C-9b (δ_C 166.0). A series of NOESY experiments was conducted to identify the relative configuration of the dimethyldihydrofuran moiety at C-2 and C-3. Cross-peaks were observed from the pairs H-3/ H-1′, indicating a *cis* relationship between 2- and 3-Me. The configuration of the double bond of the sesquiterpene unit was assigned as 4′*E* on the basis of the chemical shift of 4′-Me, which is shifted relatively upfield (δ_C 19.6).[16] Thus, compound **5** was established as 2,3-

dihydro-7-hydroxy-2*S**,3*R**-dimethyl-2-[4,8-dimethyl-4(*E*),7-nonadien-6-onyl]furo[3,2-*c*]coumarin.

Fukanefuromarin B (**6**) was obtained as a colorless oil. 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-1′ (δ_C 35.1 for **6**, δ_C 41.5 for **5**) and 4′-Me (δ_C 26.0 for **6**, δ_C 21.0 for **5**), suggesting that **6** may be a diastereomer of **5** at chiral centers C-2 and C-3. A series of NOESY experiments was conducted to identify the relative configuration of the dimethyldihydrofuran moiety at C-2 and C-3. Cross-peaks were observed between the pairs H-3/2-Me and H-1′/3-Me, which indicated that the relationship between the 2- and 3-Me is *trans*. Thus, compound **6** is 2,3-dihydro-7-hydroxy-2*R**,3*R**-dimethyl-2-[4,8-dimethyl-4-(*E*),7-nonadien-6-onyl]-furo[3,2-*c*]coumarin.

Fukanefuromarin C (**7**) was obtained as a colorless oil. The ^{1}H and ^{13}C NMR spectra of **7** were similar to those of **5**. HMBC experiments suggested that the structure of **7** was similar to that of **5**. However, the NMR spectra of **7** differed slightly from those of **5**, especially the 4′-Me signal (δ_H 1.86 s for **7**, δ_H 2.13 s for **5**) and the H-3′ methylene signal (δ_H 2.64 m for **7**, δ_H 2.18 m for **5**). A series of NOESY experiments was conducted to identify the relative configuration of the dimethyldihydrofuran moiety at C-2 and C-3. Cross-peaks were observed from the pairs H-3/H-1′, suggesting a *cis* relationship between the 2- and 3-Me of **7**. Furthermore, the configuration of the double bond of the sesquiterpene unit was determined on the basis of the differences in NOE experiments. Compound **7** showed significant NOE correlation between 4′-Me and H-5′, indicating that the double bond of the sesquiterpene unit of compound **7** has the *Z* configuration. Thus, compound **7** is 2,3-dihydro-7-hydroxy-2*S**,3*R**-dimethyl-2-[4,8-dimethyl-4(*Z*),7-nonadien-6-only]furo[3,2-*c*]coumarin.

Fukanefuromarin D (**8**) was obtained as a colorless oil. The HMBC experiments suggested that the structure of **8** was similar to that of **7**. However, the NMR spectra of **8** differed slightly from those of **7**, especially C-1′ (δ_C 34.8 for **8**, δ_C 41.9 for **7**) and 2-Me (δ_C 25.3 for **8**, δ_C 21.0 for **7**), suggesting **8** is a diastereomer of **7** at the chiral centers C-2 and C-3. A series of NOESY experiments was conducted to identify the relative configuration of the dimethyldihydrofuran moiety at C-2 and C-3. Cross-peaks were observed between the pairs H-3/2-Me and H-1′/3-Me, indicating a *trans* relationship between the 2- and 3-Me of **8**. Thus, the structure of **8** is 2,3-dihydro-7-hydroxy-2*R**,3*R**-dimethyl-2-[4,8-dimethyl-4(*Z*),7-nonadien-6-onyl]-furo[3,2-*c*]coumarin.

Compound **4** was obtained as a colorless oil. The HMBC experiment 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 C-1′ and 2-Me, indicating **4** may be a diastereomer of **3** at the chiral centers C-2 and C-3. A series of NOESY experiments was conducted to identify the relative configulation of the dimethyldihydrofuran moiety at C-2 and C-3. Cross-peaks were observed between the pairs H-3/2-Me and H-1′/3-Me of **4**. These associations indicated a *trans* relationship between the 2- and 3-Me of **4**. The configurations of the double bonds of the sesquiterpene unit were determined as 4′*E* on the basis of the ^{13}C NMR spectral data (δ_C 17.3, 4′-Me)[16] and NOESY experiments, in which cross-peaks were observed from the H-3′/H-5′ pairs. Thus, **4** is 2,3-dihydro-7-hydroxy-2*R**,3*R**-dimethyl-2-[4,8-dimethyl-3(*E*),7-nonadien-6-onyl]furo[3,2-*c*]coumarin.

