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Labdane-type Diterpenes with Inhibitory Effects on Increase in Vascular Permeability and Nitric Oxide Production from *Hedychium coronarium*

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Abstract—The methanolic extract from the rhizome of *Hedychium coronarium* was found to inhibit the increase in vascular permeability induced by acetic acid in mice and nitric oxide production in lipopolysaccharide-activated mouse peritoneal macrophages. From the methanolic extract, three new labdane-type diterpenes, hedychilactones A, B, and C, were isolated together with six known diterpenes. The structures of hedychilactones were elucidated on the basis of chemical and physicochemical evidence. The diterpene constituents showed inhibitory effects on the increase in vascular permeability, nitric oxide production, and inducible nitric oxide synthase induction. © 2002 Published by Elsevier Science Ltd.

Introduction

The Zingiberaceae plant *Hedychium coronarium* KOEN., which has many common names including butterfly ginger, butterfly lily, cinnamon jasmine, garland flower, and ginger lily, is widely cultivated in India, Southeast Asian countries, South China, Taiwan, Japan, and Brazil, and so on. The rhizome of *H. coronarium* is used in Chinese natural medicine, and has been prescribed for the treatment of headache, lancinating pain, contusion inflammatory, and sharp pain due to rheumatism in Chinese traditional preparations, while it is used as a febrifuge, tonic, excitant, and anti-rheumatic in the Ayurvedic system of traditional Indian medicine. In chemical constituents of this plant, several labdane-type diterpenes were isolated from Brazilian *H. coronarium*.[1–3] As pharmacological studies of this natural medicine, it was reported that the chloroform extract and some diterpene constituents showed cytotoxic activity on Chinese hamster V-79 cells,[1] although no pharmacological studies for the traditional medicinal application have been reported.

In the course of our studies to characterize bioactive constituents from Zingiberaceae herbal medicines,[4] we found that the methanolic extract of the rhizome of *H. coronarium* showed inhibitory effects on the vascular permeability induced by acetic acid (AcOH) in mice and nitric oxide (NO) production in lipopolysaccharide (LPS)-activated mouse peritoneal macrophages. From the methanolic extract, we have isolated three new labdane-type diterpenes named hedychilactones A (**1**), B (**2**), and C (**3**) together with six known diterpenes, three sesquiterpenes, and a flavonol from the fresh rhizome of *H. coronarium*. This paper describes elucidation of the structures of hedychilactones (**1**–**3**) and antiinflammatory effects of the principal labdane-type diterpene constituents, coronarins D (**4**) and its methyl ether (**5**), on the increase in vascular permeability induced by AcOH in mice. In addition, we describe the inhibitory effects of labdane-type diterpene constituents including hedychilactones (**1**–**3**) against NO production and/or induction of inducible nitric oxide synthase (iNOS) in LPS-activated mouse peritoneal macrophages.

Results and Discussion

Fresh rhizomes of *H. coronarium* cultivated in Kagawa Prefecture (Japan) were extracted with methanol under reflux. The methanolic extract (1.5% from the fresh rhizome) was found to exhibit inhibitory effects on NO production in LPS-activated mouse peritoneal

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macrophages, the methanolic extract was partitioned in an ethyl acetate (AcOEt)–H_2O (1:1) mixture to give an AcOEt-soluble fraction (0.5%) and H_2O-soluble fraction (1.0%). As shown in Table 1, the AcOEt-soluble fraction showed potent inhibitory activity, whereas the H_2O-soluble fraction showed only weak activity. The AcOEt-soluble fraction was subjected to ordinary- and reversed-phase silica gel column chromatography and finally HPLC to furnish nine labdane-type diterpenes, hedychilactones A (**1**, 0.00021%), B (**2**, 0.00023%), and C (**3**, 0.00045%), coronarins D (**4**,[1] 0.0083%), D methyl ether (**5**,[2] 0.0022%), and E (**6**,[3] 0.0011%), labda-8(17),13(14)-dien-15,16-olide (**7**,[5] 0.00006%), hedychenone (**8**,[6] 0.00090%), and 7-hydroxyhedychenone (**9**,[7] 0.00027%), three farnesane-type sesquiterpene, (+)-nerolidol (**10**,[8] 0.0042%), hedychiols A (**11**,[9] 0.00084%) and B 8,9-diacetate (**12**,[9] 0.000086%) and a flavonol, 5-hydroxy-3,7,4′-trimethoxyflavone (**13**,[10] 0.00050%) (Chart 1).

Stereostructures of hedychilactones A–C (1–3)

Hedychilactone A (**1**) was isolated as a colorless oil with positive optical rotation ($[\alpha]_D^{22}$ +12.3°). Electron impact (EI)-MS of **1** showed a molecular ion (M^+) peak at *m*/*z* 318 in addition to a fragment ion peak at *m*/*z* 300 (M^+-H_2O). The molecular formula of **1**, $C_{20}H_{30}O_3$, was determined from the molecular ion peak observed

Table 1. Inhibitory effects of MeOH extract and AcOEt- and H_2O-soluble fractions from *Hedychium coronarium* on NO production in LPS–activated mouse peritoneal macrophages

	Inhibition (%)					IC_{50} (μg/mL)
	0 μg/mL	3 μg/mL	10 μg/mL	30 μg/mL	100 μg/mL	
MeOH extract	0.0±2.0	5.5±2.7	1.4±8.2	38.1±6.3**	76.0±0.9**	45
AcOEt-soluble fraction	0.0±6.0	13.8±6.7	40.6±2.3**	80.3±1.5**	106.2±1.3[a].**	13
H_2O-soluble fraction	0.0±5.1	4.7±4.2	−10.3±3.9	5.4±4.9	18.3±3.7*	>100

Each value represents the mean±SEM ($N=4$). Significantly different from the control: *$p<0.05$, **$p<0.01$.
[a]Cytotoxic effect was observed.

