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Inhibitors from the rhizomes of *Alpinia officinarum* on production of nitric oxide in lipopolysaccharide-activated macrophages and the structural requirements of diarylheptanoids for the activity

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Abstract—The 80% aqueous acetone extract from the rhizomes of *Alpinia officinarum*, a Chinese medicinal herb, were found to inhibit nitric oxide (NO) production in lipopolysaccharide (LPS)-activated mouse peritoneal macrophages. Through bioassay-guided separation, two diarylheptanoids [7-(4″-hydroxy-3″-methoxyphenyl-1-phenylhept-4-en-3-one and 3,5-dihydroxy-1,7-diphenylheptane] and a flavonol constituent (galangin) substantially inhibited LPS-induced NO production with IC_{50} values of 33–62 μM. To clarify structure–activity relationships of diarylheptanoids, related diarylheptanoids from *Curcuma zedoaria* were examined. Results indicate that the double bond or enone moiety at the 1–7 positions is important for the activity.
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1. Introduction

Nitric oxide (NO) is a short-lived free radical produced from L-arginine by nitric oxide synthase (NOS) and mediates diverse functions by acting on various cells through the interaction with different molecular targets. Although NO acts as an essential multifunctional mediator in various biological systems, excessive production of NO by inducible NOS (iNOS) is involved in various types of inflammation and multistage carcinogenesis at inflammatory sites.[1,2]

In the course of our studies on bioactive constituents from natural medicines, we found that the 80% aqueous acetone extract from the dried rhizomes of *Alpinia officinarum* HENCE showed inhibitory effects on the production of NO in lipopolysaccharide (LPS)-activated macrophages. The Zingiberaceae plant *A. officinarum* has been cultivated in southern China and Taiwan, and the rhizomes of this plant are used as a spice and as a traditional medicine for several purposes, such as stomachic and carminative, in China. In the chemical studies of the rhizomes, the isolation and structure determination of monoterpenes,[3] diarylheptanoids,[4–10] flavonoids,[11–13] phenylpropanoids,[14,15] and neolignans[16] have been reported so far. With regard to biological effects, antiemetic,[10] antioxidant,[15,16] anti-genotoxic activities,[17] and inhibitions of prostaglandin and leukotriene biosynthesis,[8,18] pancreatic lipase,[13,19] and 5α-reductase[20] have been reported. In addition, Yadav et al. reported that 7-(4″-hydroxy-3″-methoxyphenyl)-1-phenylhept-4-en-3-one (**3**), a principal constituent from this natural medicine, inhibited the production of NO stimulated by LPS in a mouse macrophage-like cell line RAW 264.7.[21] However, effects of other constituents of this natural medicines on production of NO have not been reported so far.

In the present study, we examined the effects of constituents (**1**–**9**) from the roots of *A. officinarum* on NO production in LPS-activated mouse peritoneal macrophages. In addition, to clarify structure–activity relationships of diarylheptanoids for the activity, the effects of the related diarylheptanoids (**10**–**18**) were examined.

Keywords: Nitric oxide production; Inhibitor; *Alpinia officinarum*; diarylheptanoid; Structure–activity relationships.
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2. Results and discussion

2.1. Extraction and isolation

The dried rhizomes of *A. officinarum* (3.0 kg) were extracted with 80% aqueous acetone three times at room temperature. The extract (9.6% from this natural medicine) significantly inhibited the production of NO with an IC_{50} of 35 μg/mL (Table 1).

The extract was partitioned into an ethyl acetate (EtOAc)–water mixture to furnish the EtOAc-soluble fraction (5.2%) and water-soluble fraction (4.4%). The EtOAc-soluble fraction, which showed potent activity (IC_{50} = 24 μg/mL), was subjected to normal-phase silica gel (SiO_2) [*n*-hexane → *n*-hexane–EtOAc (9:1 → 8:2 → 7:3 → 1:1 → 3:7) → EtOAc → MeOH] to give 7-(4″-hydroxy-3″-methoxyphenyl)-1-phenylhept-4-en-3-one (**3**,[5,8] 0.43%) and seven fractions (Fr. 1–7). Fractions 3–6 were subjected to reversed-phase silica-gel (ODS) column chromatography (MeOH–H_2O) and finally HPLC (YMC-Pack ODS-A, 250 × 20 mm i.d., MeOH–H_2O) to give three diarylheptanoids [5-hydroxy-1,7-diphenyl-3-heptanone (**1**,[5,7,22] 0.50%), 5-hydroxy-7-(4″-hydroxy-3″-methoxyphenyl)-1-phenyl-3-heptanone (**2**,[5,6] 0.88%), and 3,5-dihydroxy-1,7-diphenylheptane (**4**,[6] 0.0005%)], three flavonoids [galangin (**5**,[23] 1.0%), kaempheride (**6**,[10] 0.017%), and pinobaksin (**7**,[11] 0.010%)], 3-phenylpropanoic acid (**8**, 0.004%), and zingerone (**9**,[8] 0.0005%) (see Fig. 1).

