Inhibitors of Nitric Oxide Production from the Flowers of *Angelica furcijuga*: Structures of Hyuganosides IV and V

Hisashi MATSUDA, Toshio MORIKAWA, Teruki OHGUSHI, Tomoko ISHIWADA, Norihisa NISHIDA, and Masayuki YOSHIKAWA*

Kyoto Pharmaceutical University; Misasagi, Yamashina-ku, Kyoto 607–8412, Japan.
Received November 19, 2004; accepted January 12, 2005

The methanolic extract from the flowers of *Angelica furcijuga* KITAGAWA was found to inhibit nitric oxide production in lipopolysaccharide-activated mouse peritoneal macrophages. From the methanolic extract, two new glycosides, hyuganosides IV and V, were isolated together with 28 known constituents. The structures of the new constituents were determined on the basis of chemical and physicochemical evidence. Furthermore, the inhibitory effects of 11 coumarin constituents on nitric oxide production were examined. Among them, 3′-angeloyl-*cis*-khellactone (IC_{50}=82 μM), (*S*)-(−)-oxypeucedanin (57 μM), imperatorin (60 μM), isoepoxypteryxin (53 μM), and isopteryxin (8.8 μM) showed inhibitory activity.

Key words *Angelica furcijuga*; coumarin; nitric oxide production inhibitor; hyuganoside

Angelica furcijuga KITAGAWA (Umbelliferae) is indigenous to Japan and the whole plant has been used as a Japanese folk medicine for the treatment of hepatopathy, allergosis, inflammation, diabetes, and hypertension. Previously, we have reported four acylated khellactone-type coumarins called hyuganins A—D and four glycosides, hyuganosides I, II, IIIa, and IIIb, from the roots of *A. furcijuga*.[1—3] In addition, the methanolic extract and principal constituents from the roots of *A. furcijuga* were found to show hepatprotective and vasorelaxant activities.[2,3] However, chemical and pharmacologic studies on the flower parts of this plant were not performed. During the course of our characterization studies on medicinal flowers,[4—8] we found that the methanolic extract (IC_{50}=71 μg/ml) of the flower part of *A. furcijuga* showed an inhibitory effect on nitric oxide (NO) production in lipopolysaccharide (LPS)-activated mouse peritoneal macrophages (Table 1). Two new glycosides called hyuganosides IV (**1**) and V (**2**) were isolated from the water-soluble fraction of the methanolic extract. In this paper, we describe the isolation and structure elucidation of new constituents (**1**, **2**) and inhibitory effects of the 11 coumarin constituents (**3**—**13**) from the flowers of *A. furcijuga* on NO production in LPS-activated mouse peritoneal macrophages.

The methanolic extract from the flowers of *A. furcijuga* (cultivated in Miyazaki Prefecture, Japan) was partitioned in an EtOAc–H_2O (1 : 1) mixture to give an EtOAc-soluble fraction and H_2O-soluble fraction. Since the EtOAc-soluble fraction exhibited inhibitory effects on NO production (IC_{50}=39 μg/ml), it was subjected to ordinary- and reverse-phase silica gel column chromatography and finally HPLC to furnish 3′-angeloyl-*cis*-khellactone[9,10] (**3**, 0.018% from dried flowers), laserpitin[10] (**4**, 0.075%), xanthotoxol[11] (**5**, 0.0015%), (*R*)-(+)-oxypeucedanin hydrate[12,13] (**6**, 0.0037%), (*S*)-(−)-oxypeucedanin hydrate[14] (**7**, 0.0037%), (*S*)-(−)-oxypeucedanin[12] (**8**, 0.0016%), imperatorin[11] (**9**, 0.019%), isoimperatorin[14] (**10**, 0.0083%), umbelliferone[15] (**11**, 0.0035%), isoepoxypteryxin[1,3,16] (**12**, 0.14%), isopteryxin[1,3] (**13**, 0.016%), 1α-angeloyloxycarotol[17] (**14**, 0.012%), (+)-4-epi-α-bisabolol[18] (**15**, 0.0047%), 4-hydroxybenzyl cyanide[15] (**16**, 0.0019%), 4-ethoxy-3-methoxybenzoic acid[19] (**17**, 0.0029%), ferulic acid[15] (**18**, 0.0017%), and kaempferol[15] (**19**, 0.030%). On the other hand, the H_2O-soluble fraction, which showed weak activity, was subjected to Diaion HP-20 column chromatography (H_2O→MeOH →$CHCl_3$) to give H_2O-, MeOH-, and $CHCl_3$-eluted fractions, respectively. The MeOH-eluted fraction was further purified using ordinary- and reverse-phase silica gel column chromatography and finally HPLC to furnish hyuganosides IV (**1**, 0.0013%) and V (**2**, 0.0051%) together with praeroside II[1,3] (**20**, 0.0049%), apiosylskimmin[1,3] (**21**, 0.0026%), (*R*)-peucedanol 7-*O*-β-D-glucopyranoside[1,3] (**22**, 0.0073%), apterin[20] (**23**, 0.039%), cnidioside A[21] (**24**, 0.0023%), (4*R*)-*p*-menth-1-ene-7,8-diol 8-*O*-β-D-glucopyranoside[22] (**25**, 0.0065%), arbutin[23] (**26**, 0.0021%), chlorogenic acid[15] (**27**, 0.0093%), 3-*O*-feruloylquinic acid[24] (**28**, 0.012%), kaempferol 3-*O*-β-D-glucopyranoside[25] (**29**=astragalin, 0.020%), and quercetin 3-*O*-β-D-glucopyranoside[26] (**30**, 0.021%) (Charts 1, 2).

Structures of Hyuganosides IV (1) and V (2) Hyuganoside IV (**1**) was isolated as a white powder with

Table 1. Inhibitory Effects of the MeOH Extract and EtOAc- and H_2O-Soluble Fractions from the Flowers of *A. furcijuga* on NO Production in LPS-Activated Mouse Peritoneal Macrophages

	Inhibition (%)[a]						IC_{50} (μg/ml)
	0 μg/ml	3 μg/ml	10 μg/ml	30 μg/ml	100 μg/ml	300 μg/ml	
MeOH extract	0.0±5.5	10.3±8.7	−3.7±4.6	19.9±2.9[b]	64.8±1.5[c]	99.0±0.4[c,d]	71
EtOAc-soluble fraction	0.0±0.9	−6.8±2.7	18.5±3.3[c]	32.7±4.2[c]	82.8±0.5[c]	95.7±0.5[c,d]	39
H_2O-soluble fraction	0.0±1.4	−11.2±4.0	−6.5±2.5	0.6±1.8	−0.9±6.8	38.6±3.3[c]	—

a) Each value represents the mean±S.E.M. (n=4). Significantly different from the control, *b*) $p<0.05$, *c*) $p<0.01$. *d*) Cytotoxic effects were observed.