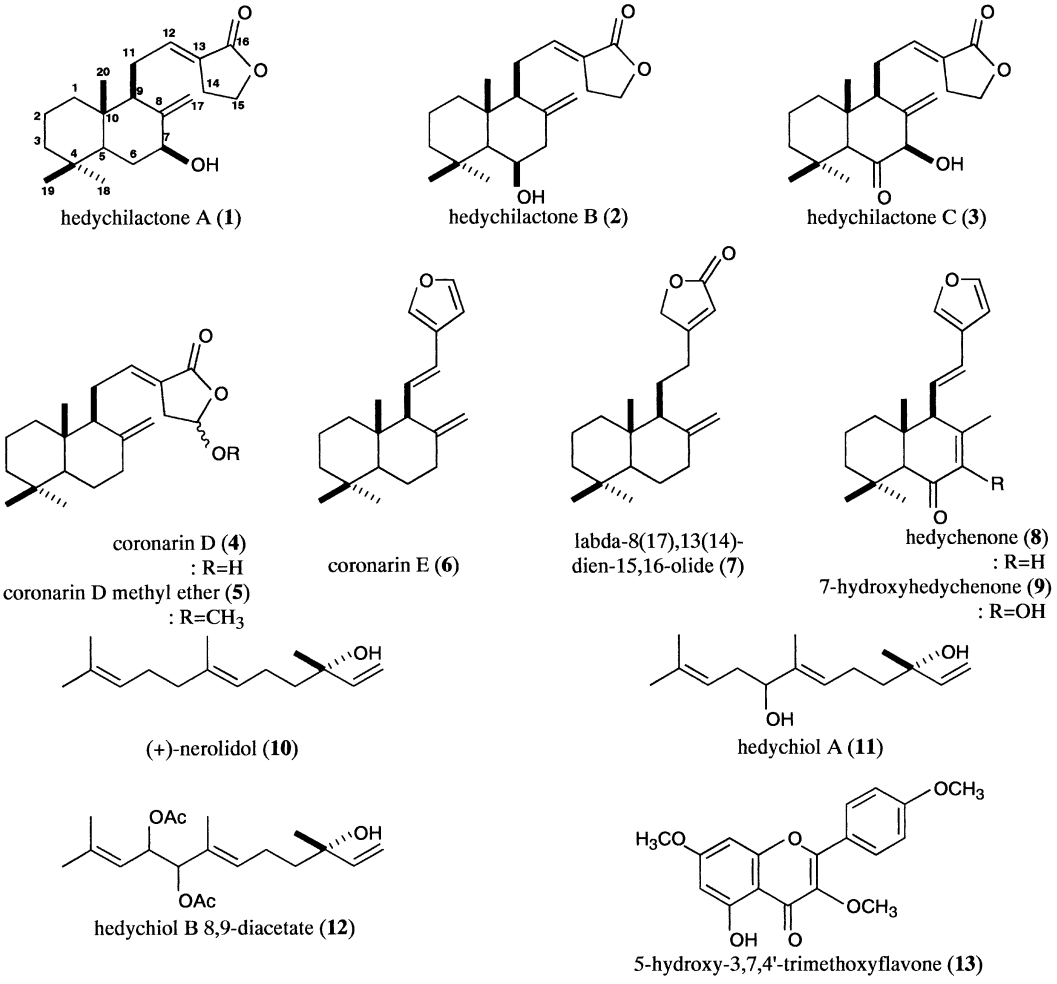

Chart 1.

on EI–MS and by high-resolution MS measurement. The IR spectrum of **1** showed absorption bands ascribable to hydroxyl, lactone carbonyl, and olefin functions at 3393, 1752 and 1676 cm^{-1}, respectively, while the absorption maximum of the conjugated enone chromophore was observed at 226 (log ε 4.08) nm in the UV spectrum. The ^1H NMR ($CDCl_3$) and ^{13}C NMR (Table 2) spectra of **1** showed signals assignable to three methyls [δ 0.72, 0.83, 0.92 (both s, 20, 19, and 18-H_3)], a methylene [δ 4.39 (t-like, 15-H_2)] and a methine bearing a hydroxyl group [δ 4.00 (dd-like, 7-H)], an *exo*-methylene [δ 4.58, 5.20 (both br s, 17-H_2)], and an olefin [δ 6.68 (m, 12-H)] together with six methylenes (1, 2, 3, 6, 11, 14-H_2), two methines (5, 9-H), and five quaternary carbons (4, 8, 10, 13, 16-C).

The planar structure of **1** was constructed on the basis of ^1H–^1H correlation spectroscopy (H–H COSY) and heteronuclear multiple bond correlation (HMBC) experiments. Thus, the H–H COSY experiment on **1** indicated the presence of four partial structures shown as bold lines in Figure 1 (1-C–3-C, 5-C–7-C, 9-C–12-C and 14-C–15-C). In the HMBC experiment, long-range correlations were observed between the following protons and carbons of **1** (3-H_2, 5-H, 18-H_3, 19-H_3 and 4-C; 1-H_2, 9-H, 20-H_3 and 10-C; 6-H, 9-H and 8-C; 14-H_2, 15-H_2 and 13-C), so that the connectivities of the quaternary carbons (4, 8, 10, 13-C) in **1** were clarified. The above observations confirmed the skeleton of hedychilactone A (**1**) to be 7-hydroxy-8(17),12-labdadien-16,15-olide.

The relative structure of **1** was characterized on the basis of the nuclear Overhauser effect spectroscopy (NOESY) experiment shown in Figure 1, in which NOE correlations were observed between the following proton pairs of **1** (19-H_3 and 20-H_3; 5-H and 7-H, 9-H, 18-H_3).