2.2. Effects of the constituents and related diarylheptanoids on the production of NO

Among the constituents, two diarylheptanoids [7-(4″-hydroxy-3″-methoxyphenyl)-1-phenylhept-4-en-3-one (**3**) and 3,5-dihydroxy-1,7-diphenylheptane (**4**)] and a flavonol [galanin (**5**) substantially inhibited the production of NO with IC_{50} values of 33, 62, and 55 μM, respectively. Pinobaksin (**7**) also showed moderate activity (inhibition at 100 μM: 43.3%). In agreement with the previous study,[21] compound **3** showed the strongest activity among the isolated compounds and an enone moiety at the 3–5 positions suggested to be important for the activity [**3** (IC_{50} = 33 μM) > **2** (>100 μM)].

With regard to the inhibitory effects of diarylheptanoids on the production of NO in LPS-stimulated macrophages, various diarylheptanoids, such as 2-dihydrobis(de-*O*-methyl)curcumin and diarylheptanoids with a chalcone or flavanone moiety (calyxins A–H, epicalyxins B–D, hydroxycalyxin F, and blepharocalyxins A and B) from *Alpinia blepharocalyx*,[24] oregonin and hirsutanonol from *Alnus hirsute*,[25] and curcumin (**10**) from *Curcuma longa*[26,27] were reported to be active principles. In our previous studies, curcumin (**10**) and bisdemethoxy-

Table 1. Inhibitory effects of the 80% aqueous acetone extract and its fractions and constituents (**1**–**9**) from the rhizomes of *A. officinarum* and related diarylheptanoids (**10**–**18**) on NO production in LPS-activated mouse peritoneal macrophages