* To whom correspondence should be addressed. e-mail: shoyaku@mb.kyoto-phu.ac.jp

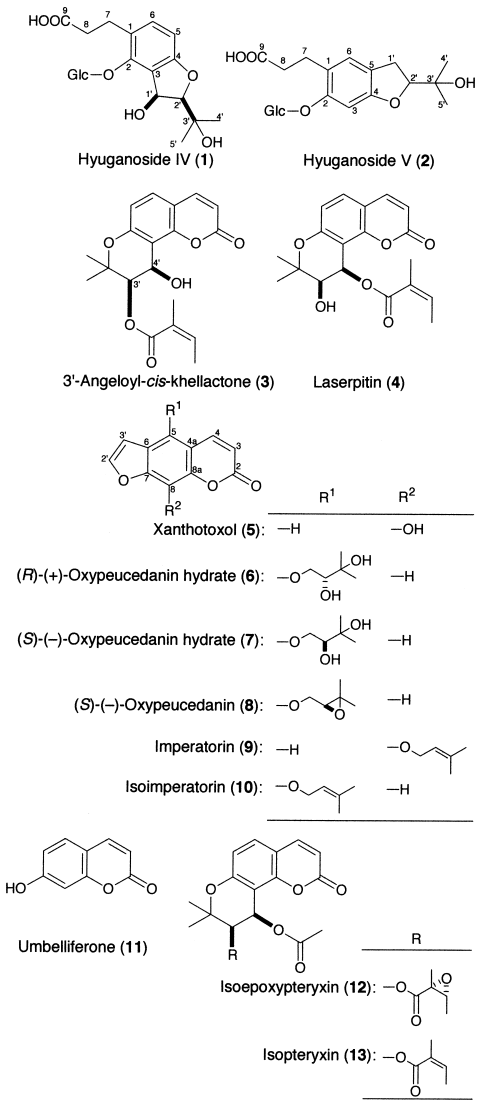

Chart 1

negative optical rotation ($[\alpha]_D^{27}$ −5.5° in MeOH). The molecular formula $C_{20}H_{28}O_{11}$ was confirmed from the quasimolecular ion peaks at *m/z* 467 $(M+Na)^+$ and 443 $(M-H)^-$ in the positive- and negative-ion fast atom bombardment (FAB)-MS of **1** and with high-resolution FAB-MS. The IR spectrum of **1** showed absorption bands at 1719, 1638, 1478, and 1266 cm^{-1} ascribable to a carbonyl function and an aromatic ring, and strong absorption bands at 3410 and 1075 cm^{-1} suggestive of a glycosidic moiety. The UV spectrum of **1** showed an absorption maximum at 287 (log ε 3.60) nm. Acid hydrolysis of **1** with 1.0 M hydrochloric acid (HCl) liberated D-glucose, which was identified in HPLC analysis using an optical rotation detector.[1,8] The ^{1}H- and ^{13}C-NMR (CD_3OD, Table 2) spectra[27] of **1** showed signals assignable to two methyls [δ 1.42, 1.47 (both s, CH_3)], two methylenes [δ 2.61 (t, J=7.6 Hz, 8-H_2), 2.98 (m, 7-H_2)], two methines bearing an oxygen function [δ 4.14 (d, J=5.6 Hz, 2′-H), 5.57 (d, J=5.6 Hz, 1′-H)], and two aromatic protons [δ 6.57 (d, J=8.1 Hz, 5-H), 7.10 (d, J=8.1 Hz, 6-H)] together with a β-D-glucopyranosyl part [δ 5.05 (d, J=7.3 Hz, Glc-1)]. The planar structure of **1** was constructed on the basis of the ^{1}H–^{1}H correlation spectroscopy (^{1}H–^{1}H COSY) and heteronuclear multiple bond correlation (HMBC) experiments, as shown in Fig. 1. Thus the ^{1}H–^{1}H COSY experiment of **1** indicated the presence of partial structures indicated in bold lines (from 5-C–6-C, from 7-C–8-C, from 1′-C–2′-C, *etc.*). The connectivities of the quaternary carbons and the position of the glucoside linkage in **1** were clarified by HMBC experiments on **1**, which showed long-range correlations between the following proton and carbon pairs: 5-H and 1, 3, 4-C; 6-H and 1, 2, 4-C; 7-H_2 and 1, 9-C; 8-H_2 and 1, 9-C; 1′-H and 3, 3′-C; 2′-H and 4, 3′-C; 4′-H_3 and 2′, 3′, 5′-C; 5′-H_3 and 2′, 3′, 4′-C; and Glc-1-H and 2-C (Fig. 1). Furthermore, in a nuclear Overhauser enhancement spectroscopy (NOESY) experiment on **1**, a nuclear Overhauser effect (NOE) correlation was observed between the 1′- and 2′-proton pair. Consequently, the structure of **1** was determined to be as shown in Fig. 1.