Next, the absolute stereostructure of **1** was determined by the following procedure. To characterize the geometry of the conjugated enone position, **1** was treated with diisobutylalminum hydride (DIBAL) to give the reductant (**1a**). As NOE correlation of **1a** was observed between the 12-olefin proton [δ 5.48 (dd, $J = 6.4$, 6.7 Hz, 12-H)] and the 16-hydroxymethyl proton [δ 4.01 (s, 16-H_2)] as shown in Figure 2, the geometry of the conjugated enone position in **1a** was confirmed to be 12*E*-form. In addition, the absolute configuration on the 7-hydroxyl position of **1** was determined by application of the allylic beznoate rule[11] for the 7-*O*-*p*-bromobenzoate derivative (**1b**), prepared by treatment of **1** with *p*-bromobenzoyl chloride in the presence of 4-dimethylaminopyridine (4-DMAP). As shown in Figure 2, the CD spectrum of **1b** showed positive Cotton effects at 245 nm (Δε +6.71) and 226 nm (Δε −2.25), indicating the absolute configuration of the 7-position to be *S* orientation. The structure of hedychilactone A (**1**) was determined on the basis of the above observations.

Hedychilactone B (**2**) was isolated as a colorless oil with positive optical rotation ($[\alpha]_D^{28}$ +10.6°). The molecular formula $C_{20}H_{30}O_3$ of **2** was determined from the quasi-molecular ion peak at m/z 319 $(M+H)^+$ on positive-ion fast atom bombardment (FAB)-MS and by high-resolution MS measurement, which was the same on that of **1**. The IR spectrum of **2** showed absorption bands at 3496, 1750, and 1674 cm^{-1} ascribable to hydroxyl, lactone carbonyl, and olefin functions. In addition, the UV

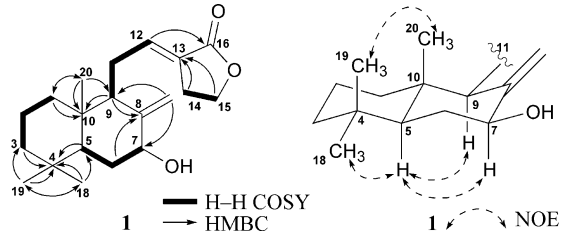

Figure 1.

Table 2. ^{13}C NMR data for hedychilactones A (**1**), B (**2**), and C (**3**)

	1	2	3
C-1	39.1	42.2	38.8
C-2	19.3	19.5	18.8
C-3	41.8	43.8	42.2
C-4	33.5	34.4	32.7
C-5	53.0	57.3	64.1
C-6	33.5	69.1	207.9
C-7	73.6	47.3	79.9
C-8	150.1	143.8	145.7
C-9	54.4	56.8	53.7
C-10	39.2	40.6	42.4
C-11	25.2	25.4	25.4
C-12	141.5	141.6	140.2
C-13	124.9	124.9	125.7
C-14	25.3	25.2	25.3
C-15	65.3	65.3	65.3
C-16	171.2	171.2	171.1
C-17	104.3	111.2	107.7
C-18	33.5	33.6	32.5
C-19	21.6	23.6	21.9
C-20	14.4	17.1	15.6

Measured in $CDCl_3$, at 125 MHz.

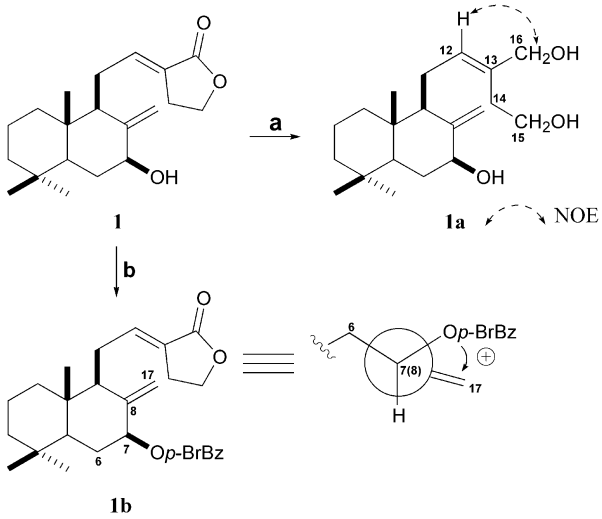

Figure 2. Reagents and conditions: (a) DIBAL/CH_2Cl_2, 0 °C, 30 min, quant.; (b) *p*-BrBzCl, 4-DMAP/pyridine, rt, 24 h, 80%.

spectrum showed an absorption maximum at 227 nm (log ε 4.08) ascribable to a conjugated enone chromophore. The ^{1}H NMR ($CDCl_3$) and ^{13}C NMR (Table 2) spectra of **2** showed the same functional groups as those of **1**: three methyls [δ 1.02, 1.05, 1.22 (all s, 18, 20, and 19-H_3)], a methylene [δ 4.39 (t-like, 15-H_2)] and a methine bearing a hydroxyl group [δ 4.40 (ddd, J = 0.6, 7.0, 7.3 Hz, 6-H)], an *exo*-methylene [δ 4.67, 5.00 (both br s, 17-H_2)], and an olefin [δ 6.72 (m, 12-H)] together with six methylenes (1, 2, 3, 7, 11, 14-H_2), two methines (5, 9-H), and five quaternary carbons (4, 8, 10, 13, 16-C). The partial structure of **2** shown in bold was clarified by H–H COSY and long-range correlations were observed between the following protons and carbons of **2** (3-H, 5-H, 18-H_3, 19-H_3 and 4-C; 7-H, 9-H and 8-C; 1-H_2, 9-H, 20-H_3 and 10-C; 14-H_2 and 13-C) in the HMBC experiment (Fig. 3). In addition, the relative stereostructure of **2** was elucidated on the basis of NOE correlation between the following proton pairs (19-H_3 and 20-H_3; 5-H and 6-H, 9-H, 18-H_3). Furthermore, the geometric structure of **2** was determined by the same procedure as described for **1**. Treatment of **2** with DIBAH gave 6,15,16-triol derivative (**2a**), and NOE correlation was observed between the 12-olefin proton [δ 5.53 (dd, J = 6.4, 6.4 Hz, 12-H)] and 16-hydroxymethyl proton [δ 4.03 (2H, s, 16-H_2)] pair as shown in Figure 3.