	Concentration of test sample (μg/mL)						
	0	1	3	10	30	100	IC_{50} (μg/mL)
aq Acetone extract	0.0 ± 7.0	0.9 ± 5.1	4.7 ± 5.1	24.0 ± 1.3**	54.5 ± 3.9**	99.7 ± 0.1**,a	35
EtOAc-soluble fraction	0.0 ± 4.8	−1.0 ± 4.8	−2.3 ± 5.0	19.2 ± 2.1*	58.1 ± 4.7**	101.4 ± 0.1**,a	24
H_2O-soluble fraction	0.0 ± 5.3	−9.9 ± 8.0	3.0 ± 2.1	−9.7 ± 5.2	5.0 ± 1.1	−3.1 ± 6.5	—
	Concentration of test sample (μM)						
	0	1	3	10	30	100	IC_{50} (μM)
5-Hydroxy-1,7-diphenyl-3-heptanone (**1**)	0.0 ± 5.7	0.0 ± 7.3	−6.2 ± 5.8	−1.5 ± 4.7**	−1.2 ± 5.0	31.3 ± 1.3**	—
5-Hydroxy-7-(4″-hydroxy-3″-methoxyphenyl)-1-phenyl-3-heptanone (**2**)	0.0 ± 6.1	4.4 ± 3.5	15.2 ± 5.6	3.7 ± 5.6	1.7 ± 2.8	32.0 ± 2.9**	
7-(4″-Hydroxy-3″-methoxyphenyl)-1-phenylhept-4-en-3-one (**3**)	0.0 ± 5.4	−1.9 ± 2.4	4.8 ± 3.2	10.1 ± 4.0	28.3 ± 1.0**	94.4 ± 1.2**	33
3,5-Dihydroxy-1,7-diphenylheptane (**4**)	0.0 ± 4.1	−0.5 ± 7.9	2.0 ± 7.0	5.3 ± 5.0	20.3 ± 5.2*	72.7 ± 0.8**	62
Galangin (**5**)	0.0 ± 0.6	−4.2 ± 1.1	2.6 ± 2.6	−1.1 ± 4.3	10.6 ± 1.5*	85.5 ± 2.5**	55
Kaempheride (**6**)	0.0 ± 6.7	−5.6 ± 1.8	−3.8 ± 4.5	6.2 ± 1.8	27.6 ± 3.0**	23.0 ± 3.7**	—
Pinobaksin (**7**)	0.0 ± 5.0	−5.9 ± 3.8	6.6 ± 3.9	−1.2 ± 2.5	13.7 ± 4.6	43.3 ± 2.4**	—
3-Phenylpropanoic acid (**8**)	0.0 ± 2.7	−9.0 ± 4.8	−8.3 ± 6.3	1.3 ± 6.7	1.2 ± 3.3	11.6 ± 3.9	—
Zingerone (**9**)	0.0 ± 2.9	−6.3 ± 1.8	4.0 ± 5.7	9.1 ± 1.9	13.3 ± 4.6	29.4 ± 6.8**	—
Curcumin (**10**)	0.0 ± 2.1	5.2 ± 1.1	10.0 ± 1.2**	36.7 ± 3.0**	94.3 ± 1.9**	99.5 ± 0.0**,a	11
Dihydrocurcumin (**11**)	0.0 ± 2.0	−0.1 ± 4.5	0.3 ± 2.1	4.7 ± 2.7	58.2 ± 3.2**	100.5 ± 0.1**,a	25
Tetrahydrodemethoxycurcumin (**12**)	0.0 ± 2.2	2.4 ± 2.1	0.0 ± 1.5	1.9 ± 1.7	8.6 ± 3.0*	45.9 ± 1.2**	—
Tetrahydrobisdemethoxycurcumin (**13**)	0.0 ± 1.7	1.3 ± 6.2	−0.3 ± 4.2	0.5 ± 3.1	7.3 ± 2.9	30.3 ± 0.7**	—
Bisdemethoxycurcumin (**14**)	0.0 ± 1.1	1.2 ± 1.7	8.6 ± 2.2	30.7 ± 4.5**	93.6 ± 0.9**	99.0 ± 0.2**,a	14
Tetrahydrocurcumin (**15**)	0.0 ± 2.2	2.7 ± 5.5	4.8 ± 6.0	6.1 ± 4.2	9.2 ± 2.7	58.5 ± 0.9**	ca. 90
(±)-Hexahydrocurcumin (**16**)	0.0 ± 4.1	8.5 ± 4.8	2.7 ± 3.7	−4.5 ± 5.8	13.0 ± 1.3	25.6 ± 2.7**	—
Monomethylcurcumin (**17**)	0.0 ± 4.5	8.8 ± 3.7	8.1 ± 4.0	30.8 ± 1.8**	78.5 ± 1.8**	96.2 ± 0.3**,a	14
Dimethylcurcumin (**18**)	0.0 ± 1.3	5.7 ± 5.2	8.2 ± 1.6	25.0 ± 0.4**	57.7 ± 1.5**	93.4 ± 0.5**	18
L-NMMA	0.0 ± 4.0	5.9 ± 0.9	10.3 ± 3.7	15.0 ± 1.6**	34.1 ± 3.2	63.1 ± 1.2**	57
CAPE	0.0 ± 0.7	3.8 ± 0.1	1.4 ± 0.1	68.2 ± 0.0**	93.7 ± 0.2**	99.6 ± 0.0**,a	15

Values represent means ± SEM (N = 4). Significantly different from controls, $^{*}p < 0.05$, $^{**}p < 0.01$.

[a] Cytotoxic effect was observed.