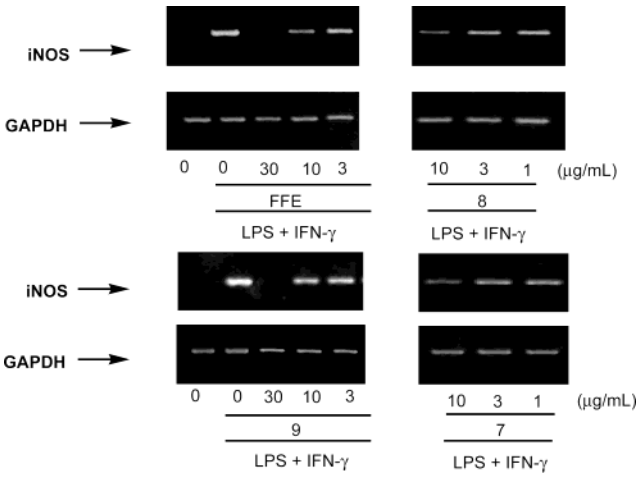

Figure 1. Inhibitory effect of FFE and compounds **7**−**9** on gene expression stimulated by LPS and IFN-γ.

Compounds **1**−**3** are known compounds, whose structures were elucidated by comparison with literature reports.[14]

When the RAW 264.7 cells were incubated with LPS and IFN-γ, NO production increased sharply. The addition of FFE caused concentration-dependent inhibition of NO production by macrophages during incubation (IC_{50} = 21.9 μg/mL). The chloroform and water fractions showed 60% and 33.3% inhibition, respectively, at 30 μg/mL. The ethyl acetate fraction showed cytotoxic effects at 30 μg/mL. Quercetin is reported to have an inhibitory effect on the production of NO by LPS and IFN-γ stimulated macrophage cell RAW 264.7 (IC_{50} = 26.8 μM).[17−19] Compounds **1** and **2** did not inhibit NO production at 10 μg/mL and showed no cytotoxic effects, while **3** and **5**−**9** had stronger inhibition than did quercetin on NO production (**3**, IC_{50} = 24.6 μM; **5**, IC_{50} = 13.0 μM; **6**, IC_{50} = 16.2 μM; **7**, IC_{50} = 11.1 μM; **8**, IC_{50} = 8.9 μM; **9**, IC_{50} = 19.5 μM). In contrast, compound **4** inhibited NO production more weakly than did quercetin (IC_{50} = 31.2 μM). By comparing the inhibitory activities of these compounds, we identified some interesting features that may affect the activity level. Compounds **1** and **2**, neither of which contains a ketone in the sesquiterpene unit, did not inhibit NO production. However, **3**−**9**, which have α,β-unsaturated ketones, inhibited NO production. This suggests that the α,β-unsaturated ketone moiety of the sesquiterpene unit may be important for sesquiterpene coumarin inhibitory activity. Compounds **3**−**6** have the same molecular backbone, but differ in the position of the double bond in the sesquiterpene unit. Inhibitory activities of **5** and **6** were stronger than those of **3** and **4**, suggesting that the double-bond position of the sesquiterpene enhances sesquiterpene coumarin inhibitory activity. Furthermore, compounds **7** and **8** have the *Z* configuration in the sesquiterpene, which resulted in greater inhibitory effects on NO production than those caused by compounds **5** and **6**. This suggests that a sesquiterpene with *Z* configuration enhances sesquiterpene coumarin inhibitory activity. NO is synthesized by a family of enzymes termed NOS, which utilize arginine as a substrate in the generation of NO. Of the three NOS isoforms, the isoform expressed in the macrophage is termed iNOS. Its activity is regulated at the transcription level by cytokines as well as through cell exposure to immune and inflammatory stimuli. FFE and compounds **7**−**9** inhibited iNOS mRNA expression in a dose-dependent manner (Figure 1). The cytotoxic effects of FFE and these compounds were measured using the MTT assay. FFE,

compound **9** (1−30 μg/mL), and compounds **3**−**8** (0.3−10 μg/mL) did not demonstrate any significant cytotoxicity upon LPS/IFN-γ treatment for 24 h. The RT-PCR analysis in the present study indicated that LPS/IFN-γ treatment increased the level of iNOS mRNA expression, and the sesquiterpene coumarin derivatives inhibited this increase. Therefore, inhibition of iNOS induction by the sesquiterpene coumarin derivatives may be mediated through the expression of these transcription-activating factors, thereby inhibiting iNOS transcription.