Hedychilactone C (**3**) was isolated as a colorless oil with positive optical rotation ($[\alpha]_D^{23}$ +23.8°). The molecular formula $C_{20}H_{28}O_4$ of **3** was determined from the molecular ion peak at m/z 332 (M^+) in addition to the fragment ion peak at m/z 314 ($M^+ - H_2O$) in the EI–MS and by high-resolution MS measurement. In the UV spectrum of **3**, an absorption maximum was observed at 222 nm (log ε 3.92), suggestive of a conjugated enone chromophore. The IR spectrum of **3** showed absorption bands ascribable to hydroxyl, lactone carbonyl, carbonyl, and olefin functions at 3492, 1750, 1717, and 1675 cm^{-1}, respectively. The ^{1}H NMR ($CDCl_3$) and ^{13}C NMR (Table 2) spectra of **3** showed signals assignable to three tertiary methyls [δ 0.67, 1.00, 1.26 (all s, 20, 18, and 19-H_3)], a methylene [δ 4.42 (t-like, 15-H_2)] and a methine bearing a hydroxyl group [δ 4.49 (br s, 7-H)], an *exo*-methylene [δ 4.67, 5.44 (both br s, 17-H_2)], and an olefin [δ 6.75 (m, 12-H)], which were superimposable on those of **1**, except for the signals due to the carbonyl group [δ c 207.9 (6-C)]. Next, the connectivities of the ^{1}H–^{1}H and the quaternary carbons in **3** was clarified by H–H COSY and HMBC experiments as shown in Figure 4. Furthermore, NOE correlations were observed between the following proton pairs of **3**: 19-H_3 and 20-H_3; 5-H and 7-H, 9-H, 18-H_3. On the basis of these observations, the stereostructure of **3** was determined to be the 6-oxo-hedychilactone A.

Effects on the increase in vascular permeability induced by acetic acid in mice

The development of the increase in vascular permeability induced by acetic acid is known to correspond to the early exudative stage of inflammation, one of the most important processes in inflammatory pathology.[12] Histamine and serotonin are presumed to play an important roles in the first stage of the acetic acid-induced increase of vascular permeability. In the course of characterization studies on antiinflammatory principles, we reported several natural medicines using the above screening test.[13] To clarify the antiinflammatory activity on this herbal medicine, we examined the effects of the methanolic extract and principal labdane-type diterpene constituents, coronarins D (**4**) and D methyl ether (**5**), on the acetic acid-induced increase in vascular permeability in mice. As shown in Table 3, the methanolic extract (250–500 mg/kg, po) and **4** and **5** (25–50 mg/kg, po) dose-dependently inhibited the leakage of dye.

Inhibitory effects on NO production and iNOS induction in LPS-activated mouse peritoneal macrophages

The inorganic free radical NO 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 NO synthase (NOS). In the family of NOS, iNOS in particular is involved in pathological aspects 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 cell types including macrophages, endothelial cells, and smooth muscle cells.

As a part of our characterization studies on bioactive components of natural medicines, we previously reported various NO production inhibitors: higher unsaturated

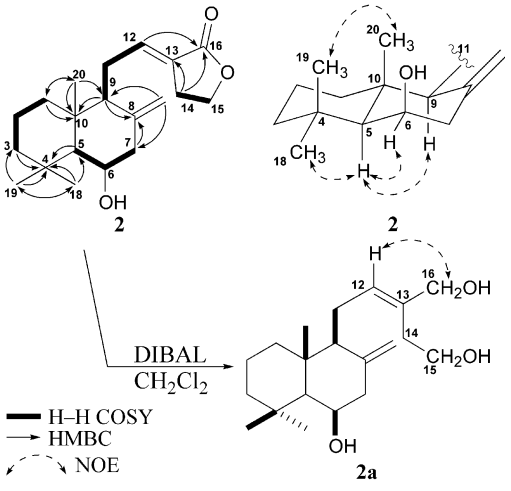

Figure 3.

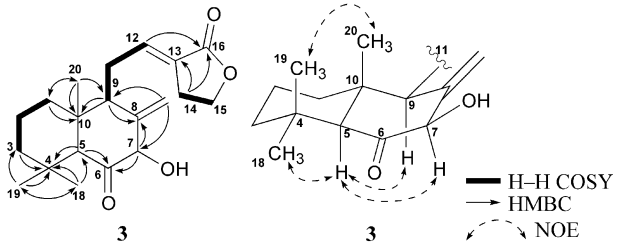

Figure 4.

fatty acids,[14] polyacetylenes,[15,16] coumarins,[15] flavonoids,[16] stilbenes,[17] lignans,[18] sesquiterpenes,[4c,4f,4 h,4i,16,19] and triterpenes.[20] As a continuation of these studies, the effects of the constituents from *H. coronarium* on NO production from LPS-activated macrophages were examined, and the results were summarized in Table 4. The isolated constituents (**1**–**10**, **12**) significantly inhibited the accumulation of nitrite, a product of NO, in the medium. Among them, five labdane-type diterpenes, hedychilactone A (**1**, $IC_{50}=18$ μM), coronarins D (**4**, 16 μM) and D methyl ether (**5**, 21 μM), labda-8(17),13(14)-dien-15,16-olide (**7**, 15 μM), and hedychenone (**8**, 7.9 μM) showed stronger inhibitory effects.

