Neolignans from *Piper futokadsura* and Their Inhibition of Nitric Oxide Production

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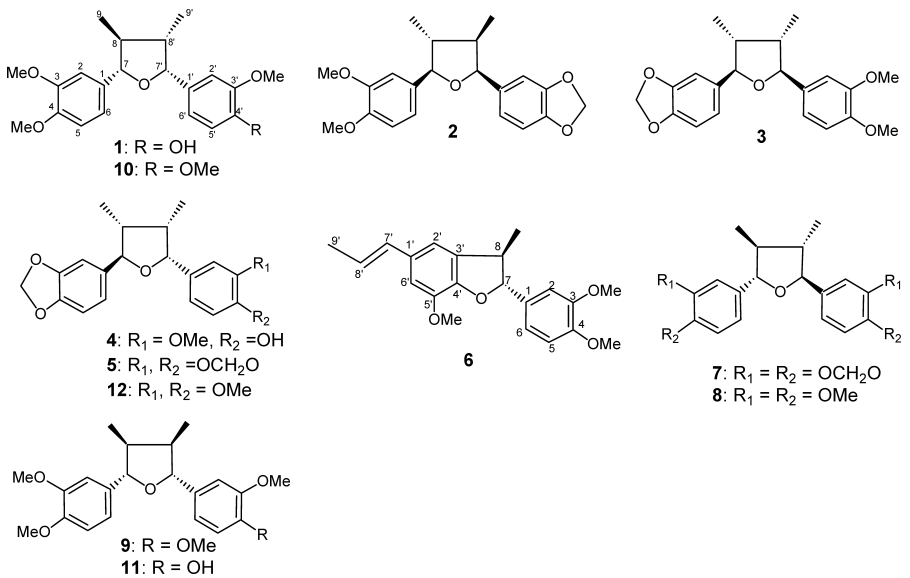
Received August 20, 2004; accepted September 29, 2004

From a MeOH extract of the aerial part of *Piper futokadsura*, the tetrahydrofuran lignans, futokadsurin A [(7*S*,8*S*,7′*S*,8′*R*)-3,4,3′-trimethoxy-4′-hydroxy-7,7′-epoxylignan], futokadsurin B [(7*R*,8*R*,7′*R*,8′*S*)-3,4-dimethoxy-3′,4′-methylenedioxy-7,7′-epoxylignan], and futokadsurin C [(7*R*,8*R*,7′*S*,8′*S*)-3,4-methylenedioxy-3′,4′-dimethoxy-7,7′-epoxylignan] were isolated, together with nine known neolignans. In addition, L-tryptophan, pellitorine, phytol, elemicin, and 1,2,4-trimethoxyphenyl-5-aldehyde were isolated. The structures of the new compounds were elucidated using spectroscopic methods. These lignans inhibited nitric oxide production by a murine macrophage-like cell line (RAW 264.7), which was activated by lipopolysaccharide and interferon-γ.

Key words *Piper futokadsura*; Piperaceae; tetrahydrofuran lignan; nitric oxide

Piper futokadsura SIEB. *et* ZUCC. (Piperaceae) grows in coastal forests and is a vine-like plant that covers rocks and trees. Although this plant belongs to the same genus as pepper, there is no sharp taste. In China, the leaf and stem of *P. futokadsura* are used to treat colds, neuralgia, and rheumatism.[1)] In Okinawa, Japan, the fruit is used for cooking, similar to pepper. It is also used as a stomachic, for intestinal regulation, and to stimulate appetite.[2)] The following compounds were previously reported from this plant: futoamide,[3)] futoenone,[4—6)] piperinone, and futoxide (crotepoxide).[3)] Piperinone inhibits insect feeding[7)] and kadsurenone and futoxide show inhibitory effects against platelet aggregation induced by platelet-activating factor.[8)] Several neolignans, such as kadsurenone, (−)-acuminatine, (+)-licarin A, (−)-denudatin B, and kazurenin M, were also isolated from the leaf and stem.[9)]