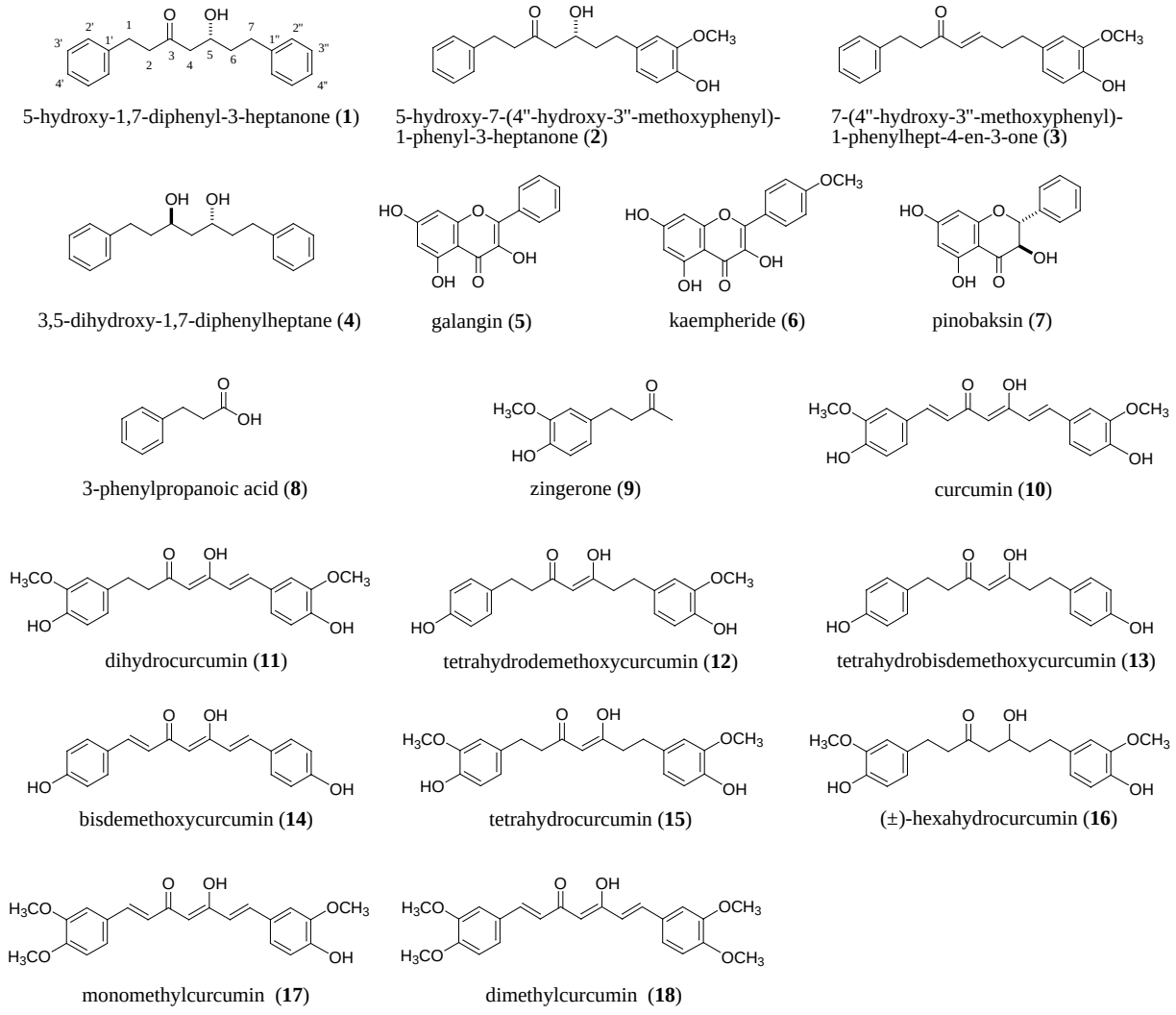

Figure 1. Chemical structures of compounds **1**–**9** isolated from the rhizomes of *A. officinarum* and related diarylheptanoids **10–18**.

curcumin (**14**) from *Curcuma zedoaria*, cyclic diarylheptanoids [myricanol, (+)-*S*-myricanol, myricanone, myricanenes A and B, myricanane, and acerogenins A, B, E, and K] from *Myrica rubra* and *Acer nikoense* were reported as active constituents.[27–29] However, the effects of these compounds have not been compared in the same experimental conditions and their structure–activity relationships have been discussed insufficiently.

To clarify the structural requirements of diarylheptanoids for the activity, effects of curcumin (**10**), dihydrocurcumin (**11**), tetrahydrodemethoxycurcumin (**12**), tetrahydrobisdemethoxycurcumin (**13**), and bisdemethoxycurcumin (**14**) isolated from Thai and Chinese zedoary,[27,30] and tetrahydrocurcumin (**15**), (±)-hexahydrocurcumin (**16**), monomethylcurcumin (**17**), and dimethylcurcumin (**18**) derived from **10**[30] were examined.

