Medicinal Flowers. II.[1] Inhibitors of Nitric Oxide Production and Absolute Stereostructures of Five New Germacrane-Type Sesquiterpenes, Kikkanols D, D Monoacetate, E, F, and F Monoacetate from the Flowers of *Chrysanthemum indicum* L.

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The methanolic extract and ethyl acetate-soluble portion from the flowers of *Chrysanthemum indicum* L., Chrysanthemi Indici Flos, were found to show inhibitory activity against nitric oxide (NO) production in lipopolysaccharide-activated macrophages. Five new germacrane-type sesquiterpenes, kikkanols D, D monoacetate, E, F, and F monoacetate, were isolated from the ethyl acetate-soluble portion. Their absolute stereostructures were elucidated on the basis of chemical and physicochemical evidence, which included application of the modified Mosher's method. The effects of fifteen principal components from the ethyl acetate-soluble portion of this medicinal flower against NO production were examined and, among them, acetylenic compounds and flavonoids were found to show potent inhibitory activity.

Key words kikkanol; *Chrysanthemum indicum*; NO production inhibitor; germacrane-type sesquiterpene; medicinal flower; Compositae

In the course of our studies on bioactive principles of natural medicines,[2] we have found that the methanolic extract from a medicinal flower Chrysanthemi Indici Flos, the flower of *Chrysanthemum* (*C.*) *indicum* L. (Compositae), exhibited potent inhibitory activity against rat lens aldose reductase. In the preceding paper,[1] we reported the isolation and structural elucidation of three eudesmane-type sesquiterpenes, termed kikkanols A (**6**), B (**7**), and C (**8**), from the ethyl acetate-soluble portion with the inhibitory activity on aldose reductase. Several active components: luteolin (**14**), eupatilin (**15**), luteolin 7-*O*-β-D-glucopyranoside, luteolin 7-*O*-β-D-glucopyranosiduronic acid, acacetin 7-*O*-(6″-α-L-rhamnopyranosyl)-β-D-glucopyranoside, and chlorogenic acid, were isolated through bioassay-guided separation using the inhibitory activity against rat lens aldose reductase. As a continuation of the characterization study of the flowers of *C. indicum*, we have also found that the methanolic extract and the ethyl acetate-soluble portion showed inhibitory effect against nitric oxide (NO) production in lipopolysaccharide (LPS)-activated macrophages. We recently isolated five new germacrane-type sesquiterpenes called kikkanols D (**1**), D monoacetate (**2**), E (**3**), F (**4**), and F monoacetate (**5**) from the ethyl acetate-soluble portion. This paper deals with the isolation and absolute stereostructure elucidation of new kikkanols (**1**—**5**) from the flowers of *C. indicum.* In addition, we describe the inhibitory effect of the principal components from the ethyl acetate-soluble portion of this medicinal flower against NO production.[3]

The methanolic extract of the flowers of Chinese *C. indicum*, which showed an inhibitory effect against NO production as shown in Table 1, was partitioned into a mixture of ethyl acetate and water to furnish the ethyl acetate-soluble portion and an aqueous phase. The aqueous phase was further extracted with 1-butanol to give a 1-butanol-soluble portion and a water-soluble portion as described.[1] The ethyl acetate-soluble portion was found to show inhibitory effect against NO production, while the 1-butanol-soluble and water-soluble portions lacked the activity. The ethyl acetate-soluble portion was subjected to silica gel and octadecyl silica (ODS) column chromatography and, finally, HPLC to furnish **1** (0.002% from the natural medicine), **2** (0.001%), **3** (0.003%), **4** (0.0024%), and **5** (0.0044%) together with **6**, **7**, and **8**, three sesquiterpene (**9**, **10**, **11**), two polyacetylenes (**12**, **13**), and two flavones (**14**, **15**).[1]

Absolute Stereostructures of 1—3 Kikkanol D (**1**) was isolated as a colorless oil with negative optical rotation ($[\alpha]_D^{25}$ −60.0°). The electron impact (EI)-MS of **1** showed a molecular ion (M^+) peak at *m/z* 254 in addition to fragment ion peaks at *m/z* 236 (M^+-H_2O) and *m/z* 95 (base peak). The molecular formula $C_{15}H_{26}O_3$ of **1** was determined from the molecular ion peak observed in the EI-MS and by high-resolution MS measurement. The IR spectrum of **1** showed absorption bands ascribable to hydroxyl and olefin functions at 3417 and 1646 cm^{-1}, respectively. The ^{1}H-NMR (CD_3OD) and ^{13}C-NMR (Table 2) spectra of **1** showed signals assignable to two secondary methyls [δ 0.94, 0.96 (both d, J=7.0 Hz, 12 and 13-H_3)], a methylene [δ 3.84, 3.95 (ABq, J=13.1 Hz, 15-H_2)] and two methines bearing a hydroxyl group [δ 3.92 (br d, J=*ca.* 10 Hz, 8-H), 4.09 (d, J=9.8 Hz, 9-H)], an *exo*-methylene [δ 4.89, 4.94 (both s, 14-H_2)], and an olefin [δ 5.44 (dd, J=6.1, 11.0 Hz, 3-H)] together with four methylenes (1, 2, 5, 6-H_2), two methines (7, 11-H), and two quaternary carbons (4, 10-C).

The plane structure of **1** was constructed on the basis of ^{1}H–^{1}H correlation spectroscopy (H–H COSY) and heteronuclear multiple bond correlation (HMBC) experiments. Thus, the H–H COSY experiment on **1** indicated the presence of two partial structures written in bold lines as shown in Fig. 1: from 1-C–3-C, 5-C–9-C, and 7-C–13-C. In the HMBC experiment, long-range correlations were observed between the following protons and carbons of **1** (5-H_2, 15-H_2 and 4-C; 1-H_2, 9-H and 10-C), so that the connectivities of the quaternary carbons (4, 10-C) in **1** were clarified. The abovementioned evidence led us to confirm the skeleton of **1** to be 3,10(14)-germacradien-8,9,15-triol.