We have examined the extracts of *P. futokadsura* in Okinawa and isolated three new tetrahydrofuran lignans, futokadsurin A (**1**), futokadsurin B (**2**), and futokadsurin C (**3**) as well as 14 known compounds, (−)-chicanine (**4**),[10)] (−)-zuonin-A (**5**),[11)] (+)-acuminatin (**6**),[12)] (−)-galbacin (**7**),[13,14)] (−)-galbelgin (**8**),[15,16)] (±)-galgravin (**9**), (+)-veraguensin (**10**),[17—19)] machilin F (**11**),[20)] (−)-machilusin (**12**),[21,22)] L-tryptophan, pellitorine=*N*-(2-methylpropyl)-2*E*,4*E*,(9C1)-2,4-decadienamide, elemicin, 1,2,4-trimethoxyphenyl-5-aldehyde, and phytol from the aerial parts of *P. futokadsura*. In this study, the structural elucidation of the new compounds, futokadsurin A (**1**), B (**2**), and C (**3**), and complete ^{13}C- and ^{1}H-NMR assignments of other neolignans were carried out using ^{1}H–^{1}H correlation spectroscopy (^{1}H–^{1}H COSY), heteronuclear single-quantum coherence (HSQC), ^{1}H-detected heteronuclear multiple-bond correlation (HMBC), and nuclear Overhauser effect spectroscopy (NOESY) experiments. As an integral part of the research on antioxidant effects of medicinal plants, we also investigated the effects of tetrahydrofuran lignans from this plant on nitric oxide (NO) production by activated macrophages treated with lipopolysaccharide (LPS) and interferon (IFN)-γ.



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Results and Discussion

The aerial parts of *P. futokadsura* were extracted with MeOH. The MeOH extract was suspended in distilled water and partitioned successively with *n*-hexane, $CHCl_3$, AcOEt, and *n*-BuOH. The *n*-hexane fraction (34.1 g) was fractionated by chromatography on a silica gel column using a hexane–AcOEt gradient and yielded 16 lignan-containing subfractions. These subfractions were purified by silica gel, reverse-phase silica gel, and preparative HPLC giving three new lignans (**1**—**3**), nine known lignans (**4**—**12**), and other known compounds. Their structures were established on the basis of electron-impact (EI)-MS, ^{1}H-, ^{13}C-NMR, distortionless enhancement by polarization transfer (DEPT), ^{1}H–^{1}H COSY, HSQC, and HMBC as well as NOESY. All molecular ion peaks were obtained in EI-MS and their molecular formulas were obtained with high-resolution (HR)-MS.

The EI-MS spectral data of futokadsurin A (**1**) showing a molecular ion peak at *m/z* 358 were compatible with the molecular formula $C_{21}H_{26}O_5$. The IR spectrum showed a hydroxyl group at 3546 cm^{-1} and an intense absorption band at 1140 cm^{-1}, suggesting ether functionality. Since no absorptions near 1700 cm^{-1} were observed, the presence of a carbonyl group was not considered further. The ^{1}H-NMR spectrum (Table 1) showed two sets of ABX system signals at δ 6.83 (1H, dd, J=2.0, 8.0 Hz, H-6′), 6.86 (1H, d, J=2.0 Hz, H-2′), 6.88 (2H, d, J=8.0 Hz, H-5, 5′), 7.04 (1H, dd, J=1.6, 8.0 Hz, H-6), and 7.07 (1H, d, J=1.6 Hz, H-2), and three methoxyl and one hydroxyl groups at δ 3.85, 3.89, 3.91 and 5.63 (1H, d, J=1.2 Hz) belonging to two partially methoxylated and hydroxylated aromatic moieties. The ^{13}C-NMR spectrum of **1** showed signals assignable to five methyls including three methoxyl groups, 10 methine, and six quaternary carbons. This information and the HMBC spectrum (Fig. 1) determined the two aromatic rings to be 3,4-dimethoxyphenyl and 3′-methoxy-4′-hydroxyphenyl for this lignan. Two sets of signals at δ 0.66 (3H, d, J=6.8 Hz), 2.24 (1H, ddq, J=6.8, 8.8, 9.2 Hz), and 5.12 (1H, d, J=8.8 Hz), and δ 1.06 (3H, d, J=6.8 Hz), 1.78 (1H, ddq, J=6.8, 9.2, 9.2 Hz), and 4.41 (1H, d, J=9.2 Hz), for which the connectivities were established by ^{1}H–^{1}H COSY and HMBC spectra, confirmed the tetrahydrofuran ring moiety. The relative stereochemistry in the tetrahydrofuran ring was defined as *trans* H-7/H-8, *trans* H-8/H-8′, and *cis* H-8′/H-7 based on the correlations of the NOESY spectrum (Fig. 1). All these assignments were confirmed by HMBC correlations in which mutual cross-peaks between C-7/H-2, H-6, H-8′, and H-9 and between C-7′/H-2′, H-6′, H-8, and H-9′ were observed. The chemical shifts of the proton and carbons of **1** were very similar to those of (+)-veraguensin previously isolated from *Illicium floridanum*[17,18] except for the presence of a hydroxyl group at δ 5.63 (1H, d, J=1.2 Hz) instead of one methoxyl group. The optical rotation of **1** ($[\alpha]_D$ +12.3°) showed the same positive sign as that of (+)-veraguensin ($[\alpha]_D$ +34.2°).[19] Therefore the structure of futokadsurin A (**1**) was determined to be *rel*-(7*S*,8*S*,7′*R*,8′*S*)-3,4,3′-trimethoxy-4′-hydroxy-7,7′-epoxylignan.