Experimental Section

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. The mass spectra (MS) were obtained on a JEOL GCmate spectrometer. Column chromatography was carried out silica gel (Wako gel C-300, WAKO Pure Chemical Industry Ltd.). 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 × 150 mm i.d.) columns and YMC-PAK Pro-C_{18} (10 × 150 mm i.d.) were used for preparative purposes.

Plant Materials. The dried roots of *F. fukanensis* were collected in the Urumqi, Xinjiang, People's Republic of China, in October 2002.

Extraction and Isolation. The dried roots of *F. fukanensis* (5.9 kg) were extracted with 80% MeOH. The solvent was evaporated under reduced pressure from the combined extract (448.8 g). The extract was suspended in water (3.0 L) and partitioned with chloroform (3 × 3 L) and ethyl acetate (3 × 3 L), successively. Evaporation of the solvent yielded a $CHCl_3$ fraction (272.4 g), a EtOAc fraction (142.0 g), and an aqueous fraction (96.1 g). The $CHCl_3$ 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 11 portions. Fraction 6, eluted with hexane−EtOAc (80:20), was isolated and further purified by column chromatography and reversed-phase HPLC (CH_3CN−water, 72:28, flow rate 3 mL/min, UV detector set at 210 nm) to give **1** (8.4 mg, t_R 22.3 min) and **2** (16.7 mg, t_R 23.9 min), by reversed-phase HPLC (CH_3CN−water, 60:40, flow rate 3 mL/min, UV detector set at 210 nm) to give **9** (5.4 mg, t_R 8.3 min), by reversed-phase HPLC (CH_3-CN−water, 60:40, flow rate 3 mL/min, UV detector set at 210 nm) to give **4** (20.1 mg, t_R 9.5 min), by reversed-phase HPLC (CH_3CN−water, 60:40, flow rate 3 mL/min, UV detector set at 210 nm) and normal-phase HPLC (hexane−EtOAc, 65:35, flow rate 3 mL/min, UV detector set at 254 nm) to give **8** (4.7 mg, t_R 12.3 min) and **6** (15.0 mg, t_R 13.3 min), and by reversed-phase HPLC (CH_3CN−water, 56:44, flow rate 3 mL/min, UV detector set at 210 nm) and normal-phase HPLC (hexane−EtOAc, 70:30, flow rate 3 mL/min, UV detector set at 254 nm) to give **7** (12.6 mg, t_R 13.6 min), **5** (40.0 mg, t_R 15.6 min), and **3** (41.9 mg, t_R 15.9 min).

2,3-Dihydro-7-hydroxy-2*R,3*R**-dimethyl-2-[4,8-dimethyl-3(*E*),7-nonadien-6-onyl]furo[3,2-*c*]coumarin (4):** colorless oil; $[\alpha]^{23}_D$ −8.46° (*c* 1.41, MeOH); UV (MeOH) λ_{max} (log ϵ) 338.0 (4.68), 316.5 (4.13), 288.5 (3.75), 228.0 (4.27), 209.5 (4.20); IR (LF) ν_{max} 3237, 2975, 2936, 2590, 1712, 1694, 1629, 1571, 1518, 1449, 1415, 1378, 1265, 1234, 1153, 1110, 1000, 959, 851, 825, 768 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; FABMS *m/z* 397 [M + H]$^+$; HRFABMS *m/z* 397.20145 [M + H]$^+$ (calcd for $C_{24}H_{29}O_5$ 397.20147).

Fukanefuromarin A (5): colorless oil; $[\alpha]^{23}_D$ ±0° (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 330.5 (4.07) 317.0 (4.15), 270.5 (4.27), 228.0 (4.13), 206.5 (4.45); IR (KBr) ν_{max} 3228, 2930, 1721, 1686, 1633, 1572, 1518, 1415, 1381, 1263, 1222, 1149, 1109, 1002, 956, 881, 851, 806, 767 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; FABMS *m/z* 397 [M + H]$^+$; HRFABMS *m/z* 397.20149 [M + H]$^+$ (calcd for $C_{24}H_{29}O_5$ 397.20147).

Fukanefuromarin B (6): colorless oil; $[\alpha]^{23}_D$ ±0° (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ϵ) 330.5 (3.88), 317.0 (3.97), 268.5 (4.09), 227.0 (3.99), 206.5 (4.30); IR (KBr) ν_{max} 3230, 2973, 2938, 1717, 1684, 1630, 1571, 1518, 1415,1377, 1265, 1222, 1144, 1108, 997, 958, 883, 852, 808, 754 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; FABMS *m/z* 397 [M + H]$^+$; HRFABMS *m/z* 397.20109 [M + H]$^+$ (calcd for $C_{24}H_{29}O_5$ 397.20147).