Kikkanol D monoacetate (**2**) was isolated as a colorless oil

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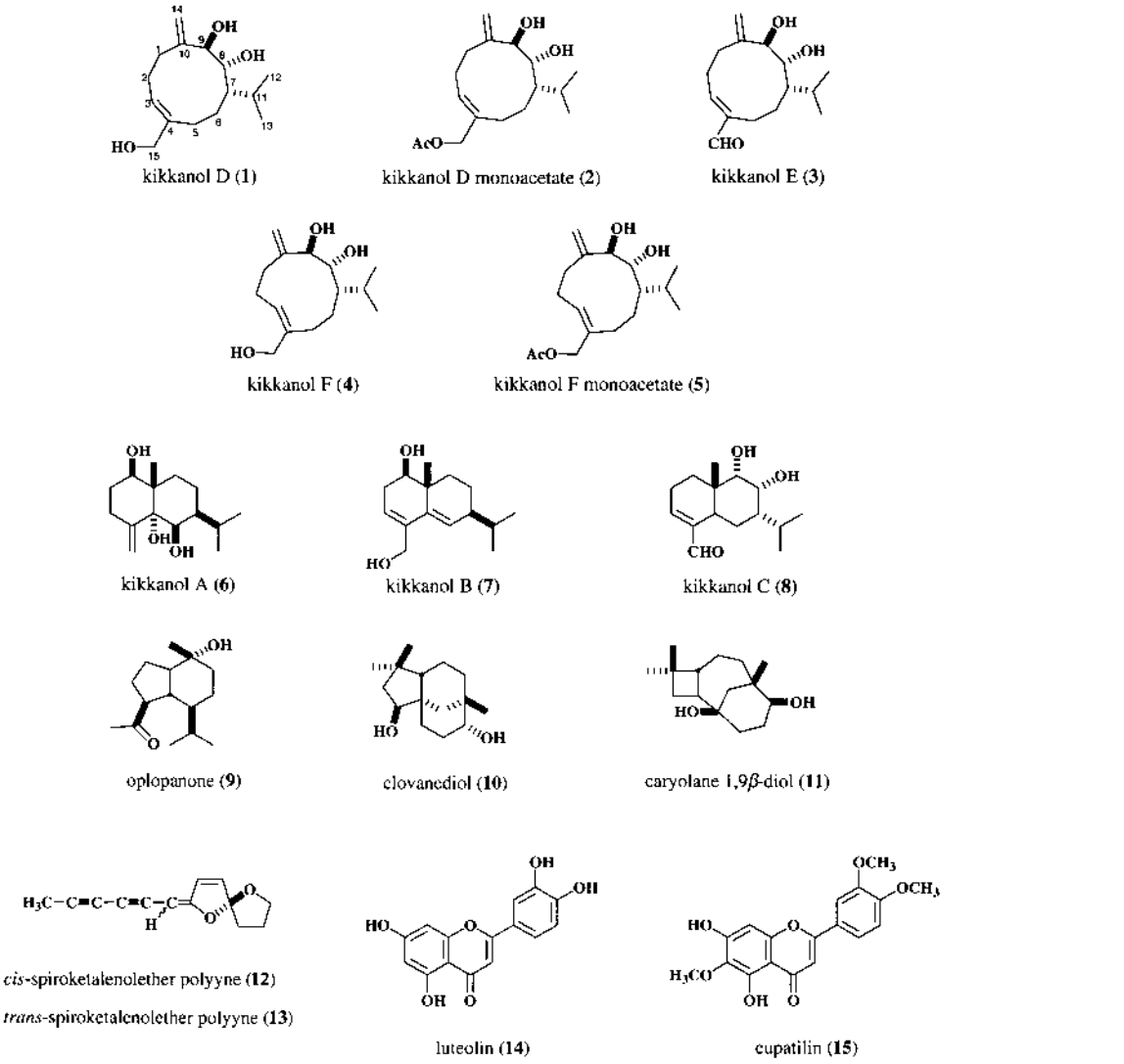

Chart 1

Table 1. Inhibitory Activity of MeOH Extract, Ethyl Acetate-, 1-Butanol-, and Water-Soluble Portions from *C. indicum* against NO Production in LPS-Activated Macrophages

	IC_{50} (μg/ml)
MeOH extract	89.2
AcOEt-soluble portion	17.3
1-Butanol-soluble portion	>300
H_2O-soluble portion	>300

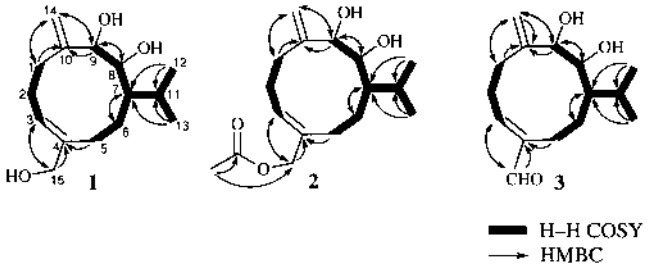

Fig. 1

with negative optical rotation ($[\alpha]_D^{24}$ −138.1°). The molecular formula $C_{17}H_{28}O_4$ of **2** has been determined from the quasimolecular ion peak at *m/z* 319 $(M+Na)^+$ in the positive-ion fast atom bombardment (FAB)-MS and by high-resolution MS measurement. The IR spectrum of **2** showed absorption bands at 3436, 1717, and 1648 cm^{-1} ascribable to hydroxyl, carbonyl, and olefin functions. The ^{1}H-NMR ($CDCl_3$) and ^{13}C-NMR (Table 2) spectra of **2** showed the presence of an acetyl group [δ 2.06 (s, 15-OAc)] and a methylene bearing an acetoxyl group [δ 4.33, 4.52 (ABq, J=12.3 Hz, 15-H_2)] together with two secondary methyls, two methines bearing a hydroxyl group, an *exo*-methylene, and an olefin. Furthermore, **1** was obtained by treatment of **2** with 0.1% sodium methoxide (NaOMe)–methanol and the HMBC experiment showed long-range correlation between the acetyl proton and the 15-carbon. On the basis of this evidence, **2** was determined to be the 15-acetate of **1**.