Next, the effects of four active constituents (**1**, **4**, **7**, **8**) on iNOS induction were examined. iNOS was detected at 130 kDa after a 12-h incubation with LPS by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)-Western blotting analysis (Fig. 5).[17–19] iNOS induction of LPS-activated macrophages was shown to be suppressed by these four active constituents (**1**, **4**, **7**, **8**) in a concentration-dependent manner. These results suggested that labdane-type diterpene constituents (**1**, **4**, **7**, **8**) inhibited NO production due to their inhibitory activities against induction of iNOS in LPS-activated macrophages.

The inhibitory activities of these components against the acetic acid-induced increase in vascular permeability and/or NO production in LPS-activated macrophages substantiated the traditional effects of this herbal medicine for the treatment of inflammation.

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; 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); reversed-phase silica gel column chromatography, Chromatorex ODS DM1020T

Table 3. Effects of MeOH extract, coronarins D (**4**), and D methyl ether (**5**) from *Hedychium coronarium* on the increase in vascular permeability induced by acetic acid in mice

	Dose (mg/kg, po)	*N*	Amount of leaking dye (μg/animal)
Control	—	9	252.6±15.8
MeOH extract	250	8	267.4±11.4
	500	7	195.1±14.9*
Control	—	11	223.9±11.7
Coronarin D (**4**)	12.5	7	188.0±15.8
	25	7	166.9±13.9*
	50	8	145.6±7.7**
Coronarin D methyl ether (**5**)	25	7	215.0±16.3
	50	7	166.0±16.4*
Aminopyrine	100	7	91.6±10.7**

Each value represents the mean±SEM. Significantly different from the control: *$p<0.05$, **$p<0.01$.

Table 4. Inhibitory effects of constituents from *Hedychium coronarium* on NO production in LPS–activated mouse peritoneal macrophages

	Inhibition (%)						IC_{50} (μM)
	0 μM	1 μM	3 μM	10 μM	30 μM	100 μM	
Hedychilactone A (**1**)	0.0±0.9	5.2±2.2	4.4±4.9	27.2±3.2**	62.6±4.7**	70.6±1.3**	18
Hedychilactone B (**2**)	0.0±3.8	−4.2±8.2	10.4±1.5	18.9±2.1*	53.1±2.5**	69.2±1.5[a,]**	28
Hedychilactone C (**3**)	0.0±4.5	−0.7±1.4	4.1±4.4	10.0±3.2	5.9±2.7	51.7±3.2**	ca. 97
Coronarin D (**4**)	0.0±1.2	−8.2±2.2	−8.8±1.7	23.2±6.6**	89.4±1.5**	101.9±0.2[a,]**	16
Coronarin D methyl ether (**5**)	0.0±4.5	−1.4±1.6	4.1±3.5	16.9±1.9**	72.0±2.9**	98.6±1.1[a,]**	21
Coronarin E (**6**)	0.0±1.1	−15.3±1.1	−2.7±3.7	9.2±3.9	47.3±2.8**	93.3±0.3**	32
Labda-8(17),13(14)-dien-15,16-olide (**7**)	0.0±5.3	−4.1±2.3	3.1±4.2	27.4±2.2**	95.4±1.0**	100.2±1.3[a,]**	15
Hedychenone (**8**)	0.0±3.1	12.5±2.0*	30.5±4.7**	54.3±3.3**	92.2±0.6**	102.3±0.1[a,]*	7.9
7-Hydroxyhedychenone (**9**)	0.0±3.1	3.3±3.8	3.1±1.1	6.0±3.8	34.6±4.2**	91.7±1.0**	43
(+)-Nerolidol (**10**)	0.0±3.1	−2.9±3.6	−3.5±4.0	22.8±2.2**	22.5±1.1**	36.3±2.2**	>100
Hedychiol A (**11**)	0.0±1.9	2.7±6.0	−2.3±5.2	−1.3±5.9	6.3±3.4	16.6±2.6	>100
Hedychiol B 8,9-diacetate (**12**)	0.0±5.8	2.5±1.5	3.9±2.1	12.5±3.3*	46.8±2.2**	86.3±0.7**	32
L-NMMA	0.0±1.1	4.4±2.0	2.0±1.6	17.7±2.8**	52.3±1.5**	79.2±0.9**	28

Each value represents the mean±SEM ($N=4$). Significantly different from the control: *$p<0.05$, **$p<0.01$.
[a]Cytotoxic effect was observed.

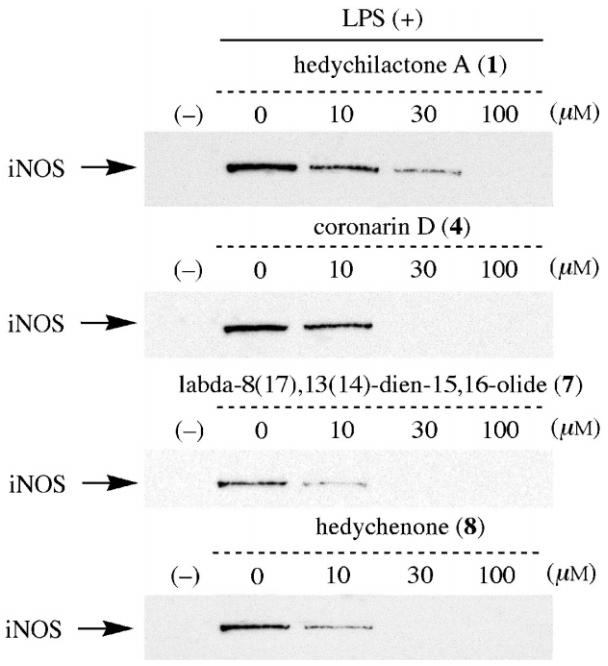

Figure 5. Effects of diterpenes (**1**, **4**, **7**, **8**) on iNOS induction in LPS-activated mouse macrophages.