Fukanefuromarin C (7): colorless oil; $[\alpha]^{23}_D$ ±0° (*c* 0.49, MeOH); UV (MeOH) λ_{max} (log ϵ) 330.5 (3.57), 317.0 (3.65), 270.0 (3.77), 227.5 (4.66), 206.5 (4.97); IR (KBr) ν_{max} 3259, 2973, 2932, 2590, 1894, 1721, 1688, 1633, 1573, 1518, 1445, 1416, 1380, 1263, 1230, 1150, 1113, 997, 957, 852, 808, 767 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; FABMS *m/z* 397 [M + H]$^+$; HRFABMS *m/z* 397.20139 [M + H]$^+$ (calcd for $C_{24}H_{29}O_5$ 397.20147).

Fukanefuromarin D (8): colorless oil; $[\alpha]^{23}_D$ −5.0° (*c* 0.16, MeOH); UV (MeOH) λ_{max} (log ϵ) 330.5 (4.04), 317.0 (4.11), 269.5 (4.23), 227.5 (4.13), 206.0 (4.44); IR (KBr) ν_{max} 3248, 2972, 2935, 1688, 1632, 1572, 1688, 1632, 1572, 1518, 1448, 1417, 1377, 1265, 1226, 1111, 998, 958, 852, 812, 767 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; FABMS *m/z* 397 [M + H]$^+$; HRFABMS *m/z* 397.20163 [M + H]$^+$ (calcd for $C_{24}H_{29}O_5$ 397.20147).

Fukanemarin A (9): yellow oil; $[\alpha]^{23}_D$ +22.5° (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 312.0 (4.12), 237.5 (4.21), 212.0 (4.47); IR (KBr) ν_{max} 3309, 2973, 2934, 1677, 1617, 1512, 1430, 1379, 1326, 1228, 1157, 1121, 1016, 950, 852, 755 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 3; FABMS *m/z* 397 [M + H]$^+$; HRFABMS *m/z* 397.20113 [M + H]$^+$ (calcd for $C_{24}H_{29}O_5$ 397.20147).

Nitrite Assay.[20] The cells were seeded at 1.2 × 10^6 cells/mL onto a 96-well flat-bottomed plate (Sumitomo Bakelite, #8096R, Tokyo) and then incubated at 37 °C for 2 h. Then the test compound was added to the culture simultaneously with both *Escherichia coli* LPS (100 ng/mL) and recombinant mouse IFN-γ (0.33 ng/mL), and the cells were incubated at 37 °C, usually for 16 h. After incubation, the cells were chilled on ice. One 100 μL of the culture supernatant was placed in a well in duplicate 96-well flat-bottomed plates. A standard solution of $NaNO_2$ was also placed in other wells on the same plate. To quantify nitrite, 50 mL of Griess reagent, 1% sulfanilamide in 5% H_3PO_4, and 0.1% *N*-1-naphthyletylenediamide dihydrochloride were added to each well. After 10 min, the reaction products were colorimetrically quantified at 550 nm with subtraction of the background absorbance at 630 nm, using a model 3550 microplate reader (BIO-RAD).

Reverse Transcription-Polymerase Chain Reaction Analysis of iNOS mRNA. The cells were cultured at 1.2 × 10^6 cells/mL onto a 96-well flat-bottomed plate at 37 °C for 2 h, then the test compound was added to the culture simultaneously with both LPS (100 ng/mL) and IFN-γ (0.33 ng/mL). The cells were incubated at 37 °C for approximately 8 h. 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 μL of the reaction mixture, comprised of 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM dNTP, 0.6 units of Ampli Taq GOLD (Applied Biosystems, CA), and 0.4 μmol of sense and antisense primers. The sense primer for iNOS was 5′-ACCTACTTCCTGGACATTACGACCC-3′, and the antisense primer was 5′-AAGGGAGCAATGCCCGTACCAGGCC-3′. The sense primer for glyceraldehydes-3-phosphatedehydrogenase (GAPDH) was 5′-ACCACAGTCCATGCCATCAC-3′, and the antisense primer was 5′-TCCACCACCCTGTTGCTGTA-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). The PCR products were run on a 2% agarose gel and visualized by ethidium bromide staining. The bands in the gel were then photographed.

Acknowledgment. This work was supported by grants to Nihon University from the Promotion and Mutual Aid Corporation for Private Schools of Japan; a Grant from the Ministry of Education, Culture, Sports, Science, and Technology to promote multidisciplinary research projects; and a Health Sciences Research Grant for Research on Eye and Ear Science, Immunology, Allergy and Organ Transplantation from the Ministry of Health, Labor and Welfare, Japan.

References and Notes

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NP030408K