Kikkanol E (**3**) was isolated as a colorless oil with negative optical rotation ($[\alpha]_D^{22}$ −94.4°). The molecular formula $C_{15}H_{24}O_3$ of **3** has been determined from the molecular ion peak at *m/z* 252 (M^+) in the EI-MS of **3** and by high-resolution MS measurement. In the UV spectrum of **3**, an absorption maximum was observed at 232 nm (log ε 3.89), suggestive of an α,β-unsaturated carbonyl function. The IR spec-

Table 2. ^{13}C-NMR Data for **1**—**5**

Position	**1**[a]	**2**[b]	**3**[a]	**4**[a]	**5**[b]
C-1	37.4	35.9	36.6	36.2	35.1
C-2	25.3	24.6	27.2	28.2	27.6
C-3	126.7	129.6	156.9	129.0	130.9
C-4	140.1	134.5	144.5	140.8	135.1
C-5	26.4	26.0	23.7	33.4	32.6
C-6	22.8	21.9	22.7	28.4	27.1
C-7	41.7	40.3	42.3	44.2	42.7
C-8	76.5	75.5	76.5	73.6	72.7
C-9	72.8	72.1	73.0	77.7	77.0
C-10	150.3	148.7	149.6	151.1	149.5
C-11	35.3	34.2	34.2	33.5	32.2
C-12	21.1[c]	20.5[c]	20.9[c]	21.5[c]	21.0[c]
C-13	21.3[c]	20.8[c]	21.4[c]	21.8[c]	21.3[c]
C-14	115.1	115.0	115.6	113.8	113.9
C-15	66.3	67.6	197.1	61.9	63.6
$\underline{C}H_3$–CO–		21.0			21.0
CH_3–$\underline{C}$O–		170.9			171.2

Measured in *a*) CD_3OD, *b*) $CDCl_3$ at 125 MHz. *c*) Assignments may be interchangeable within the same column.

trum of **3** showed absorption bands ascribable to hydroxyl, α,β-unsaturated carbonyl, and olefin functions at 3417, 1688, and 1640 cm^{-1}. The ^{1}H-NMR (CD_3OD) and ^{13}C-NMR (Table 2) spectra of **3** showed signals assignable to two secondary methyls [δ 0.74, 0.77 (both d, J=6.7 Hz, 12 and 13-H_3)], two methines bearing a hydroxyl group [δ 3.78 (br d, J=*ca.* 10 Hz, 8-H), 3.90 (d, J=9.8 Hz, 9-H)], an *exo*-methylene [δ 4.80, 4.95 (both d, J=1.2 Hz, 14-H_2)], an olefin [δ 6.54 (dd, J=5.8, 11.9 Hz, 3-H)], and an aldehyde [δ 9.22 (br s, 15-H)] together with four methylenes (1, 2, 5, 6-H_2), two methines (7, 11-H), and two quaternary carbons (4, 10-C). The partial structures of **3** written in bold lines were clarified by H–H COSY and long-range correlations were observed between the following protons and carbons of **3** (3-H and 15-C; 5-H_2, 15-H and 4-C; 1-H_2, 9-H and 10-C) in the HMBC experiment (Fig. 1). The proton and carbon signals in the NMR spectra of **3** were superimposable on those of **1**, except for the signals due to the 15-aldehyde function. Finally, reduction of **3** with sodium borohydride ($NaBH_4$) in the presence of cerium chloride ($CeCl_3$) in MeOH furnished **1** quantitatively. Thus, **3** was characterized to be the 15-aldehyde derivative of **1**.

Next, the absolute stereostructures and the geometric structures of **1**—**3** were determined by the following procedure. In order to characterize the relative stereostructure of the 7-, 8-, and 9-positions, **3** was converted to the 8,9-acetonide derivative (**3a**) by treatment of **3** with 2,2-dimethoxypropane and Dowex HCR-W2 (H^+ form). In the nuclear Overhauser effect spectroscopy (NOESY) experiment on **3a**, the nuclear Overhauser effect (NOE) correlations were observed between the signals of the following proton pairs (3-H and 15-H, 7-H and 8-H) as shown in Fig. 2. Since the NOE correlation was observed between the 3-olefin proton and the 15-aldehyde proton, the geometry of the olefin group in **3** was confirmed to be *E*-form. The NOE correlation between the 7-proton and the 8-proton and the coupling patterns of the 8-proton (dd, J=3.5, 9.5 Hz) and 9-proton (d, J=9.5 Hz) of **3a** led us to confirm the relative structure of **3a** as having 7α-isopropyl, 8α-hydroxyl, and 9β-hydroxyl groups.

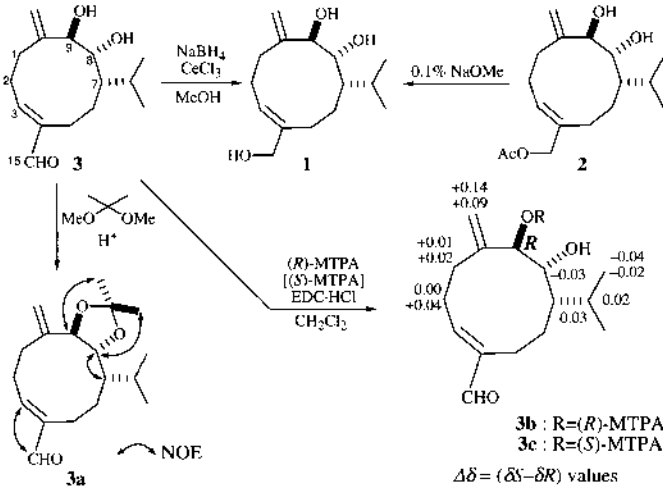

Fig. 2

The absolute configuration of **3** was determined by application of the modified Mosher's method[4] for the 9-mono-(*R*)- and (*S*)-2-methoxy-2-trifluorophenylacetate (MTPA esters, **3b** and **3c**), which were prepared by selective esterification of the 9- hydroxyl group in **3** with (*R*)- and (*S*)- 2-methoxy-2-trifluoromethylphenylacetic acid in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) and 4-dimethylaminopyridine (4-DMAP). As shown in Fig. 2, the signals due to protons attached to the 1, 2, and 14-carbons in the (*S*)-MTPA ester (**3c**) were observed at a lower field than those of the (*R*)-MTPA ester (**3b**) ($\Delta\delta$, positive), while the signals due to protons on the 7, 8, 11, 12 and 13-carbons in **3c** were observed at higher fields than those of **3b** ($\Delta\delta$, negative). Thus, the absolute configuration at the 9-carbon in **3** has been shown to be *R*. Consequently, the absolute stereostructures of **1**—**3** were determined.