Hyuganoside V (**2**) was also isolated as a white powder with negative optical rotation ($[\alpha]_D^{25}$ −43.1°, MeOH). The IR spectrum of **2** showed absorption bands at 3432, 1701, 1655, 1491, and 1076 cm^{-1} ascribable to glycosidic, carboxyl, and aromatic functions, while its UV spectrum showed an absorption maximum at 291 (log ε 3.70) nm. The molecular formula $C_{20}H_{28}O_{10}$ of **2** was characterized from the positive- and negative-ion FAB-MS and in high-resolution FAB-MS measurement. Acid hydrolysis of **2** liberated D-glucose,[1,8] whereas its aglycon (**2a**) with positive optical rotation ($[\alpha]_D^{25}$ +11.5°, MeOH) was obtained by enzymatic hydrolysis of **2** with β-glucosidase. The ^{1}H- and ^{13}C-NMR (CD_3OD, Table 2) spectra[27] of **2** indicated the presence of two methyls [δ 1.20, 1.22 (both s, CH_3)], three methylenes [δ 2.55 (dd-like, 8-H_2), 2.85 (m, 7-H_2), 3.06 (br d, J=*ca.* 9 Hz, 1′-H_2)], a methine bearing an oxygen function [δ 4.55 (dd, J=8.7, 9.0 Hz, 2′-H)], and two aromatic protons [δ 6.62, 6.93 (s, 3, 6-H)] together with an β-D-glucopyranosyl part [δ 4.81 (d, J=7.1 Hz, Glc-1)]. The ^{1}H–^{1}H COSY experiment of **2** indicated the presence of partial structures in bold lines (from 7-C–8-C, from 1′-C–2′-C, *etc.*). In the HMBC experiment of **2**, long-range correlations were observed between the following protons and carbons: 3-H and 1, 5-C; 6-H and 1, 2, 4-C; 7-H_2 and 1, 9-C; 8-H_2 and 1, 9-C; 1′-H_2 and 4, 5-C; 2′-H and 3′-C; 4′-H_3 and 2′, 3′, 5′-C; 5′-H_3 and 2′, 3′, 4′-C; and Glc-1-H and 2-C (Fig. 1), so that the connectivities of the quaternary carbons in **2** were identified. On the basis of the above-mentioned evidence and comparison of the physical data of **2** with those of **2a**, the structure of hyuganoside V (**2**) was determined to be as shown in Fig. 1.

Inhibitory Effects on NO Production in LPS-Activated Mouse Peritoneal Macrophages The inorganic free radical NO has been implicated in physiologic and pathologic processes, such as vasodilation, nonspecific host defense, ischemia-reperfusion injury, and chronic or acute inflammation. NO is produced by the oxidation of L-arginine by NO synthase (NOS). In the family of NOS, inducible NOS is specifically involved in the pathologic aspects with overproduction of NO and can be expressed in response to proinflammatory agents such as interleukin-1β, tumor necrosis factor-α, and LPS in various cell types including macrophages, endothelial cells, and smooth muscle cells. As a part of our studies to characterize the bioactive components of natural medicines, we reported various NO production inhibitors, *i.e.*, higher unsaturated fatty acids,[28] poly-

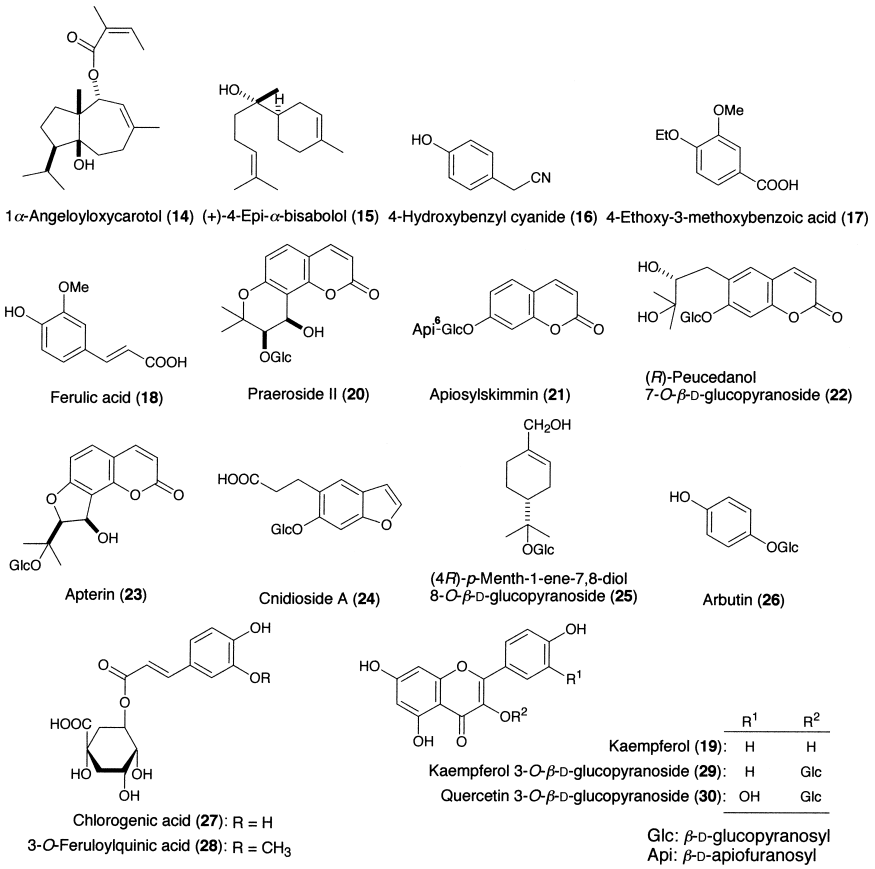

Chart 2

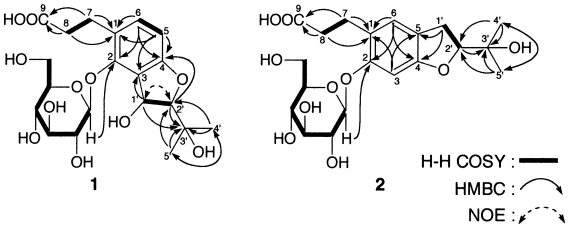

Fig. 1

acetylenes,[2,29] coumarins,[2] flavonoids,[29,30] stilbenes,[31,32] lignans,[33,34] sesquiterpenes,[35—41] diterpenes,[42,43] triterpenes,[44—47] diarylheptanoids,[48,49] cyclic peptides,[46] and alkaloids.[50,51] As a continuation of these studies, the effects of the coumarin constituents from the flowers and leaves of *A. furcijuga* on NO production from LPS-activated macrophages were examined, and the results are summarized in Table 2. The isolated constituents (**1**—**3**, **8**—**13**) significantly inhibited the accumulation of nitrite, a product of NO, in the medium. Among them, three khellactone-type coumarins, 3′-angeloyl-*cis*-khellactone (**3**, $IC_{50}=82\,\mu M$), isoepoxypteryxin (**12**, $53\,\mu M$),[2] and isopteryxin (**13**, $8.8\,\mu M$),[2] and two furanocoumarins, (*S*)-(−)-oxypeucedanin (**8**, $57\,\mu M$) and imperatorin (**9**, $60\,\mu M$), showed stronger inhibitory effects without cytotoxic effects in the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay.