Futokadsurin B (**2**), mp 102 °C, $[\alpha]_D$ +33.7°, showed a molecular ion peak at *m/z* 356 and the molecular formula was determined to be $C_{21}H_{24}O_5$. The ^{1}H- and ^{13}C-NMR spectra (Tables 1, 2) of **2** were similar to those of (+)-calopiptin.[17,23] These chemical shifts are characteristic of a tetrahydrofuran ring with a *cis*-configuration of one aryl and methyl substituent as well as a *trans*-configuration of the

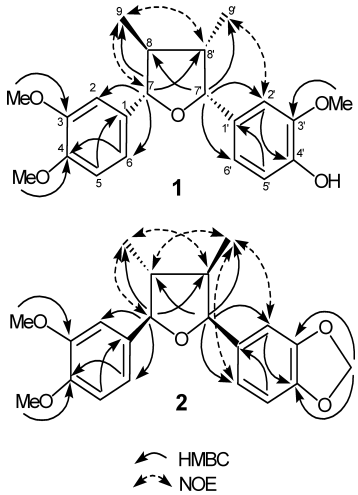

Fig. 1. Significant Correlations Observed in the HMBC and NOE Spectra of **1** and **2**

Table 1. ^{1}H-NMR Spectral Data for Neolignans **1**—**3** (δ, $CDCl_3$, J in Hz)

H	1	2	3
2	7.07 (d, 1.6)	7.06 (d, 2.0)	6.97 (d, 2.0)
5	6.88 (d, 8.0)	6.89 (d, 8.0)	6.78 (d, 8.0)
6	7.04 (dd, 1.6, 8.0)	7.02 (dd, 2.0, 8.0)	6.88 (dd, 2.0, 8.0)
7	4.41 (d, 9.2)	4.38 (d, 9.6)	4.46 (d, 7.2)
8	1.78 (ddq, 6.8, 9.2, 9.2)	1.77 (ddq, 6.4, 9.0, 9.6)	2.31 (m)
9	1.06 (d, 6.8)	1.05 (d, 6.4)	1.018 (d, 6.4)
2′	6.86 (d, 2.0)	6.78 (d, 1.6)	6.99 (d, 2.0)
5′	6.88 (d, 8.0)	6.79 (d, 8.0)	6.86 (d, 8.0)
6′	6.83 (dd, 2.0, 8.0)	6.89 (dd, 1.6, 8.0)	6.95 (dd, 2.0, 8.0)
7′	5.12 (d, 8.8)	5.10 (d, 8.8)	4.47 (d, 7.2)
8′	2.24 (ddq, 6.8, 8.8, 9.2)	2.26 (ddq, 7.2, 8.8, 9.0)	2.26 (m)
9′	0.66 (d, 6.8)	0.67 (d, 7.2)	1.022 (d, 6.4)
OCH_3-3	3.91 (s)	3.92 (s)	
OCH_3-4	3.89 (s)	3.90 (s)	
OCH_3-3′	3.85 (s)		3.89 (s)
OCH_3-4′			3.87 (s)
OH-4′	5.63 (d, 1.2)		
OCH_2O		5.95 (s, 2H)	5.93 (s, 2H)

Table 2. ^{13}C-NMR Spectral Data for Neolignans **1**—**3** (δ, $CDCl_3$)