Absolute Stereostructures of 4 and F 5 Kikkanol F (**4**) was isolated as a colorless oil with negative optical rotation ($[\alpha]_D^{25}$ −95.7°). The IR spectrum of **4** showed absorption bands due to hydroxyl and olefin functions at 3360 and 1651 cm^{-1}. The EI-MS of **4** showed a molecular ion peak at *m/z* 254 (M^+), 236 (M^+-H_2O), and 95 (base peak) and the molecular formula $C_{15}H_{26}O_3$, which is the same as that of **1**, was determined by high-resolution MS measurement. The proton signals in the ^{1}H-NMR (CD_3OD) spectrum of **4** were also found to be similar to those of **1** and indicated the presence of the same functional groups: two secondary methyls [δ 0.99, 1.01 (both d, J=6.4 Hz, 12 and 13-H_3)], a methylene [δ 3.92, 4.17 (ABq, J=11.9 Hz, 15-H_2)] and two methines bearing a hydroxyl group [δ 3.44 (d, J=10.1 Hz, 9-H), 3.92 (br d, J=*ca.* 10 Hz, 8-H)], an *exo*-methylene [δ 4.93, 5.00 (both s, 14-H_2)], and an olefin [δ 5.38 (dd, J=7.9, 7.9 Hz, 3-H)]. In addition, the connectivities of the ^{1}H–^{1}H and the quaternary carbons in **4** was clarified by H–H COSY and HMBC experiments as shown in Fig. 3. The geometric structure of **4** was characterized by comparison of the carbon signals (2-C, 5-C, 15-C) around the olefin group in the ^{13}C-NMR (Table 2) data for **4** with those for **1**. Namely, the signal due to the 5-carbon of **4** was observed at a lower field than that of **1** (δ_C 33.4 for **4**, δ_C 26.4 for **1**), while the 15-carbon signal of **4** was observed at a higher field than that of **1** (δ_C 61.9 for **4**,

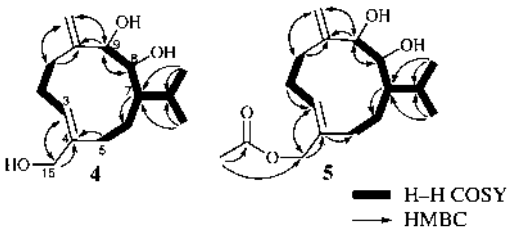

Fig. 3

δ_C 66.3 for **1**). On the basis of this evidence, **4** has been identified as the *Z*-isomer of **1**.

Kikkanol F monoacetate (**5**) was also isolated as a colorless oil with negative optical rotation ($[\alpha]_D^{22}$ −59.6°). The molecular formula $C_{17}H_{28}O_4$ of **5** has been determined for the molecular ion peak *m/z* 296 (M^+) in the EI-MS and by high-resolution MS measurement. The IR spectrum of **5** showed absorption bands at 3436, 1738, and 1651 cm^{-1} ascribable to hydroxyl, carbonyl, and olefin functions. The ^{1}H-NMR ($CDCl_3$) and ^{13}C-NMR (Table 2) spectra of **5** showed signals due to an acetyl methyl [δ 2.06 (s, 15-OAc)] and a methylene bearing an acetoxyl group [δ 4.49, 4.59 (ABq, J=12.2 Hz, 15-H_2)] together with two secondary methyls, two methines bearing a hydroxyl group, an *exo*-methylene, and an olefin. Alkaline treatment of **5** furnished **4** and the HMBC experiment showed a long-range correlation between the acetyl proton and the 15-carbon (Fig. 3). Therefore, **5** was confirmed to be the 15-acetate of **4**.

The relative stereostructures and geometric structures of **4** and **5** were also determined by the NOE experiment on the 8,9-acetonide derivative (**5a**), which was prepared by acid treatment of **5** in 2,2-dimethoxypropane. Thus, the NOE correlations were observed between the signals of the following proton pairs (2-H_2 and 15-H_2, 3-H and 5-H_2, 7-H and 8-H) as depicted in Fig. 4. This evidence led us to confirm the geometry of the olefin group in **5a** as *Z*-form. Furthermore, on the basis of the NOE correlations between the 7-proton and the 8-proton and the coupling patterns of the 8-proton (dd, J=1.8, 9.1 Hz) and 9-proton (d, J=9.1 Hz), the configurations of **5a** at the 7, 8, and 9-positions were confirmed to be the same as those of **3a**.

Finally, the absolute configuration of **5** was determined by application of the modified Mosher's method. As shown in Fig. 4, the signals due to protons attached to the 1, 2, and 14-carbons in the 9-mono-(*S*)-MTPA ester (**5c**) were observed at a lower field than those of the 9-mono(*R*)-MTPA ester (**5b**) ($\Delta\delta$, positive), while the signals due to protons on the 5, 6, 7, 8, 11, 12 and 13-carbons in **5c** were observed at higher fields than those of **5b** ($\Delta\delta$, negative). Consequently, the absolute configuration at the 9-position of **5** has been shown to be *R*, and the absolute stereostructures of **4** and **5** were determined to be as shown.

Inhibitory Activity of Constituents from Flowers of *C. indicum* against NO Production in LPS-Activated Mouse Peritoneal Macrophages NO, an inorganic free radical, has been implicated in physiological and pathological 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 a NO synthase (NOS). In the family of NOS, inducible NOS in particular is involved in a pathological aspect with overproduction of NO, and can be expressed in response to pro-inflammatory agents such as interleukin-1β, tumor necrosis factor-α, and LPS in various cells including macrophages, endothelial cells, and smooth muscle cells.