(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 F_{254S} (Merck, 0.25 mm) (reversed phase); reversed-phase HPTLC, pre-coated TLC plates with Silica gel RP-18 WF_{254S} (Merck, 0.25 mm); detection was achieved by spraying with 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4 followed by heating.

Extraction and isolation

The fresh rhizome of *H. coronarium* (6.5 kg, cultivated in Kagawa Prefecture, Japan) was finely minced and extracted with methanol under reflux. Evaporation of the solvent under reduced pressure gave the MeOH extract (99.7 g). The MeOH extract (99.7 g) was partitioned in an AcOEt–H_2O (1:1) mixture, and then removal of the solvent under reduced pressure from the AcOEt- and water-soluble portions yielded 32.9 and 66.8 g of residue, respectively.

The AcOEt-soluble portion (20.5 g) was subjected to ordinary-phase silica gel column chromatography [1.0 kg, *n*-hexane–AcOEt (20:1→10:1) → $CHCl_3$–MeOH–H_2O (65:35:10, lower layer → 6:4:1) → MeOH] to afford 10 fractions [Fr. 1 (0.3 g), Fr. 2 (0.2 g), Fr. 3 (1.4 g), Fr. 4 (0.5 g), Fr. 5 (1.7 g), Fr. 6 (1.8 g), Fr. 7 (2.8 g), Fr. 8 (3.5 g), Fr. 9 (3.0 g), Fr. 10 (5.3 g)]. Fraction 1 (0.3 g) was further subjected to ordinary-phase silica gel column chromatography [16 g, *n*-hexane–AcOEt (20:1) → $CHCl_3$–MeOH–H_2O (6:4:1)] to furnish coronarin E (**6**, 72 mg). Fraction 3 (1.4 g) was separated by reversed-phase silica gel column chromatography [MeOH–H_2O (70:30 → 80:20) → MeOH] and finally HPLC [YMC-pack ODS-A 250×20 mm i.d., MeOH–H_2O (80:20 or 90:10)] to give hedychenone (**8**, 47 mg), 7-hydroxy-hedychenone (**9**, 29 mg), and (+)-nerolidol (**10**, 199 mg). Fraction 4 (0.5 g) was purified by reversed-phase silica gel column chromatography [MeOH–H_2O (80:20 → 90:10) → MeOH] and finally HPLC [YMC-pack ODS-A 250×20 mm i.d., MeOH–H_2O (85:15)] to furnish (+)-nerolidol (**10**, 73 mg), labda-8(17),13-dien 15,16-diol (**9**, 4 mg), and coronarin D methyl ether (**5**, 143 mg). Fraction 6 (1.8 g) was subjected to ordinary-phase silica gel column chromatography [110 g, *n*-hexane–AcOEt (10:1 → 3:1) → MeOH] and reversed-phase silica gel chromatography [11 g, MeOH–H_2O (80:20 → 90:10) → MeOH] to give 5-hydroxy-3,7,4′-trimethoxyflavone (**13**, 36 mg). Fr. 7 (2.8 g) was purified by reversed-phase silica gel chromatography [84 g, MeOH–H_2O (70:30 → 80:20 → 90:10) → MeOH] and finally HPLC [YMC-pack ODS-A 250×20 mm i.d., MeOH–H_2O (65:35 or 80:20)] to furnish hedychilatones A (**1**, 13 mg), B (**2**, 14 mg), and C (**3**, 29 mg), hedychiols A (**11**, 54 mg) and B 8,9-diacetate (**12**, 6 mg). These constituents were identified by comparison of their physical data with reported values.[1–3,5–10]

Hedychilactone A (1). Colorless oil, $[\alpha]_D^{22}$ +12.3° (*c* 0.700, $CHCl_3$). High-resolution EI-MS: calcd for $C_{20}H_{30}O_3$ (M^+): 318.2195. Found: 318.2197. UV (EtOH, nm, log ε): 226 (4.08). IR (film): 3393, 2924, 1752, 1676 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 0.72, 0.83, 0.92 (3H each, both s, 20, 19, and 18-H_3), 1.06 (1H, ddd, J=4.0, 12.5, 12.5 Hz, 1α-H), 1.18 (1H, dd, J=5.5, 11.9 Hz, 5-H), 1.21 (1H, m, 3α-H), 1.29 (1H, ddd, J=11.3, 11.9, 12.8 Hz, 6β-H), 1.45 (1H, br d, J=ca. 13 Hz, 3β-H), 1.54, 1.58 (1H each, both m, 2-H_2), 1.69 (1H, br d, J=ca. 13 Hz, 1β-H), 1.81 (1H, br d, J=ca. 11 Hz, 9-H), 2.11 (1H, ddd, J=2.4, 5.5, 12.8 Hz, 6α-H), 2.29, 2.40 (1H each, both m, 11-H_2), 2.88 (2H, m, 14-H_2), 4.00 (1H, dd-like, 7-H), 4.39 (2H, t-like, 15-H_2), 4.58, 5.20 (1H each, both br s, 17-H_2), 6.68 (1H, m, 12-H). ^{13}C NMR ($CDCl_3$) δc: given in Table 2. EI-MS *m*/*z* (%): 318 (M^+, 4), 300 (M^+-H_2O, 28), 42 (100).