Recently, Wang *et al.* reported that imperatorin (**9**) isolated from the roots of *Saponshnikovia divaricata* inhibited NO production in the macrophage-like cell line RAW264.7.[52]

Table 2. ^{13}C-NMR Data for Hyuganosides IV (**1**) and V (**2**)

	1	**2**
C-1	126.2	122.9
C-2	153.7	156.6
C-3	121.3	98.6
C-4	161.5	160.6
C-5	106.8	121.6
C-6	132.9	126.3
C-7	26.8	26.8
C-8	36.0	35.9
C-9	177.1	177.5
C-1′	72.0	31.1
C-2′	91.1	91.0
C-3′	73.0	72.5
3′-*gem*-CH_3	26.6	25.2
	27.3	25.4
Glc-1	105.2	102.8
Glc-2	75.5	74.9
Glc-3	77.9	78.0
Glc-4	71.4	71.3
Glc-5	78.2	78.1
Glc-6	62.9	62.5

Measured in CD_3OD at 125 MHz.

In agreement with the previous report, **9** substantially inhibited NO production in LPS-activated mouse peritoneal macrophages.

In our previous study, we reported that the acyl groups at the 3′- and 4′-positions of khellactone-type coumarins (*e.g.*, hyuganins, anomalin, *etc.*) were essential to inhibit LPS-induced NO production in mouse peritoneal macrophages.[2]

Table 3. Inhibitory Effects of Constituents from *A. furcijuga* on NO Production in LPS-Activated Mouse Peritoneal Macrophages

	Inhibition (%)[a]						IC_{50} (μM)
	0 μM	1 μM	3 μM	10 μM	30 μM	100 μM	
3′-Angeloyl-*cis*-khellactone (**3**)	0.0±6.3	−5.0±2.1	−5.5±8.9	−7.9±6.4	20.2±3.7[c]	63.7±3.2[c]	82
Laserpitin (**4**)	0.0±7.9	−5.0±6.5	0.6±5.4	8.7±6.2	11.8±6.9	24.9±4.0[c]	
Xanthotoxol (**5**)	0.0±2.2	16.9±5.5	20.2±8.8	13.3±6.1	14.4±7.2	45.1±4.9[c]	
(*R*)-(+)-Oxypeucedanin hydrate (**6**)	0.0±4.9	2.0±4.0	4.8±3.6	−9.4±1.5	−1.0±2.5	5.5±3.4	
(*S*)-(−)-Oxypeucedanin hydrate (**7**)	0.0±6.7	4.5±7.0	−8.8±4.6	2.8±10.0	16.1±5.3	15.1±3.0	
(*S*)-(−)-Oxypeucedanin (**8**)	0.0±7.8	−3.3±13.2	−18.4±8.2	12.8±6.2	49.6±5.5[c]	79.6±3.2[c]	57
Imperatorin (**9**)	0.0±3.4	20.8±6.2	10.0±7.5	1.0±3.0	40.5±3.3[c]	82.8±3.2[c]	60
Isoimperatorin (**10**)	0.0±8.7	1.8±10.6	18.3±6.4	43.2±6.1[b]	72.3±2.1[c,d]	82.5±4.5[c,d]	
Umbelliferone (**11**)	0.0±8.2	−10.9±13.0	−12.7±6.0	15.1±10.5	24.1±2.1[c]	44.7±9.6[c]	
Isoepoxypteryxin (**12**)	0.0±2.7	14.2±7.4	15.7±5.2	21.4±6.2[b]	44.6±2.1[c]	58.2±2.5[c]	53[2]
Isopteryxin (**13**)	0.0±1.2	20.4±2.1[c]	32.4±3.0[c]	47.8±3.4[c]	75.5±2.2[c]	91.3±1.0[c]	8.8[2]
L-NMMA	0.0±1.1	4.4±2.0	2.0±1.6	17.7±2.8[c]	52.3±1.5[c]	79.2±0.9[c]	28

a) Each value represents the mean±S.E.M. (n=4). Significantly different from the control, *b*) $p<0.05$, *c*) $p<0.01$. *d*) Cytotoxic effects were observed.

However, the effects of the 3′- or 4′-monoacyl derivative have not been clarified. In the present study, 3′-angeloyl-*cis*-khellactone (**3**, 82 μM) with the 3′-angeloyl group showed stronger activity than laserpitin (**4**, >100 μM) with the 4′-angeloyl group, but both compounds (**3**, **4**) showed less activity than anomalin (3.4 μM)[2] with angeloyl groups at the 3′- and 4′-positions. These findings suggest that the 3′-acyl group contributes to the activity more than the 4′-acyl group and provide evidence that both the 3′- and 4′-acyl groups of khellactone-type coumarins are essential for the potent activity.

With regard to the activity of furanocoumarins (**5**—**10**), imperatorin (**9**, 60 μM) with the 5-*O*-prenyl group showed substantial inhibitory activity and its activity was stronger than that of xanthotoxol (**5**, >100 μM). Isoimperatorin (**10**) with the 5-*O*-prenyl group also showed stronger activity, but cytotoxic effects were observed. Thus the prenyl ether moiety at the 5-*O*- or 8-*O*-position in furanocoumarins is suggested to enhance the inhibition of NO production. On the other hand, (*S*)-(−)-oxypeucedanin (**8**, 57 μM) with the oxidized prenyl group showed less activity than **10**, but did not show cytotoxic effects, while (*R*)-(+)- and (*S*)-(−)-oxypeucedanin hydrate (**6**, **7**) lacked activity.

In conclusion, two new glycosides, hyuganosides IV (**1**) and V (**2**), were isolated from the flowers of *A. furcijuga* and their structures were determined on the basis of chemical and physicochemical evidence. Among the coumarin constituents, 3′-angeloyl-*cis*-khellactone (**3**), (*S*)-(−)-oxypeucedanin (**8**), imperatorin (**9**), isoepoxypteryxin (**12**), and isopteryxin (**13**) inhibited NO production in LPS-activated mouse peritoneal macrophages, and some structural requirements of khellactone-type coumarins and furanocoumarins for the activity were clarified.

Experimental

The following instruments were used to obtain physical data: specific rotations, Horiba SEPA-300 digital polarimeter (l=5 cm); UV spectra, Shimadzu UV-1600 spectrometer; IR spectra, Shimadzu FTIR-8100 spectrometer; EI-MS and high-resolution MS, JEOL JMS-GCMATE mass spectrometer; FAB-MS and high-resolution MS, JEOL JMS-SX 102A mass spectrometer; ^{1}H-NMR spectra, JNM-LA500 (500 MHz) spectrometer; ^{13}C-NMR spectra, JNM-LA500 (125 MHz) spectrometer with tetramethylsilane as an internal standard; and HPLC detector, Shimadzu RID-6A refractive index detector.