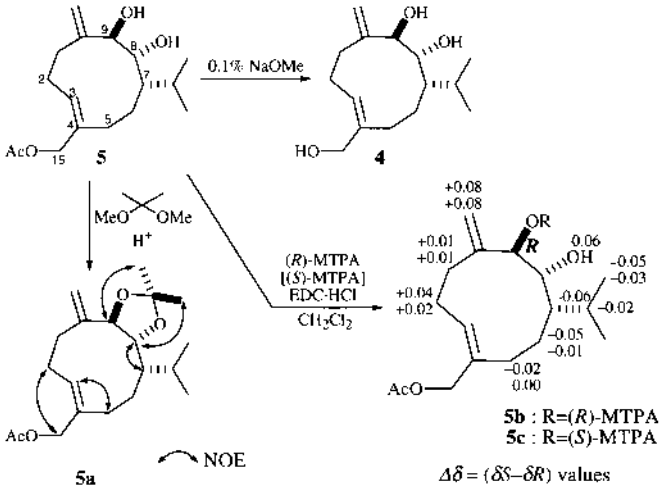

Fig. 4

Table 3. Inhibitory Activity of Constituents from *C. indicum* against NO Production in LPS-Activated Macrophages

Compounds	IC_{50} (μM)
Kikkanol A (**6**)	>100 (19.5)
Kikkanol B (**7**)	>100 (49.8)
Kikkanol C (**8**)	>100 (16.5)
Kikkanol D (**1**)	>100 (18.3)
Kikkanol D monoacetate (**2**)	>100 (29.8)
Kikkanol E (**3**)	>100 (36.2)
Kikkanol F (**4**)	>100 (14.0)
Kikkanol F monoacetate (**5**)	91.3
Oplopanone (**9**)	>100 (36.5)
Clovanediol (**10**)	>100 (27.4)
Caryolane 1,9β-diol (**11**)	>100 (40.7)
cis-Spiroketalenolether polyyne (**12**)	38.3
trans-Spiroketalenolether polyyne (**13**)	59.5
Luteolin (**14**)	19.7[a]
Eupatilin (**15**)	42.4
L-NMMA	27.9

Values in parentheses represent the (%) inhibition at 100 μM. *a*) Cytotoxic effect was observed at 100 μM.

As a part of our characterization studies on bioactive components of natural medicines,[2] we previously reported several NO production inhibitors: higher unsaturated fatty acids,[5] polyacetylenes,[6] coumarins,[6] stilbenes,[7] sesquiterpenes,[8] and triterpenes.[9] In the course of our continuing survey on NO production inhibitors from natural medicines, we have found that the methanolic extract and the ethyl acetate-soluble portion from the flowers of *C. indicum*, which is prescribed for antiinflammatory, analgesic, and antipyretic purposes and the treatment of eye disease in Chinese traditional preparations, showed inhibitory activity against NO production in LPS-activated macrophages (Table 1). Next, we examined the inhibitory activity of principal components (**1**—**15**) from the ethyl acetate-soluble portion of this natural medicine. As shown in Table 3, two acetylenic compounds, *cis*-spiroketalenolether polyyne (**12**) and *trans*-spiroketalenolether polyyne (**13**), and two flavones, **14**, and **15**, were found

to inhibit the NO production. The inhibitory activity of these components and the extract from the flower of *C. indicum* against NO production may be important evidence substantiating the traditional effects of this medicinal flower.

Experimental

The following instruments were used to obtain physical data: specific rotations, Horiba SEPA-300 digital polarimeter (l=5 cm); UV spectra, Shimadzu UV-1200 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.

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); reversed-phase silica gel column chromatography, Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., 100—200 mesh); TLC, pre-coated TLC plates with Silica gel 60F_{254} (Merck, 0.25 mm) (ordinary phase) and Silica gel RP-18 60F_{254} (Merck, 0.25 mm) (reversed phase); reversed-phase HPTLC, pre-coated TLC plates with Silica gel RP-18 60WF_{254S} (Merck, 0.25 mm); detection was achieved by spraying with 1% Ce(SO_4)$_2$–10% aqueous H_2SO_4 and heating.

Isolation of Kikkanols D (1), D Monoacetate (2), E (3), F (4), and F Monoacetate (5) from the Flowers of *C. indicum* L. Fraction 8 (3.7 g) obtained from the ethyl acetate–soluble portion of the flowers of *C. indicum* L. (5.8 kg, cultivated in China and purchased from Koshiro Co., Ltd., Osaka) and isolated kikkanols A—C (**6**—**8**), oplopanone (**9**), and eupatilin (**15**) as reported previously,[1] was further separated by reversed-phase silica gel column chromatography (100 g, MeOH–H_2O) and HPLC [YMC-Pack ODS-A, MeOH–H_2O (70 : 30, v/v)] to furnish kikkanols D monoacetate (**2**, 19 mg), and F monoacetate (**5**, 86 mg). Fraction 10 (6.3 g), from which clovanediol (**10**), caryolane 1,9β-diol (**11**), and luteolin (**14**) were previously isolated,[1] was subjected to reversed-phase silica gel column chromatography (300 g, MeOH–H_2O) and HPLC [YMC-Pack ODS-A, MeOH–H_2O (50 : 50, v/v)] to furnish kikkanols D (**1**, 38 mg), E (**3**, 57 mg), and F (**4**, 46 mg).

Kikkanol D (**1**): Colorless oil, $[\alpha]_D^{25}$ −60.0° (c=0.7, $CHCl_3$). High-resolution EI-MS: Calcd for $C_{15}H_{26}O_3$ (M^+): 254.1882. Found: 254.1889. IR (film): 3417, 2958, 1646, 909 cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 0.94, 0.96 (3H each, both d, J=7.0 Hz, 12 and 13-H_3), 1.42 (1H, m, 6β-H), 1.55 (1H, br ddd, J=*ca.* 2, 7, 9 Hz, 7-H), 1.64 (1H, dq, J=1.8, 7.0 Hz, 11-H), 1.90 (1H, dddd, J=4.3, 5.2, 6.8, 14.4 Hz, 6α-H), 2.04 (1H, m, 2-H), 2.07 (1H, m, 5-H), 2.21 (1H, ddd, J=4.3, 13.4, 15.4 Hz, 1β-H), 2.45 (1H, ddd, J=5.2, 12.5, 14.4 Hz, 5-H), 2.58 (1H, m, 1α-H), 2.64 (1H, m, 2-H), 3.84, 3.95 (ABq, J=13.1 Hz, 15-H_2), 3.92 (1H, br d, J=*ca.* 10 Hz, 8-H), 4.09 (1H, d, J=9.8 Hz, 9-H), 4.89, 4.94 (1H each, both s, 14-H_2), 5.44 (1H, dd, J=6.1, 11.0 Hz, 3-H). ^{13}C-NMR (CD_3OD) δ_C: given in Table 2. EI-MS *m/z* (%): 254 (M^+, 1), 236 (M^+-H_2O, 8), 95 (100).