C	**1**	**2**	**3**
1	133.4	133.2	136.2
2	109.9	118.7	106.8
3	148.9	148.9	147.8
4	148.5	148.5	147.0
5	110.9	111.0	107.9
6	118.6	109.8	119.8
7	87.2	87.3	87.4
8	47.8	48.1	44.5
9	14.95	14.9	12.78
1′	133.1	135.2	134.8
2′	109.6	120.2	110.0
3′	146.1	147.4	149.1
4′	144.5	146.5	148.6
5′	113.8	107.7	111.3
6′	119.8	107.5	118.6
7′	83.1	83.0	87.4
8′	45.9	45.9	44.4
9′	14.90	15.0	12.85
OCH_3-3	55.8	55.8	
OCH_3-4	55.8	55.9	
OCH_3-3′	55.9		55.9
OCH_3-4′			56.0
OCH_2O		100.9	100.9

other aryl and methyl group, whereas the two methyl groups are *trans*-oriented to each other.[24,25] Due to the asymmetric stereochemistry of the tetrahydrofuran substituents it had to be determined which of the two aryl substituents showed a *cis* orientation to the neighboring methyl group. This was deduced from the correlations of HMBC and NOESY spectra (Fig. 2). The HMBC correlations between the H-7 signal at δ 4.38 and the signals at δ 14.9, 109.8, and 118.7, and between the H-7′ signal at δ 5.10 and the signals at δ 15.0, 107.5, and 120.2 led to the assignment of C-9, C-6, and C-2, and C-9′, C-6′, and C-2′, respectively. The NOE spectrum showed correlations between the methyl group at δ 1.05 (H-9) and the signals for H-7 (δ 4.38) and H-8′ (δ 2.26) and between the other methyl group at δ 0.67 (H-9′) and the aromatic protons at δ 6.78 (H-2′) and 6.89 (H-6′) and the methine proton at δ 1.77 (H-8). Therefore the two aromatic substituents and the methyl group at δ 0.67 (H-9′) are located on the same side of the tetrahydrofuran ring, while the second methyl group (H-9) is situated on the opposite side (Fig. 1). Moreover, an HMBC experiment on **2** displayed cross-signals between the methylene proton at δ 5.95 and carbons at δ 146.5 (C-4′) and 147.4 (C-3′), and between the methoxyl signals at δ 3.90 and 3.92 and the quaternary carbons at δ 148.5 (C-4) and 148.9 (C-3), respectively, indicating that the methylenedioxy group is located at C-3′, -4′ and two methoxyl groups are C-3 and -4. This evidence demonstrated that the dimethoxyphenyl group and methyl group (C-9) had *trans*-orientations and the other aromatic group and C-9′ methyl group had *cis*. The replacements of two methyl groups in this conformation which have the same orientations of the two aromatic groups did not affect the optical rotation.[11] Thus futokadsurin B (**2**) has the *R* and *R* configuration at C-8 and -8′ in contrast with (+)-calopiptin, which has the *S* and *S* configuration at C-8 and -8′, respectively. Therefore the structure of **2** was determined to be (7*R*,8*R*,7′*S*,8′*R*)-3′,4′-methylenedioxy-3,4-dimethoxy-7,7′-epoxylignan.

Table 3. Inhibitory Effect of Compounds **1**—**12** on NO Production by RAW 264.6 Cells Activated by LPS/IFN-γ

Compounds	IC_{50} (μM)
1	47.2
2	55.0
3	79.2
4	44.1
5	88.5
6	56.7
7	47.7
8	>100
9	33.4
10	35.1
11	85.9
12	39.2
Quercetin[a]	26.8

a) Positive control.

Futokadsurin C (**3**) was obtained as a colorless oil, with the molecular formula $C_{21}H_{24}O_5$. The ^{1}H-NMR spectrum showed the presence of two *sec*-methyls (δ 1.018, 1.022), two methoxyls (δ 3.87, 3.89), four methines (δ 2.26, 2.31, 4.46, 4.47), methylenedioxy protons (δ 5.93), and two sets of ABX-type aromatic protons at δ 6.78 (d, J=8.0 Hz, H-5), 6.88 (dd, J=2.0, 8.0 Hz, H-6), and 6.97 (d, J=2.0 Hz, H-2), and δ 6.86 (d, J=8.0 Hz, H-5′), 6.95 (dd, J=2.0, 8.0 Hz, H-6′), and 6.99 (d, J=2.0 Hz, H-2′). These spectral data were similar in chemical shifts and coupling constants to those of machilin G.[20] The structure was confirmed by the HMBC and NOESY spectra. In the HMBC experiment, cross-peaks between a methine proton (H-7) at δ 4.46 and two aromatic carbons at δ 106.8 (C-2) and 119.8 (C-6), and a methyl carbon at δ 12.78 (C-9) as well as between the other methine proton at δ 4.47 (H-7′) and aromatic carbons at δ 110.0 (C-2′) and 118.6 (C-6′), and a methyl carbon at δ 12.85 (C-9′) were observed. Moreover, the HMBC experiment on **3** displayed cross-signals between the methylene proton at δ 5.93 and carbons at δ 147.0 (C-4) and 147.8 (C-3), and between the methoxyl signals at δ 3.87 and 3.89 and the quaternary carbons at δ 148.6 (C-4′) and 149.1 (C-3′), respectively, indicating that the methylenedioxy group is located at C-3, -4 and two methoxyl groups are at C-3′ and C-4′. NOE correlations of **3** were observed between H-9 (δ 1.018) and H-7/H-8 and between H-9′ (δ 1.022) and H-7′/H-8′, indicating that the two aromatic groups and methyl groups were in the *trans*-orientation with respect to each other. These observations suggest that the relative structure of **3** is the same as that of machilin G. The optical rotation of **3** showed a negative value ($[\alpha]_D$ −11.7°), which confirmed that the structure of **3** is an enantiomer of machilin G ($[\alpha]_D$ +3.9°). Therefore futokadsurin C (**3**) was determined to be (7*R*,8*R*,7′*S*,8′*S*)-3′,4′-dimethoxy-3,4-methylenedioxy-7,7′-epoxylignan.