As a result, the inhibitions of NO production by 5-hydroxy-1,7-diphenyl-3-heptanone (**1**) lacking the hydroxyl and methoxyl groups in the benzene rings, 5-hydroxy-7-(4″-hydroxy-3″-methoxyphenyl)-1-phenyl-3-heptanone (**2**) having the 4″-hydroxyl and 3″-methoxyl groups, and (±)-hexahydrocurcumin (**16**) having the 4′,4″-hydroxyl and 3′,3″-methoxyl groups were similar values (inhibitions by **1**, **2**, and **16** at 100 μM were 31%, 32%, and 26%, respectively), and curcumin (**10**) and bisdemethoxycurcumin (**14**) lacking the 3′,3″-methoxyl groups also showed similar activities with IC_{50} values of 11 and 14 μM, respectively. These findings suggest that substitution of the 3′,3″-methoxyl and 4′,4″-hydroxyl groups in benzene rings is not so important. However, methylation of the 4′,4″-hydroxyl group tended to reduce the activity [**10** (11 μM) > **17** (14 μM) > **18** (18 μM)].

Pan et al. reported that tetrahydrocurcumin (**15**), (±)-hexahydrocurcumin (**16**), and octahydrocurcumin showed less activity than curcumin (**10**) in LPS-activated RAW 264.7 cells.[26] In agreement with this report, as double bonds at the 1–7 positions of curcumin (**10**) were hydrogenated, the activities were reduced [**10** (11 μM) > **11** (25 μM) > **15** (ca. 90 μM) > **16** (>100 μM)]. These findings indicate that the double bonds and/or enone moiety at the 1–7 positions are important for the activity.

With regard to mechanisms of the action of diarylheptanoids, 7-(4″-hydroxy-3″-methoxyphenyl)-1-phenylhept-4-en-3-one (**3**) and curcumin (**10**) were reported to suppress the activation of nuclear factor-κB (NFκB),[21,26] but the detailed mechanism of action of the active phenylpropanoids should be studied further.

3. Experimental section

3.1. General

Specific rotations: Horiba SEPA-300 digital polarimeter (l = 5 cm). UV spectra: Shimadzu UV-1600 spectrometer. IR spectra: Shimadzu FTIR-8100 spectrometer. EIMS and HREIMS: JEOL JMS-GCMATE mass spectrometer. FABMS and HRFABMS: JEOL JMS-SX 102A mass spectrometer. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra: JEOL JNM-LA500, and ^{1}H NMR (270 MHz) and ^{13}C NMR (68 MHz) spectra: JEOL JNM-EX270 spectrometers with tetramethylsilane as an internal standard. Normal-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) (normal-phase) and Silica gel RP-18 F_{254S} (Merck, 0.25 mm) (reversed-phase). HPTLC: pre-coated TLC plates with Silica gel RP-18 WF_{254S} (Merck, 0.25 mm) (reversed-phase). The spots were detected by spraying with 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4, followed by heating on a hot plate. HPLC was performed on a Shimadzu LC-6AD apparatus equipped with a Shimadzu RID-6A refractive index detector. YMC-Pack ODS-A (250 × 4.6 mm i.d.) and (250 × 20 mm i.d.) columns were used for analytical and preparative purposes, respectively.

3.2. Plant material

The dried rhizomes of *A. officinarum* cultivated in China were obtained from Tochimoto Tenkaido Co. Ltd. (Osaka, Japan) and were identified by one of the authors (M.Y.). A voucher of the plant is on file in our laboratory.