Kikkanol D Monoacetate (**2**): Colorless oil, $[\alpha]_D^{24}$ −138.1° (c=0.1, $CHCl_3$). High-resolution positive-ion FAB-MS: Calcd for $C_{17}H_{28}O_4Na$ (M+Na)$^+$: 319.1886. Found: 319.1895. IR (film) 3436, 2957, 1717, 1648, 909 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.96, 0.98 (3H each, both d, J=7.1 Hz, 12 and 13-H_3), 1.40 (1H, m, 6β-H), 1.51 (1H, br ddd, J=*ca.* 2, 6, 10 Hz, 7-H), 1.68 (1H, dq, J=2.4, 7.1 Hz, 11-H), 1.97 (1H, dddd, J=2.7, 5.2, 9.8, 15.3 Hz, 6α-H), 2.06 (3H, s, C<u>H</u>$_3$–CO–), 2.06 (1H, m, 5-H), 2.10 (1H, m, 2-H), 2.21 (1H, m, 1β-H), 2.46 (1H, ddd, J=5.2, 12.5, 16.6 Hz, 5-H), 2.61 (1H, m, 2-H), 2.63 (1H, m, 1α-H), 3.95 (1H, br d, J=*ca.* 10 Hz, 8-H), 4.10 (1H, d, J=10.0 Hz, 9-H), 4.33, 4.52 (ABq, J=12.3 Hz, 15-H_2), 4.94 (1H, br s, 14-H), 5.01 (1H d, J=1.2 Hz, 14-H), 5.50 (1H, dd, J=8.8, 13.0 Hz, 3-H). ^{13}C-NMR ($CDCl_3$) δ_C: given in Table 2. Positive-ion FAB-MS *m/z*: 319 (M+Na)$^+$.

Kikkanol E (**3**): Colorless oil, $[\alpha]_D^{22}$ −94.4° (c=0.1, MeOH). High-resolution EI-MS Calcd for $C_{15}H_{24}O_3$ (M^+): 252.1725. Found: 252.1731. UV [MeOH, nm, (log ε)]: 232 (3.89). IR (film): 3417, 2952, 1688, 1640, 912 cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 0.74, 0.77 (3H each, both d, J=6.7 Hz, 12 and 13-H_3), 1.17 (1H, m, 7-H), 1.48 (1H, dq, J=0.6, 6.7 Hz, 11-H), 1.55 (1H, dddd, J=3.3, 6.4, 9.7, 13.8 Hz, 6β-H), 1.76 (1H, dddd, J=5.8, 8.0, 11.6, 13.8 Hz, 6α-H), 2.25 (1H, m, 5-H), 2.28 (1H, m, 2-H), 2.34 (1H, m, 1β-H), 2.37 (1H, m, 5-H), 2.64 (1H, br d-like, 1α-H), 2.82 (1H, dddd, J=4.3, 8.9, 11.9, 12.8 Hz, 2-H), 3.78 (1H, br d, J=*ca.* 10 Hz, 8-H), 3.90 (1H, d, J=9.8 Hz, 9-H), 4.80, 4.95 (1H each, both d, J=1.2 Hz, 14-H_2), 6.54 (1H, dd, J=5.8, 11.9 Hz, 3-H), 9.22 (1H, br s, 15-C<u>H</u>O). ^{13}C-NMR (CD_3OD) δ_C: given in Table 2. EI-MS: *m/z* (%): 252 (M^+, 4), 234 (M^+-H_2O, 8), 96 (100).

Kikkanol F (**4**): Colorless oil, $[\alpha]_D^{25}$ −95.7° (c=0.5, $CHCl_3$). High-resolution EI-MS: Calcd for $C_{15}H_{26}O_3$ (M^+): 254.1882. Found: 254.1885. IR (film): 3360, 2930, 1651, 904 cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 0.99, 1.01 (3H each, both d, J=6.4 Hz, 12 and 13-H_3), 1.30 (1H, m, 7-H), 1.36 (1H, dddd, J=1.5, 3.7, 7.3, 14.3 Hz, 6β-H), 1.66 (1H, dddd, J=1.8, 4.0, 13.4, 14.3 Hz, 6α-H), 1.75 (1H, dq, J=1.5, 6.4 Hz, 11-H), 1.85 (1H, ddd-like, 5-H), 2.19 (1H, ddd, J=8.5, 11.3, 12.0 Hz, 1α-H), 2.37 (1H, m, 5-H), 2.40, 2.41 (1H each, both m, 2-H_2), 2.44 (1H, m, 1β-H), 3.44 (1H, d, J=10.1 Hz, 9-H), 3.92 (1H, br d, J=*ca.* 10 Hz, 8-H), 3.92, 4.17 (ABq, J=11.9 Hz, 15-H_2), 4.93, 5.00 (1H each, both s, 14-H_2), 5.38 (1H, dd, J=7.9, 7.9 Hz, 3-H). ^{13}C-NMR (CD_3OD) δ_C: given in Table 2. EI-MS *m/z* (%): 254 (M^+, 1), 236 (M^+-H_2O, 9), 95 (100).