Hedychilactone B (2). Colorless oil, $[\alpha]_D^{28}$ +10.6° (*c*=0.600, $CHCl_3$). High-resolution positive-ion FAB-MS: calcd for $C_{20}H_{31}O_3$ $(M+H)^+$: 319.2266. Found: 319.2273. UV (EtOH, nm, log ε): 227 (4.08). IR (film): 3496, 2926, 1750, 1674 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 1.02, 1.05 (3H each, both s, 18 and 20-H_3), 1.11 (1H, d-like, 5-H), 1.12 (1H, ddd, J=3.7, 13.4, 13.7 Hz, 1α-H), 1.18 (1H, ddd, J=3.1, 13.4, 13.7 Hz, 3α-H), 1.22 (3H, s, 19-H_3), 1.39 (1H, br d, J=ca. 14 Hz, 3β-H), 1.52, 1.65 (1H each, both s, 2-H_2), 1.72 (1H, br d, J=ca. 14 Hz, 1β-H), 1.93 (1H, br s, J=ca. 11 Hz, 9-H), 2.28, 2.38 (1H each, both m, 11-H_2), 2.35 (2H, m, 7-H_2), 2.88 (2H, m, 14-H_2), 4.39 (2H, t-like, 15-H_2), 4.40 (1H, ddd, J=0.6, 7.0, 7.3 Hz, 6-H), 4.67, 5.00 (1H each, both br s, 17-H_2), 6.72 (1H, m, 12-H). ^{13}C NMR ($CDCl_3$) δc: given in Table 2. Positive-ion FAB-MS *m*/*z*: 319 $(M+H)^+$.

Hedychilactone C (3). Colorless oil, $[\alpha]_D^{23}$ +23.8° (*c* 0.700, $CHCl_3$). High-resolution EI-MS calcd for $C_{20}H_{28}O_4$ (M^+): 332.1987. Found: 332.1982. UV (EtOH, nm, log ε): 222 (3.92). IR (film): 3492, 2928, 1750, 1717, 1675 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 0.67, 1.00 (3H each, both s, 20 and 18-H_3), 1.15 (1H, ddd, J=4.3, 13.1, 13.1 Hz, 3α-H), 1.26 (3H, s, 19-H_3), 1.28 (1H, m,

1α-H), 1.42 (1H, ddd, J = 2.4, 2.4, 13.1 Hz, 3β-H), 1.61 (2H, m, 2-H_2), 1.80 (1H, ddd, J = 3.1, 3.1, 12.5 Hz, 1β-H), 2.26 (1H, br s, 5-H), 2.36, 2.50 (1H each, both m, 11-H_2), 2.39 (1H, br d, J = ca. 11 Hz, 9-H), 2.91 (2H, m, 14-H_2), 4.42 (2H, t-like, 15-H_2), 4.49 (1H, br s, 7-H), 4.67, 5.44 (1H each, both br s, 17-H_2), 6.75 (1H, m, 12-H). ^{13}C NMR ($CDCl_3$) δc: given in Table 2. EI-MS: m/z (%): 332 (M^+, 21), 314 ($M^+ - H_2O$, 8), 112 (100).

Diisobutylalminum hydride reduction of 1 and 2. A solution of **1** (1.4 mg, 4.4 μmol) or **2** (1.6 mg, 5.0 μmol) in CH_2Cl_2 (0.5 mL) was treated with diisobutylalminum hydride (DIBAL, 1.0 M in *n*-hexane, 50 μL) and the mixture was stirred at 0 °C for 30 min. The reaction mixture was poured into saturated aqueous NH_4Cl, and the whole was extracted with AcOEt. The AcOEt extract was washed with brine, then dried over $MgSO_4$ powder and filtered. After removal of the solvent from the filtrate under reduced pressure, the residue was purified by silica gel column chromatography (0.2 g, *n*-hexane–acetone = 1:1 or 5:2) to give **1a** (1.4 mg, quant.) or **2a** (1.6 mg, quant.).

1a. Colorless oil. ^{1}H NMR ($CDCl_3$) δ: 0.70, 0.82, 0.91 (3H each, both s, 20, 19, and 18-H_3), 1.03 (1H, ddd, J = 4.0, 12.8, 12.8 Hz, 1α-H), 1.16 (1H, dd, J = 2.4, 12.8 Hz, 5-H), 1.18 (1H, ddd, J = 4.6, 13.1, 13.7 Hz, 3α-H), 1.28 (1H, m, 6β-H), 1.43 (1H, br d, J = ca. 14 Hz, 3β-H), 1.53, 1.57 (1H each, both m, 2-H_2), 1.64 (1H, br d, J = ca. 12 Hz, 9-H), 1.74 (1H, br d, J = ca. 13 Hz, 1β-H), 2.10 (1H, ddd, J = 2.4, 5.5, 11.0 Hz, 6α-H), 2.13, 2.33 (1H each, both m, 11-H_2), 2.45 (2H, m, 14-H_2), 3.75 (2H, m, 15-H_2), 3.99 (1H, dd, J = 5.5, 11.0 Hz, 7-H), 4.01 (2H, s, 16-H_2), 4.64, 5.18 (1H each, both br s, 17-H_2), 5.48 (1H, dd, J = 6.4, 6.7 Hz, 12-H). Positive-ion FAB-MS m/z (%): 345 $(M + Na)^+$.