3.3. Extraction and isolation

The dried rhizomes of *A. officinarum* (3.0 kg) were finely cut and extracted with 80% aqueous acetone three times at room temperature. Evaporation of the solvent under reduced pressure provided the 80% aqueous acetone extract (228 g, 9.6%). The extract (221.4 g) was partitioned into an EtOAc–H_2O (1:1, v/v) mixture, and removal of the solvent in vacuo from the EtOAc- and H_2O-soluble portions yielded 119.8 g (5.2%) and 101.6 g (4.4%). The EtOAc-soluble fraction (100.0 g) was subjected to normal-phase SiO_2 [3.0 kg, *n*-hexane → *n*-hexane–EtOAc (9:1 → 8:2 → 7:3 → 1:1 → 3:7) → EtOAc → MeOH] to give 7-(4″-hydroxy-3″-methoxyphenyl)-1-phenylhept-4-en-3-one (**3**, 8.21 g, 0.43%) and seven fractions [Fr. 1 (0.80 g), Fr. 2 (7.9 g), Fr. 3 (18.5 g), Fr. 4 (25.7 g), Fr. 5 (22.6 g), Fr. 6 (7.5 g), and Fr. 7 (11.7 g)]. Fraction 3 (18.5) was subjected to ODS column chromatography [MeOH–H_2O (1:1 → 7:3 → 9:1) → MeOH] to give 5-hydroxy-1,7-diphenyl-3-heptanone (**1**, 9.53 g, 0.50%). Fraction 4 (25.7 g) was subjected to ODS column chromatography [MeOH–H_2O (6:4 → 7:3 → 8:2 → 9:1) → MeOH] to give galangin (**5**, 19.4 g, 1.0%) and four fractions [Fr. 4-1 (98 mg), Fr. 4-2 (524 mg), Fr. 4-3 (4.1 g), and Fr. 4-4 (1.1 g)]. Fractions 4-1 (98 mg) and 4-2 (524 mg) were separated further by HPLC [YMC-Pack ODS-A, 250 × 20 mm i.d., MeOH–H_2O (4:6) for Fr. 4-1 and MeOH–H_2O (1:1) for Fr. 4-2] to give zingerone (**9**, 10 mg, 0.0005%) from Fr. 4-1 and pinobaksin (**7**, 197 mg, 0.010%) and 3-phenylpropanoic acid (**8**, 84 mg, 0.004%) from Fr. 4-2. Fraction 5 (22.6 g) was subjected to ODS column chromatography [MeOH–H_2O (6:4 → 7:3 → 8:2 → 9:1) → MeOH] to give 5-hydroxy-7-(4″-hydroxy-3″-methoxyphenyl)-1-phenyl-3-heptanone (**2**, 16.9 g, 0.88%) and five fractions [Fr. 5-1 (0.50 g), Fr. 5-2 (0.60 g), Fr. 5-3 (1.79 g), Fr. 5-4 (0.93 g), and Fr. 5-5 (0.52 g)]. Fraction 5-2 (0.60 g) was repeatedly separated by ODS column chromatography and finally HPLC [YMC-Pack ODS-A, 250 × 20 mm i.d., MeOH–H_2O (3:1)] to give 3,5-dihydroxy-1,7-diphenylheptane (**4**, 9.5 mg, 0.0005%) and kaempheride (**6**, 323 mg, 0.017%).

Known compounds **1**–**9** were identified by comparison of their physical data ([α]$_D$, IR, ^{1}H NMR, ^{13}C NMR, and MS) with reported values[5–8,10,11,22,23] or those of a commercially obtained sample (**8**).

3.4. Related compounds

Curcumin (**10**), dihydrocurcumin (**11**), tetrahydrodemethoxycurcumin (**12**), tetrahydrobisdemethoxycurcumin (**13**), and bisdemethoxycurcumin (**14**) were isolated from Chinese and Thai zedoary,[27,30] and tetrahydrocurcumin (**15**), (±)-hexahydrocurcumin (**16**), monomethylcurcumin (**17**), and dimethylcurcumin (**18**) were derived from **10** as described in the previous report.[30]

3.5. Bioassay methods

3.5.1. Reagents. Lipopolysaccharide (LPS, from *Salmonella enteritidis*) and N^G-monomethyl-L-arginine (L-NMMA) were purchased from Sigma; 3-(4,5-dimethyl-2-thiazolyl) 2,5-diphenyl tetrazolium bromide (MTT) was from Dojin; RPMI 1640 was from Sigma; fetal calf serum (FCS) was from Gibco; thioglycolate (TGC) medium was from Nissui Seiyaku; other reagents were from Wako Pure Chemical.

3.5.2. Effects on the production of NO in LPS-stimulated macrophages. Screening test for NO production using TGC-induced mouse peritoneal macrophages was performed, as described previously.[31] Briefly, peritoneal exudate cells (5×10^5 cells per well) were collected from the peritoneal cavities of male ddY mice and were suspended in 100 μL RPMI 1640 supplemented with 5% fetal calf serum (FCS), penicillin (100 U/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 with PBS, and the adherent cells were cultured in fresh medium (200 μL) containing 10 μg/mL LPS and various concentrations of the test compound for 20 h. NO production in each well was assessed by measuring the accumulation of nitrite (NO_2^-) in the culture medium using the Griess reagent. Cytotoxicity was determined by the MTT colorimetric assay, after a 20-h incubation with test compounds. Each test compound was dissolved in DMSO, and the solution was added to the medium (final DMSO concentration was 0.5%). Inhibition (%) was calculated using 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 (−)].

3.5.3. Statistics. Values are expressed as means ± SEM. One-way analysis of variance, followed by Dunnett's test, was used for statistical analysis.

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