Kikkanol F Monoacetate (**5**): Colorless oil, $[\alpha]_D^{22}$ −59.6° (c=0.8, $CHCl_3$). High-resolution EI-MS: Calcd for $C_{17}H_{28}O_4$ (M^+): 296.1987. Found: 296.1993. IR (film) 3436, 2977, 1738, 1651, 903 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.00, 1.00 (3H each, both d, J=6.8 Hz, 12 and 13-H_3), 1.27 (1H, m, 7-H), 1.41 (1H, dddd, J=3.7, 4.1, 8.6, 15.0 Hz, 6β-H), 1.66 (1H, dddd, J=2.1, 4.1, 4.1, 15.0 Hz, 6α-H), 1.78 (1H, dq, J=1.8, 6.8 Hz, 11-H), 1.81 (1H, ddd, J=4.1, 4.1, 13.9 Hz, 5-H), 2.06 (3H, s, C<u>H</u>$_3$–CO–), 2.23 (1H, m, 1α-H), 2.37 (1H, m, 5-H), 2.43, 2.46 (1H each, both m, 2-H_2), 2.50 (1H, m, 1β-H), 3.50 (1H, d, J=9.8 Hz, 9-H), 3.94 (1H, br d, J=*ca.* 10 Hz, 8-H), 4.49, 4.59 (ABq, J=12.2 Hz, 15-H_2), 4.99, 5.04 (1H each, both s, 14-H_2), 5.51 (1H, dd, J=6.7, 10.4 Hz, 3-H). ^{13}C-NMR ($CDCl_3$) δ_C: given in Table 2. EI-MS *m/z*: 296 (M^+, 1), 278 (M^+-H_2O, 3), 95 (100).

Deacetylation of 2, and 5 A solution of **2** (1.2 mg, 4.1 μmol) or **5** (3.5 mg, 11.8 μmol) in 0.1% NaOMe–MeOH (1.0 ml) was stirred at 40 °C for 1 h. The reaction mixture was neutralized with Dowex HCR-W2 (H^+ form) and the resin was removed by filtration. Evaporation of the solvent from the filtrate under reduced pressure gave a residue, which was purified by silica gel column chromatography (0.2 g, n-hexane–acetone=3 : 1) to furnish **1** (0.9 mg, 87%) or **4** (3.0 mg, quant.).

$NaBH_4$–$CeCl_3$ Reduction of 3 A solution of **3** (2.0 mg, 7.9 μmol) in MeOH (2.0 ml) was treated with $NaBH_4$ (1.5 mg) in the presence of $CeCl_3$ (10 mg) and the mixture was stirred at 0 °C for 45 min. The reaction mixture was quenched at acetone (1.0 ml), then poured into ice-water and the whole was extracted with AcOEt. The AcOEt extract was washed with brine, then dried over $MgSO_4$ powder and filtered. Removal of the solvent from the filtrate under reduced pressure furnished a residue, which was purified by silica gel column chromatography (0.2 g, n-hexane–acetone=2 : 1) to give **1** (2.1 mg, quant.).

Preparation of Acetonide Derivatives 3a and 5a from 3 and 5 A solution of **3** (0.9 mg, 3.6 μmol) or **5** (2.5 mg, 8.4 μmol) in 2,2-dimethoxypropane (1.0 ml) was treated with Dowex HCR-W2 (H^+ form, 10 mg) and the mixture was stirred at room temperature for 2 h. The resin was removed by filtration. Removal of the solvent from the filtrate under reduced pressure yielded a residue, which was subjected to silica gel column chromatography (0.2 g, n-hexane–AcOEt=5 : 1) to give **3a** (1.1 mg, quant.) or **5a** (2.9 mg, quant.).

3a: Colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 0.88, 0.90 (3H each, both d, J=7.0 Hz, 12 and 13-H_3), 1.25 (1H, m, 7-H), 1.40, 1.43 (3H each, both s, (C<u>H</u>$_3$)$_2$-C), 1.49 (1H, m, 6β-H), 1.76 (1H, dq, J=1.6, 7.0 Hz, 11-H), 1.82 (1H, dddd, J=2.2, 10.1, 10.6, 12.6 Hz, 6α-H), 2.27, 2.54 (1H each, both m, 5-H_2), 2.57 (1H, m, 2-H), 2.60, 2.62 (1H each, both m, 1-H_2), 2.70 (1H, dddd, J=5.8, 6.4, 8.5, 12.5 Hz, 2-H), 4.03 (1H, dd, J=3.5, 9.5 Hz, 8-H), 4.29 (1H, d, J=9.5 Hz, 9-H), 5.09, 5.21 (1H each, both s, 14-H_2), 6.51 (1H, dd, J=8.5, 8.6 Hz, 3-H), 9.39 (1H, s, 15-C<u>H</u>O). EI-MS *m/z*: 292 (M^+, 5), 252 ($M^+-C_3H_4$, 5), 234 ($M^+-C_3H_4-H_2O$, 19), 148 (100).

5a: Colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 0.98, 1.00 (3H each, both d, J=6.4 Hz, 12 and 13-H_3), 1.18 (1H, m, 7-H), 1.35, 1.38 (3H each, both s, (C<u>H</u>$_3$)$_2$–C–), 1.43 (1H, m, 6β-H), 1.64 (1H, dddd, J=3.0, 3.7, 6.7, 13.8 Hz, 6α-H), 1.73 (1H, dq, J=2.7, 6.4 Hz, 11-H), 1.81 (1H, ddd, J=3.3, 3.4, 13.4 Hz, 5-H), 2.08 (3H, s, C<u>H</u>$_3$–CO–), 2.20 (1H, m, 2-H), 2.26 (1H, m, 1α-H), 2.37 (1H, ddd, J=3.6, 3.7, 13.4 Hz, 5-H), 2.46 (1H, m, 1β-H), 2.49 (1H, m, 2-H), 3.73 (1H, d, J=9.1 Hz, 9-H), 3.97 (1H, dd, J=1.8, 9.1 Hz, 8-H), 4.50, 4.64 (ABq, J=12.2 Hz, 15-H_2), 5.22, 5.26 (1H each, both s, 14-H_2), 5.56 (1H, dd, J=3.9, 4.0 Hz, 3-H). EI-MS *m/z*: 336 (M^+, 38), 120 (100).

Preparation of the (*R*)-MTPA Ester (3b) and the (*S*)-MTPA Ester (3c) from 3 A solution of **3** (1.2 mg, 4.8 μmol) in CH_2Cl_2 (1.0 ml) was treated with (*R*)-MTPA (5.6 mg, 24 μmol) in the presence of EDC·HCl (4.9 mg, 24 μmol) and 4-DMAP (1.7 mg, 15 μmol) and the mixture was stirred at room temperature for 24 h. The reaction mixture was poured into ice-water and the whole was extracted with AcOEt. The AcOEt extract was treated in the usual manner to give a residue, which was purified by silica gel column chromatography (0.2 g, n-hexane–AcOEt=5 : 1) to give **3b** (1.5 mg, 67%).