2a. Colorless oil. ^{1}H NMR ($CDCl_3$) δ: 1.01, 1.02 (3H each, both s, 18 and 20-H_3), 1.10 (1H, ddd, J = 3.7, 13.0, 13.1 Hz, 1α-H), 1.10 (1H, d-like, 5-H), 1.18 (1H, ddd, J = 3.7, 12.5, 13.0 Hz, 3α-H), 1.21 (3H, s, 19-H_3), 1.38 (1H, br d, J = ca. 13 Hz, 3β-H), 1.51, 1.63 (1H each, both m, 2-H_2), 1.76 (1H, br d, J = ca. 13 Hz, 1β-H), 1.77 (1H, br d, J = ca. 10 Hz, 9-H), 2.11, 2.33 (1H each, both m, 11-H_2), 2.35 (2H, br s, 7-H_2), 2.45 (2H, m, 14-H_2), 3.76 (2H, m, 15-H_2), 4.03 (2H, s, 16-H_2), 4.38 (1H, br s, 6-H), 4.73, 5.01 (1H each, both br s, 17-H_2), 5.53 (1H, dd, J = 6.4, 6.4 Hz, 12-H). Positive-ion FAB-MS m/z: 345 $(M + H)^+$.

Preparation of *p*-bromobenzoyl ester (1b) from 1. A solution of **1** (2.0 mg, 6.3 μmol) in pyridine (0.8 mL) was treated with *p*-bromobenzoyl chloride (13.8 mg, 63 μmol) in the presence of 4-dimethylaminopyridine (4-DMAP, 3.0 mg) 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 washed with 5% aqueous HCl, aqueous saturated $NaHCO_3$, and 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–AcOEt = 5:1 → 1:1) to give **1b** (1.0 mg, 80%, recover 1.4 mg).

1b. Colorless oil. CD [EtOH, nm (Δε)]: 226 (−2.25), 245 (+6.71). UV [EtOH, nm (log ε)]: 242 (4.03). ^{1}H NMR ($CDCl_3$) δ: 0.80, 0.85, 0.92 (3H each, both s, 20, 19, and 18-H_3), 1.11 (1H, ddd, J = 4.3, 12.5, 12.8 Hz, 1α-H), 1.21 (1H, ddd, J = 4.3, 11.2, 13.1 Hz, 3α-H), 1.23 (1H, dd, J = 4.3, 13.1 Hz, 5-H), 1.30 (1H, ddd, J = 11.6, 13.1, 13.1 Hz, 6β-H), 1.47 (1H, br d, J = ca. 13 Hz, 3β-H), 1.51, 1.60 (1H each, both m, 2-H_2), 1.72 (1H, br d, J = ca. 13 Hz, 1β-H), 1.96 (1H, br d, J = ca. 11 Hz, 9-H), 2.18 (1H, m, 6α-H), 2.31, 2.44 (1H each, both m, 11-H_2), 2.88 (2H, m, 14-H_2), 4.38 (2H, t-like, 15-H_2), 4.60, 5.12 (1H each, both br s, 17-H_2), 5.39 (1H, dd, J = 5.5, 11.6 Hz, 7-H), 6.70 (1H, m, 12-H), 7.60, 7.97 (2H each, both d, J = 8.5 Hz, Ph). Positive-ion FAB-MS m/z (%): 523, 525 $(M + Na)^+$.

Bioassay

Effects on increase in vascular permeability induced by acetic acid in mice. Male ddY mice weighing 21–23 g were used. Two percent (w/v) Evans Blue solution in saline was injected intravenously (10 mL/kg) into the tail vein 55 min after administration of test compound. Five minutes later, 1% (w/v) acetic acid solution in saline was injected intraperitoneally (10 mL/kg), and 20 min later the mice were sacrificed by cervical dislocation and the abdomen was immediately opened. After washing of the peritoneal cavity with 8 mL of saline, the washed solution was filtered through glass wool, and 0.1 mL of 1 M NaOH was added. The solution was filled up to 10 mL with saline, and the absorbance was measured at 620 nm. Vascular permeability was assessed by measuring the amount of the dye that leaked into the peritoneal cavity.

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 phosphate-buffered saline (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 (1–100 μM) for 20 h. NO production in each well was assessed by measuring the accumulation of nitrite in the culture medium using Griess reagent.[21] 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 solution (10 μL, 5 mg/mL in PBS) 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). 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 (−)].

Detection of iNOS. In this experiment, peritoneal exudate cells were obtained from the peritoneal cavities of male ddY mice that had been injected intraperitoneally with 4% TGC medium 4 days previously to obtain a large numbers of cells. Cells (7.5×10^6 cells/3 mL/dish) were pre-cultured in culture dishes (6 cm i.d.) for 1 h, and the adherent cells (more than 95% macrophages) were obtained as described above. After washing, the culture medium was exchanged for fresh medium containing 5% FCS, 20 μg/mL LPS and test compound for 12 h. Cells were collected in lysis buffer [100 mM NaCl, 10 mM Tris, Complete Mini (1 tab/10 mL), 0.1% Triton X-100, 2 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)] and sonicated. After determination of protein concentration of each suspension by the BCA method (BCA™ Protein Assay Kit, Pierce), the suspension was boiled in Laemmli buffer.[22] For SDS-PAGE, aliquots of 50 μg of protein from each sample were subjected to electrophoresis in 10% polyacrylamide gels. Following electrophoresis, the proteins were transferred electrophoretically onto nitrocellulose membranes. The membranes were incubated with 5% nonfat dried milk in Tris-buffered saline (TBS, 100 mM NaCl, 10 mM Tris, 0.1% Tween 20, pH 7.4) and probed with mouse monoclonal IgG (dilution of 1:1000) against iNOS. The blots were washed in TBS and probed with secondary antibody, anti-mouse IgG antibody conjugated to horseradish peroxidase (dilution of 1:5000). Detection was performed using an ECL kit and X-ray film (Hyper Film, Amersham).

Statistics

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

References and Notes

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