The following experimental conditions were used for chromatography: ordinary-phase silica gel column chromatography, Silica gel BW-200 (Fuji Silysia Chemical, Ltd., 150—350 mesh); reverse-phase silica gel column chromatography, Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., 100—200 mesh); TLC, precoated TLC plates with Silica gel 60F_{254} (Merck, 0.25 mm) (ordinary phase) and Silica gel RP-18 F_{254S} (Merck, 0.25 mm) (reverse phase); reverse-phase HPTLC, precoated TLC plates with Silica gel RP-18 WF_{254S} (Merck, 0.25 mm); and detection was achieved by spraying with 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4, followed by heating.

Extraction and Isolation The dried flowers of *A. furcijuga* (950 g, collected in Miyazaki Prefecture, Japan) were finely cut and extracted three times with MeOH under reflux for 3 h. Evaporation of the solvent under reduced pressure provided a MeOH extract (295 g, 31.1% from the dried flowers). The MeOH extract (275 g) was partitioned in an EtOAc–H_2O (1 : 1) mixture to give EtOAc- and H_2O-soluble fractions (94 g, 181 g), respectively.

The EtOAc-soluble fraction (88 g) was subjected to ordinary-phase silica gel column chromatography [2.0 kg, *n*-hexane–EtOAc (10 : 1→5 : 1→1 : 1)→MeOH] to give seven fractions [fr. 1 (8.6 g), fr. 2 (7.8 g), fr. 3 (10.9 g), fr. 4 (10.3 g), fr. 5 (10.9 g), fr. 6 (9.5 g), fr. 7 (30.0 g)]. Fraction 2 (7.8 g) was subjected to reverse-phase silica gel column chromatography [230 g, MeOH–H_2O (70 : 30→80 : 20→90 : 10)→MeOH] to furnish eight fractions [fr. 2-1 (45 mg), fr. 2-2 (100 mg), fr. 2-3 (95 mg), fr. 2-4 (710 mg), fr. 2-5 (150 mg), fr. 2-6 (2.0 g), fr. 2-7 (2.5 g), fr. 2-8 (2.2 g)]. Fraction 2-2 (100 mg) was purified by HPLC [detection: RI, column: YMC-Pack ODS-A (250×20 mm i.d., YMC Co., Ltd.), mobile phase: MeOH–H_2O (90 : 10, v/v)] to give isoimperatorin (**10**, 69 mg). Fraction 2-4 (710 mg) was purified by HPLC [MeOH–H_2O (75 : 25, v/v)] to give 1α-angeloyloxycarotol (**14**, 99 mg) and (+)-4-*epi*-α-bisabolol (**15**, 39 mg). Fraction 4 (9.4 g) was subjected to reverse-phase silica gel column chromatography [300 g, MeOH–H_2O (60 : 40→75 : 25)→MeOH] to give five fractions [fr. 4-1 (0.1 g), fr. 4-2 (0.2 g), fr. 4-3 (2.0 g), fr. 4-4 (4.5 g), fr. 4-5 (2.6 g)]. Fraction 4-1 (100 mg) was further purified by HPLC [MeOH–H_2O (55 : 45, v/v)] to give 4-hydroxybenzyl cyanide (**16**, 14 mg) and 4-ethoxy-3-methoxybenzoic acid (**17**, 22 mg). Fraction 4-3 (570 mg) was also purified by HPLC [MeOH–H_2O (75 : 25, v/v)] to give 3′-angeloyl-*cis*-khellactone (**3**, 10 mg), laserpitin (**4**, 164 mg), (*S*)-(−)-oxypeucedanin (**8**, 4 mg), and isoepoxypteryxin (**12**, 22 mg). Fraction 4-4 (480 mg) was subjected to HPLC [MeOH–H_2O (85 : 15, v/v)] to give **3** (29 mg), imperatorin (**9**, 15 mg), and isopteryxin (**13**, 13 mg). Fraction 5 (10.1 g) was subjected to reverse-phase silica gel column chromatography [300 g, MeOH–H_2O (50 : 50→60 : 40→75 : 25)→MeOH] to give 10 fractions [fr. 5-1 (0.2 g), fr. 5-2 (0.3 g), fr. 5-3 (0.2 g), fr. 5-4 (0.2 g), fr. 5-5 (0.1 g), fr. 5-6 (=**4**, 1.0 g), fr. 5-7 (2.3 g), fr. 5-8 (1.7 g), fr. 5-9 (1.2 g), fr. 5-10 (2.9 g)]. Fraction 5-2 (300 mg) was subjected to HPLC [MeOH–H_2O (50 : 50, v/v)] to give umbelliferone (**11**, 27 mg) and ferulic acid (**18**, 13 mg). Fraction 5-3 (200 mg) was purified by HPLC [MeOH–H_2O (50 : 50, v/v)] to give xanthotoxol (**5**, 12 mg). Fraction 5-4 (200 mg) was purified by HPLC [MeOH–H_2O (60 : 40, v/v)] to give (*R*)-(+)-oxypeucedanin hydrate (**6**, 28 mg). Fraction 5-8 (690 mg) was purified by HPLC [MeOH–H_2O (75 : 25, v/v)] to give (*S*)-(−)-oxypeucedanin hydrate (**7**, 12 mg), and kaempferol (**19**, 94 mg).