Through a similar procedure, **3c** (1.4 mg, 63%) was prepared from **3** (1.2 mg) using (*S*)-MTPA (5.6 mg), EDC·HCl (4.9 mg) and 4-DMAP (1.7 mg).

3b: Colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 0.83, 0.85 (3H each, both d, J=7.0 Hz, 12 and 13-H_3), 1.36 (1H, m, 7-H), 1.60 (1H, dq, J=1.5, 7.0 Hz, 11-H), 1.69 (1H, m, 6β-H), 1.95 (1H, m, 6α-H), 2.42 (1H, m, 5-H), 2.44 (1H, m, 1β-H), 2.46 (1H, m, 2-H), 2.60 (1H, ddd, J=5.8, 6.7, 14.4 Hz, 5-H), 2.91 (1H, br d-like, 1α-H), 3.04 (1H, dddd, J=1.2, 8.9, 11.6, 14.6 Hz, 2-H), 3.54 (3H, s, –OCH_3), 4.09 (1H, dd, J=3.6, 10.4 Hz, 8-H), 4.94, 5.17 (1H each, both s, 14-H_2), 5.47 (1H, d, J=10.4 Hz, 9-H), 6.55 (1H, dd, J=5.8, 11.6 Hz, 3-H), 7.40—7.54 (5H, m, Ph), 9.38 (1H, s, 15-C<u>H</u>O).

3c: Colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 0.79, 0.83 (3H each, both d, J=7.0 Hz, 12 and 13-H_3), 1.33 (1H, m, 7-H), 1.58 (1H, dq, J=1.8, 7.0 Hz, 11-H), 1.69 (1H, m, 6β-H), 1.95 (1H, m, 6α-H), 2.42 (1H, m, 5-H), 2.45 (1H, m, 1β-H), 2.46 (1H, m, 2-H), 2.59 (1H, ddd, J=5.8, 6.8, 12.5 Hz, 5-H), 2.93 (1H, br d-like, 1α-H), 3.08 (1H, dddd, J=5.7, 11.4, 11.7 Hz, 2-H), 3.56 (3H, s, –O<u>C</u>H_3), 4.06 (1H, dd, J=3.2, 10.1 Hz, 8-H), 5.08, 5.26 (1H each, both s, 14-H_2), 5.49 (1H, d, J=10.1 Hz, 9-H), 6.54 (1H, dd, J=6.2, 11.7 Hz, 3-H), 7.39—7.54 (5H, m, Ph), 9.38 (1H, s, 15-C<u>H</u>O).

Preparation of the (*R*)-MTPA Ester (5b) and the (*S*)-MTPA Ester (5c) from 5 A solution of **5** (2.0 mg, 6.8 μmol) in CH_2Cl_2 (1.0 ml) was treated with (*R*)-MTPA (7.9 mg, 34 μmol) in the presence of EDC·HCl (7.0 mg, 34 μmol) and 4-DMAP (2.5 mg, 20 μmol) and the mixture was heated under reflux for 6 h. The reaction mixture was poured into ice-water and the whole was extracted with AcOEt. Work-up of the AcOEt extract as described above gave a product which was purified by silica gel column chromatography (0.5 g, *n*-hexane–AcOEt=5 : 1) to furnish **5b** (1.0 mg, recovered **5**, 0.4 mg, 23%). Through a similar procedure, **5c** (1.8 mg, recovered **5**, 0.4 mg, 33%) was obtained from **5** (2.5 mg) using (*S*)-MTPA (10 mg), EDC·HCl (8.8 mg) and 4-DMAP (3.1 mg) by the same procedure.

5b: Colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 0.97, 0.99 (3H each, both d, J=6.7 Hz, 12 and 13-H_3), 1.42 (1H, m, 7-H), 1.48 (1H, m, 6β-H), 1.75 (1H, m, 11-H), 1.77 (1H, m, 6α-H), 1.84 (1H, ddd, J=3.3, 3.4, 13.8 Hz, 5-H), 2.07 (3H, s, C<u>H</u>$_3$–CO–), 2.33 (1H, m, 2-H), 2.39 (1H, m, 5-H), 2.40, 2.42 (1H each, both m, 1-H_2), 2.53 (1H, m, 2-H), 3.52 (3H, s, –O<u>C</u>H_3), 4.16 (1H, dd, J=4.9, 9.7 Hz, 8-H), 4.61, 4.66 (ABq, J=12.2 Hz, 15-H_2), 5.08, 5.16 (1H each, both s, 14-H_2), 5.20 (1H, d, J=9.7 Hz, 9-H), 5.58 (1H, dd, J=7.0, 7.0 Hz, 3-H), 7.39—7.54 (5H, m, Ph).

5c: Colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 0.92, 0.96 (3H each, both d, J=6.7 Hz, 12 and 13-H_3), 1.36 (1H, m, 7-H), 1.43 (1H, m, 6β-H), 1.73 (1H, m, 11-H), 1.76 (1H, m, 6α-H), 1.82 (1H, m, 5-H), 2.07 (3H, s, C<u>H</u>$_3$–CO–), 2.37 (1H, ddd, J=4.3, 7.0, 12.2 Hz, 2-H), 2.39 (1H, m, 5-H), 2.41 (1H, m, 1-H), 2.43 (1H, ddd, J=5.8, 7.3, 14.7 Hz, 1-H), 2.55 (1H, m, 2-H), 3.54 (3H, s, –O<u>C</u>H_3), 4.10 (1H, dd, J=4.3, 9.4 Hz, 8-H), 4.65, 4.66 (ABq, J=12.5 Hz, 15-H_2), 5.16, 5.24 (1H each, both s, 14-H_2), 5.21 (1H, d, J=9.4 Hz, 9-H), 5.58 (1H, dd, J=7.7, 9.8 Hz, 3-H), 7.39—7.54 (5H, m, Ph).

Bioassay: NO Production from Macrophages Stimulated by LPS 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 (FCS), penicillin (100 units/ml) and streptomycin (100 μg/ml), and pre-cultured 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 (more than 95% macrophages as determined by Giemsa staining) were cultured in fresh medium containing 10 μg/ml LPS and test compound (1, 3, 10, 30 and 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 by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (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 N 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). 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 by the following formula and 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 (−)].

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