We examined the inhibitory effect of the tetrahydrofuran lignans on NO production in murine macrophage-like RAW 264.7 cells activated by LPS and IFN-γ.[26,27] The IC_{50} values of the tetrahydrofuran lignan derivatives were found to be less than 100 μM except for **8** (Table 3). Compounds **9**, **10**, and **12** had potent NO inhibitory activity, but the IC_{50} values of these compounds were less than that of quercetin, which was used as a positive control.

Experimental

Melting points were determined on a Yanagimoto micromelting point apparatus and are uncorrected. IR spectra were recorded using a Shimadzu FTIR-8100A in a KBr disk. Optical rotations were recorded in $CHCl_3$ using a Jasco DIP-370 digital polarimeter. ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra were recorded on Varian INOVA-400 spectrometers in $CDCl_3$ with tetramethylsilane as an internal standard. Coupling constants (J) are given in Hz. MS were obtained with a JEOL MS-BU 20 (70 eV) using a direct inlet system. Chromatographic separations were achieved on column chromatography using silica gel 60 (70—230 mesh, Merck), reverse-phase silica gel (Cosmosil 75C_{18}-OPN, Nacalai Tesque), Lichroprep Rp-18 (40—63 μm, 200 mm×10 mm, i.d., Merck), or Sephadex LH-20 (Pharmacia) and on HPLC. Preparative HPLC was carried out on an LC-09 instrument (Nihon Bunseki Kogyo). For HPLC column chromatography, GS-310 (20 mm×500 mm, Nihon Bunseki Kogyo) was used. Analytical TLC was performed on precoated silica gel 60 F_{254} plates (Merck) and precoated octadecyl-functionalized silica gel F_{254S} plates (Merck 15389), and detection was achieved by spraying with anisaldehyde reagent, followed by heating.

Plant Material The fresh aerial part of *P. futokadsura* was collected in August 2001, on Okinawa Island, Japan. A voucher specimen (KPU 001961) has been deposited in the Herbarium of the Department of Pharmaceutical Sciences of Natural Resources, Kyoto Pharmaceutical University, Japan.