The H_2O-soluble fraction (170 g) was subjected to Diaion HP-20 column chromatography [H_2O→MeOH→$CHCl_3$] to afford H_2O-, MeOH-, and $CHCl_3$-eluted fractions (120, 38, 12 g), respectively. The MeOH-eluted frac-

tion (31 g) was subjected to ordinary-phase silica gel column chromatography [1.0 kg, $CHCl_3$–MeOH (10 : 1)→$CHCl_3$–MeOH–H_2O (10 : 3 : 0.5→6 : 4 : 1)→MeOH] to give seven fractions [fr. 1 (2.2 g), fr. 2 (5.6 g), fr. 3 (3.7 g), fr. 4 (6.5 g), fr. 5 (6.6 g), fr. 6 (3.4 g), fr. 7 (3.0 g)]. Fraction 2 (5.6 g) was subjected to reverse-phase silica gel column chromatography [180 g, MeOH–H_2O (70 : 30→80 : 20→90 : 10)→MeOH] to furnish 10 fractions [fr. 2-1 (110 mg), fr. 2-2 (93 mg), fr. 2-3 (120 mg), fr. 2-4 (572 mg), fr. 2-5 (578 mg), fr. 2-6 (775 mg), fr. 2-7 (1546 mg), fr. 2-8 (916 mg), fr. 2-9 (807 mg), fr. 2-10 (83 mg)]. Fraction 2-5 (300 mg) was purified by HPLC [MeOH–H_2O (45 : 55, v/v)] to give chlorogenic acid (**27**, 19 mg). Fraction 2-6 (364 mg) was purified by HPLC [MeOH–H_2O (45 : 55, v/v)] to give 3-*O*-feruloylquinic acid (**28**, 40 mg). Fraction 2-7 (1546 mg) was purified by HPLC [MeOH–H_2O (50 : 50, v/v)] to give praeroside II (**20**, 33 mg). Fraction 3 (400 mg) was purified by HPLC [MeOH–H_2O (45 : 55, v/v)] to give apterin (**23**, 28 mg), cnidioside A (**24**, 17 mg), and kaempferol 3-*O*-β-D-glucopyranoside (**29**, 10 mg). Fraction 4 (6.1 g) was subjected to reverse-phase silica gel column chromatography [180 g, MeOH–H_2O (20 : 80→30 : 70→50 : 50)→MeOH] to give seven fractions [fr. 4-1 (0.3 g), fr. 4-2 (0.3 g), fr. 4-3 (0.4 g), fr. 4-4 (1.1 g), fr. 4-5 (1.1 g), fr. 4-6 (2.5 g), fr. 4-7 (0.4 g)]. Fraction 4-2 (300 mg) was purified by HPLC [MeOH–H_2O (35 : 65, v/v)] to give apiosylskimmin (**21**, 17 mg), arbutin (**26**, 13 mg), and **27** (25 mg). Fraction 4-4 (1100 mg) was purified by HPLC [MeOH–H_2O (45 : 55, v/v)] to give hyuganosides IV (**1**, 8 mg) and V (**2**, 33 mg), (*R*)-peucedanol 7-*O*-β-D-glucopyranoside (**22**, 47 mg), and (4*R*)-*p*-menth-1-ene-7,8-diol 8-*O*-β-D-glucopyranoside (**25**, 42 mg). Fraction 4-6 (1.0 g) was purified by HPLC [MeOH–H_2O (55 : 45, v/v)] to give kaempferol 3-*O*-β-D-glucopyranoside (**29**, 15 mg) and quercetin 3-*O*-β-D-glucopyranoside (**30**, 54 mg).

The known compounds were identified by comparison of their physical data ($[\alpha]_D$, IR, ^{1}H-, ^{13}C-NMR) with reported values.[1,3,9—14,16—26] or authentic samples.[15]

Hyuganoside IV (**1**): A white powder; $[\alpha]_D^{27}$ −5.5° (*c*=1.00, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{20}H_{28}O_{11}Na$ $(M+Na)^+$: 467.1529. Found: 467.1523. UV [MeOH, nm (log ε)]: 287 (3.60). IR (KBr): 3410, 1719, 1638, 1478, 1266, 1075 cm^{-1}. ^{1}H-NMR (500 MHz, CD_3OD) δ: 1.42, 1.47 (3H each, both s, CH_3), 2.61 (2H, t, *J*=7.6 Hz, 8-H_2), 2.98 (2H, m, 7-H_2), 4.14 (1H, d, *J*=5.6 Hz, 2′-H), 5.05 (1H, d, *J*=7.3 Hz, Glc-1), 5.57 (1H, d, *J*=5.6 Hz, 1′-H), 6.57 (1H, d, *J*=8.1 Hz, 5-H), 7.10 (1H, d, *J*=8.1 Hz, 6-H). ^{13}C-NMR (125 MHz, CD_3OD) δ_C: see Table 2. Positive-ion FAB-MS: *m*/*z* 467 $(M+Na)^+$. Negative-ion FAB-MS: *m*/*z* 443 $(M-H)^-$, 281 $(M-C_6H_{11}O_5)^-$.

Hyuganoside V (**2**): A white powder; $[\alpha]_D^{27}$ −43.1° (*c*=1.20, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{20}H_{28}O_{10}Na$ $(M+Na)^+$: 451.1580. Found: 451.1586. UV [MeOH, nm (log ε)]: 291 (3.70). IR (KBr): 3432, 1701, 1655, 1491, 1076 cm^{-1}. ^{1}H-NMR (500 MHz, CD_3OD) δ: 1.20, 1.22 (3H each, both s, CH_3), 2.55 (2H, dd-like, 8-H_2), 2.85 (2H, m, 7-H_2), 3.06 (2H, br d, *J*=*ca.* 9 Hz, 1′-H), 4.55 (1H, dd, *J*=8.7, 9.0 Hz, 2′-H), 4.81 (1H, d, *J*=7.1 Hz, Glc-1), 6.62 (1H, s, 3-H), 6.93 (1H, s, 6-H). ^{13}C-NMR (125 MHz, CD_3OD) δ_C: see Table 2. Positive-ion FAB-MS: *m*/*z* 451 $(M+Na)^+$. Negative-ion FAB-MS: *m*/*z* 427 $(M-H)^-$, 265 $(M-C_6H_{11}O_5)^-$.

Acid Hydrolysis of 1 and 2 A solution of **1** or **2** (2.0 mg each) in 1.0 M HCl (0.1 ml) was heated under reflux for 1 h. After cooling, the reaction mixture was extracted with EtOAc (0.1 ml). The H_2O layer was analyzed by HPLC under the following conditions: [detection: optical rotation, column: Kaseisorb LC NH_2-60-5 250×4.6 mm i.d., 5 μm (Tokyo Kasei Kogyo Co., Ltd., Japan), mobile phase: CH_3CN–H_2O (3 : 1, v/v), flow rate: 0.8 ml/min, injection volume: 10 μl, column temperature: room temperature]. Identification of D-glucose present in the H_2O layer was carried out by comparison of its retention time and optical rotation with that of authentic sample. t_R: 12.3 min (D-glucose, positive optical rotation).

Enzymatic Hydrolysis of 2 A solution of **2** (5.8 mg, 0.014 mmol) in 0.2 M acetate buffer (pH 4.4, 2.0 ml) was treated with β-glucosidase (15.0 mg, Sigma) and the solution was stired at 38 °C for 24 h. After treatment of the reaction mixture with EtOH, the mixture was evaporated to dryness under reduced pressure and the residue was purified by reverse-phase silica gel column chromatography [500 mg, (H_2O→MeOH)] to give **2a** (3.1 mg, 91%).

2a: A white powder, $[\alpha]_D^{27}$ +11.5° (*c*=0.35, MeOH). High-resolution EI-MS: Calcd for $C_{14}H_{18}O_5$ (M^+): 266.1154. Found: 266.1142. UV [MeOH, nm (log ε)]: 294 (3.40). IR (KBr): 3453, 1709, 1655, 1082 cm^{-1}. ^{1}H-NMR (500 MHz, CD_3OD) δ: 1.19, 1.21 (3H each, both s, CH_3), 2.51 (2H, t, *J*=7.3 Hz, 8-H_2), 2.85 (2H, t, *J*=7.3 Hz, 7-H_2), 3.01 (2H, br d, *J*=*ca.* 9 Hz, 1′-H), 4.51 (1H, dd, *J*=8.5, 9.2 Hz, 2′-H), 6.20 (1H, s, 3-H), 6.85 (1H, s, 6-H). ^{13}C-NMR (125 MHz, CD_3OD) δ_C: 120.5 (1-C), 156.2 (2-C), 98.0 (3-C), 160.7 (4-C), 118.7 (5-C), 126.6 (6-C), 27.0 (7-C), 36.3 (8-C), 178.0 (9-C), 31.2 (1′-C), 91.0 (2′-C), 72.6 (3′-C), 25.2, 25.4 (4′, 5′-C). EI-MS (%): *m*/*z* 266 (M^+, 53), 190 (100).

Bioassay. NO Production from LPS-Stimulated Macrophages Inhibitory effects on NO production by mouse macrophages were evaluated using the method reported previously.[2,49] Peritoneal exudate cells were collected from the peritoneal cavities of male ddY mice by washing with 6—7 ml of ice-cold phosphate-buffered saline (PBS), and cells (5×10^5 cells/well) were suspended in 200 μl of RPMI 1640 supplemented with 5% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml), and precultured in 96-well microplates at 37 °C in 5% CO_2 in air for 1 h. Nonadherent cells were removed by washing the cells with PBS, and the adherent cells were cultured in fresh medium containing LPS (10 μg/ml) and test compound (1—100 μM) for 20 h. NO production in each well was assessed by measuring the accumulation of nitrite in the culture medium using Griess reagent. Cytotoxicity was determined using the MTT colorimetric assay. Briefly, after 20-h incubation with test compounds, MTT (10 μl, 5 mg/ml in PBS) solution was added to the wells. After 4-h culture, the medium was removed, and isopropanol containing 0.04 M HCl was then added to dissolve the formazan produced in the cells. The optical density of the formazan solution was measured with a microplate reader at 570 nm (reference, 655 nm). When the optical density of the sample-treated group was reduced to less than 80% of that in the vehicle-treated group, the test compound was considered to exhibit cytotoxic effects. N^G-monomethyl-L-arginine (L-NMMA) was used as a reference compound. Each test compound was dissolved in dimethyl sulfoxide (DMSO), and the solution was added to the medium (final DMSO concentration was 0.5%). Inhibition (%) was calculated using the following formula and the IC_{50} was determined graphically (*n*=4):

$$\text{inhibition (\%)} = \frac{A-B}{A-C}\times 100$$

A−*C*: NO_2^- concentration (μM) [*A*: LPS (+), sample (−); *B*: LPS (+), sample (+); *C*: LPS (−), sample (−)].

Statistical Analysis Values are expressed as mean±S.E.M. One-way analysis of variance followed by Dunnett's test was used for statistical analysis.

Acknowledgments Part of this work was supported by the 21st COE program and Academic Frontier Project from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References and Notes

1) Morikawa T., Matsuda H., Nishida N., Ohgushi T., Yoshikawa M., *Chem. Pharm. Bull.*, **52**, 1387—1390 (2004).
2) Matsuda H., Murakami T., Kageura T., Ninomiya K., Toguchida I., Nishida N., Yoshikawa M., *Bioorg. Med. Chem. Lett.*, **8**, 2191—2196 (1998).
3) Matsuda H., Murakami T., Nishida N., Kageura T., Yoshikawa M., *Chem. Pharm. Bull.*, **48**, 1429—1435 (2000).
4) Yoshikawa M., Morikawa T., Fujiwara E., Ohgushi T., Asao Y., Matsuda H., *Heterocycles*, **55**, 1653—1658 (2001).
5) Matsuda H., Morikawa T., Toguchida I., Harima S., Yoshikawa M., *Chem. Pharm. Bull.*, **50**, 972—975 (2002).
6) Yoshikawa M., Murakami T., Ishiwada T., Morikawa T., Kagawa M., Higashi Y., Matsuda H., *J. Nat. Prod.*, **65**, 1151—1155 (2002).
7) Matsuda H., Morikawa T., Ishiwada T., Managi H., Kagawa M., Higashi Y., Yoshikawa M., *Chem. Pharm. Bull.*, **51**, 440—443 (2003).
8) Yoshikawa M., Morikawa T., Kashima Y., Ninomiya K., Matsuda H., *J. Nat. Prod.*, **66**, 922—927 (2003).
9) Bellino A., Venturella P., Marino M. L., Servettaz O., Venturella G., *Phytochemistry*, **25**, 1195—1199 (1986).
10) Swager T. M., Cardellina J. H. II, *Phytochemistry*, **24**, 805—813 (1985).
11) Elgamal M. H. A., Elewa N. H., Elkhrisy E. A. M., Duddeck H., *Phytochemistry*, **18**, 139—143 (1979).
12) Nielsen B. E., Lemmich J., *Acta Chem. Scand.*, **23**, 962—966 (1969).
13) Fujioka T., Furumi K., Fujii H., Okabe H., Mihashi K., Nakano Y., Matsunaga H., Katano M., Mori M., *Chem. Pharm. Bull.*, **47**, 96—100 (1999).
14) Baba K., Kido T., Yoneda Y., Taniguchi M., Kozawa M., *Shoyakugaku Zasshi*, **44**, 235—239 (1990).
15) These known compounds were identified by comparison of their