Extraction and Isolation The aerial part of *P. futokadsura* (4.57 kg) was extracted three times with methanol at room temperature. Removal of the solvent from the combined methanol extracts gave a brown syrup (180 g). The brown syrup was suspended in H_2O and partitioned successively with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH. The *n*-hexane extract (34.1 g) was subjected to silica gel column chromatography using solvent of increasing polarity from *n*-hexane through EtOAc to give 16 fractions. Fraction 3 (5.19 g) was chromatographed on reverse-phase silica gel with MeOH–H_2O (3 : 1) and MeOH to yield fractions 17—24. Fraction 18 (490 mg) was subjected to silica gel column chromatography with *n*-hexane–EtOAc (8 : 1) and preparative HPLC ($CHCl_3$) to give compound **6** (26.9 mg), elemicin (10.5 mg), and 1,2,4-trimethoxyphenyl-5-aldehyde (4.3 mg). Fraction 20 (160.8 mg) was further purified by silica gel column chromatography with *n*-hexane–EtOAc (7 : 1) and recrystallized with MeOH to afford compound **8** (125.8 mg). Fraction 23 (366.4 mg) was further purified by silica gel column chromatography with *n*-hexane–EtOAc (10 : 1) and Sephadex LH-20 (MeOH) to give phytol (96.0 mg). Fraction 4 (646.7 mg) was subjected to silica gel column chromatography and eluted with *n*-hexane–EtOAc (8 : 1) and preparative HPLC ($CHCl_3$) to give compound **5** (78.2 mg), **7** (76.0 mg), and **12** (38.8 mg). Fraction 5 (935.8 mg) was subjected to silica gel column chromatography with *n*-hexane–EtOAc (8 : 1) to afford four fractions (25—28). Fraction 26 (230 mg) was subjected to preparative HPLC ($CHCl_3$) to give pellitorine. Fraction 27 (440 mg) was purified by preparative HPLC ($CHCl_3$) to give compounds **4** (8.1 mg) and **11** (10.2 mg). Compounds **2** (52.1 mg) and **3** (35.3 mg) were isolated from fraction 28 (407 mg) by Lichroprep Rp-18 with MeOH–H_2O (7 : 2) and preparative HPLC ($CHCl_3$). Fraction 10 (1.759 g) was chromatographed on reverse-phase silica gel with MeOH–H_2O (3 : 1) to yield fractions 29—32. Fraction 29 (452 mg) was chromatographed on preparative HPLC ($CHCl_3$) to give compound **10** (153.6 mg). Fraction 31 (269.9 mg) was subjected to silica gel column chromatography with *n*-hexane–EtOAc (4 : 1) and preparative HPLC ($CHCl_3$) to give compounds **1** (76.7 mg) and **9** (29.1 mg).

Futokadsurin A (**1**): Colorless oil, $[\alpha]_D^{25}$ +12.3° (c=1.09, $CHCl_3$). IR (KBr) cm^{-1}: 3546, 2959, 1607, 1518, 1464, 1267, 1236, 1161, 1140, 1028, 858, 826, 762. UV λ_{max} (EtOH) nm: 233.0 (log ε 5.19), 279.6 (log ε 4.76). EI-MS *m*/*z* (rel. int.): 358 (27.4) $[M]^+$, 206 (100), 192 (77.5), 191 (60.9), 175 (59.6), 160 (20.0). HR-EI-MS *m*/*z*: 358.1783 (Calcd 358.1780 for $C_{21}H_{26}O_5$). ^{1}H- and ^{13}C-NMR: see Tables 1 and 2.

Futokadsurin B (**2**): Colorless needles, mp 102 °C; $[\alpha]_D^{23}$ +33.7° (c=1.18, $CHCl_3$). IR (KBr) cm^{-1}: 1593, 1520, 1258, 1238, 1167, 1136, 1128, 1101, 1026, 866, 804, 763. EI-MS *m*/*z* (rel. int.): 356 (33.0) $[M]^+$, 206 (89.8), 190 (79.0), 175 (100). HR-EI-MS *m*/*z*: 356.1628 (Calcd 356.1624 for $C_{21}H_{24}O_5$). ^{1}H- and ^{13}C-NMR: see Tables 1 and 2.

Futokadsurin C (**3**): Colorless oil, $[\alpha]_D^{23}$ −11.7° (c=3.26, $CHCl_3$). IR (KBr) cm^{-1}: 1590, 1515, 1263, 1243, 1135, 1133, 1098, 1035, 870, 810, 770. UV λ_{max} (EtOH) nm (log ε): 230 (5.01), 279 (4.83). EI-MS *m*/*z* (rel. int.): 356 (31.5) $[M]^+$, 206 (59.6), 190 (100.0), 175 (78.1). HR-EI-MS *m*/*z*: 356.1619 (Calcd 356.1624 for $C_{21}H_{24}O_5$). ^{1}H- and ^{13}C-NMR: see Tables 1 and 2.

Nitrite Assay The murine macrophage-like cell line RAW 264.7 was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). The cells were seeded at 1.2×10^6 cells/ml in 96-well flat-bottomed plates (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 hundred microliters 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 μl of Griess reagent [1 : 1 mixture (v/v) of 1% sulfanilamide and 0.1% *N*-1-naphthylethylenediamide dihydrochloride in 5% H_3PO_4] was 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).

Acknowledgments The authors thank Drs. K. Masuda and A. Takano of Showa Pharmaceutical University for the collection of the plant material on Okinawa, Japan, and Ms. M. Manabe of Kyoto Pharmaceutical University for MS measurements.

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