authentic samples.
16) Morikawa T., Matsuda H., Ohgushi T., Nishida N., Ishiwada T., Yoshikawa M., *Heterocycles*, **63**, 2211—2215 (2004).
17) Lu T., Parodi F. J., Vargas D., Quijano L., Mertooetomo E. R., Hjortso M. A., Fischer H., *Phytochemistry*, **33**, 113—116 (1993).
18) Hashidoko Y., Tahara S., Mizutani J., *Phytochemistry*, **35**, 325—329 (1994).
19) Kuo Y.-H., Lin S.-T., *Chem. Pharm. Bull.*, **41**, 1507—1512 (1993).
20) Shimomura H., Sashida Y., Nakata H., Kawasaki J., Ito Y., *Phytochemistry*, **21**, 2213—2215 (1982).
21) Yahara S., Sugimura C., Nohara T., Niiho Y., Nakajima Y., Ito H., *Shoyakugaku Zasshi*, **47**, 74—78 (1993).
22) Ishikawa T., Kitajima J., Tanaka Y., *Chem. Pharm. Bull.*, **46**, 1603—1606 (1998).
23) Perold G. W., Rosenberg M. E. K., Howard A. S., Huddle P. A., *J. Chem. Soc. Perkin I*, **1978**, 239—243 (1978).
24) Corse J., Lundin R. E., Sondheimer E., Waiss A. C., Jr., *Phytochemistry*, **5**, 767—776 (1966).
25) Okuyama T., Hosoyama K., Hiraga Y., Kurono G., Takemoto T., *Chem. Pharm. Bull.*, **26**, 3071—3074 (1978).
26) Lei Z.-H., Yahara S., Tai B.-S., Tian R.-H., Takiguchi Y., Nohara T., *Nat. Med.*, **49**, 475—477 (1995).
27) The ^{1}H- and ^{13}C-NMR spectra of **1**, **2**, and **2a** were assigned with the aid of distortionless enhancement by polarization transfer (DEPT) and homo- and heterocorrelation spectroscopy (^{1}H–^{1}H, ^{13}C–^{1}H COSY), and HMBC experiments.
28) Yoshikawa M., Murakami T., Shimada H., Yoshizumi S., Saka M., Yahamara J., Matsuda H., *Chem. Pharm. Bull.*, **46**, 1008—1014 (1998).
29) Yoshikawa M., Morikawa T., Toguchida I., Harima S., Matsuda H., *Chem. Pharm. Bull.*, **48**, 651—656 (2000).
30) Matsuda H., Morikawa T., Ando S., Toguchida I., Yoshikawa M., *Bioorg. Med. Chem.*, **11**, 1995—2000 (2003).
31) Matsuda H., Kageura T., Morikawa T., Toguchida I., Harima S., Yoshikawa M., *Bioorg. Med. Chem. Lett.*, **10**, 323—327 (2000).
32) Kageura T., Matsuda H., Morikawa T., Toguchida I., Harima S., Oda M., Yoshikawa M., *Bioorg. Med. Chem.*, **9**, 1887—1893 (2001).
33) Matsuda H., Kageura T., Oda M., Morikawa T., Sakamoto Y., Yoshikawa M., *Chem. Pharm. Bull.*, **49**, 716—720 (2001).
34) Yoshikawa M., Morikawa T., Xu F., Ando S., Matsuda H., *Heterocycles*, **60**, 1787—1792 (2003).
35) Matsuda H., Ninomiya K., Morikawa T., Yoshikawa M., *Bioorg. Med. Chem. Lett.*, **8**, 339—344 (1998).
36) Matsuda H., Morikawa T., Toguchida I., Ninomiya K., Yoshikawa M., *Heterocycles*, **55**, 841—846 (2001).
37) Matsuda H., Morikawa T., Toguchida I., Ninomiya K., Yoshikawa M., *Chem. Pharm. Bull.*, **49**, 1558—1566 (2001).
38) Matsuda H., Kageura T., Toguchida I., Ueda H., Morikawa T., Yoshikawa M., *Life Sci.*, **66**, 2151—2157 (2000).
39) Muraoka O., Fujimoto M., Tanabe G., Kubo M., Minematsu T., Matsuda H., Morikawa T., Toguchida I., Yoshikawa M., *Bioorg. Med. Chem. Lett.*, **11**, 2217—2220 (2001).
40) Morikawa T., Matsuda H., Toguchida I., Ueda K., Yoshikawa M., *J. Nat. Prod.*, **65**, 1468—1474 (2002).
41) Matsuda H., Toguchida I., Ninomiya K., Kageura T., Morikawa T., Yoshikawa M., *Bioorg. Med. Chem.*, **11**, 709—715 (2003).
42) Matsuda H., Morikawa T., Sakamoto Y., Toguchida I., Yoshikawa M., *Heterocycles*, **56**, 45—50 (2002).
43) Matsuda H., Morikawa T., Sakamoto Y., Toguchida I., Yoshikawa M., *Bioorg. Med. Chem.*, **10**, 2527—2534 (2002).
44) Matsuda H., Kageura T., Toguchida I., Murakami T., Kishi A., Yoshikawa M., *Bioorg. Med. Chem. Lett.*, **9**, 3081—3086 (1999).
45) Morikawa T., Tao J., Ando S., Matsuda H., Yoshikawa M., *J. Nat. Prod.*, **66**, 638—645 (2003).
46) Tao J., Morikawa M., Ando S., Matsuda H., Yoshikawa M., *Chem. Pharm. Bull.*, **51**, 654—662 (2003).
47) Matsuda H., Morikawa T., Ando S., Oominami H., Murakami T., Kimura I., Yoshikawa M., *Bioorg. Med. Chem.*, **12**, 3037—3046 (2004).
48) Tao J., Morikawa T., Toguchida I., Ando S., Matsuda H., Yoshikawa M., *Bioorg. Med. Chem.*, **10**, 4005—4012 (2002).
49) Morikawa T., Tao J., Toguchida I., Matsuda H., Yoshikawa M., *J. Nat. Prod.*, **66**, 86—91 (2003).
50) Shimoda H., Nishida N., Ninomiya K., Matsuda H., Yoshikawa M., *Heterocycles*, **55**, 2043—2050 (2001).
51) Abdel-Halim O. B., Morikawa T., Ando S., Matsuda H., Yoshikawa M., *J. Nat. Prod.*, **67**, 1119—1124 (2004).
52) Wang C. C., Chen L. G., Yang L. L., *Cancer Lett.*, **145**, 151—157 (1999).