A New Lignan from *Balanophora abbreviata* and Inhibition of Lipopolysaccharide (LPS)-induced Inducible Nitric Oxide Synthase (iNOS) Expression

Akihiro HOSOKAWA,[a] Megumi SUMINO,[a] Tomonori NAKAMURA,[a] Shingo YANO,[a] Toshikazu SEKINE,[a] Nijsiri RUANGRUNGSI,[b] Kazuko WATANABE,[d] and Fumio IKEGAMI*,[a,c]

[a] *Graduate School of Pharmaceutical Sciences, Chiba University; 1–33 Yayoi-cho, Inage-ku, Chiba 263–8522, Japan:* [b] *Faculty of Pharmaceutical Sciences, Chulalongkorn University; Bangkok 10330, Thailand:* [c] *Center for Environment, Health and Field Sciences, Chiba University; 6–2–1 Kashiwanoha, Kashiwa 277–0882, Japan: and* [d] *Laboratory of Biology, Nihon University College of Pharmacy; 7–7–1 Narashinodai, Funabashi 274–8555, Japan.*

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Six lignans including a new lignan (1), β-sitosterol glucopyranoside and phenylpropanoids were isolated from the whole plants of *Balanophora abbreviata* Bl. (Balanophoraceae). Their structures were determined by NMR, MS analysis and other spectroscopic methods. Lignans (1, 2 and 4) showed potent inhibitory activities on the lipopolysaccharide (LPS)-induced inducible nitric oxide synthase (iNOS) expression in RAW 264.7 cells.

Key words *Balanophora abbreviata*; Balanophoraceae; lignan; inducible nitric oxide synthase (iNOS); anti-inflammatory effect

Balanophora abbreviata BLUME (Balanophoraceae) is a parasitic plant distributed throughout Southeast Asia such as Thailand, Myanmar and China.[1] It is known by the local name "Hora teen mah" and is thought to be a toxic herb which causes leprosy and lymph-node cancer in the central of Thailand.[2] In the same genus *Balanophora*, *B. japonica* MAKINO and *B. polyandra* GRIFFITH have been used as a folk medicine for an antipyretic, antidote or anemic in China,[3] and for an antiasmatic in Thailand.[2] An earlier phytochemical study on *B. abbreviata* reported the isolation of amyrin acetates and lupeol acetate,[4] however, chemical constituents have not been studied in detail.

As part of our studies on the research and development of the Thai medicinal resources,[5,6] we now studied on the chemical constituents and their biological activities of the ethanolic extract of *B. abbreviata* collected at Nakorn Rachasima in Thailand.

In this paper, the isolation and structural elucidation of newly isolated lignan (**1**) and 10 known compounds from this plant were described. Effects of the isolated lignans on the lipopolysaccharide (LPS)-induced inducible nitric oxide synthase (iNOS) expression in RAW 264.7 cells were also investigated.

Results and Discussion

The 75% ethanol extract of the dried materials of *B. abbreviata* was separated into *n*-hexane, EtOAc, *n*-BuOH and H_2O-soluble fractions, successively. They were fractionated by silica gel, Sephadex LH-20, aluminium oxide and ODS column chromatographies (CC) according to the pharmacological assay on the LPS-induced iNOS expression in RAW 264.7 cells. The EtOAc-soluble fraction showing a strong activity yielded lignans (**1**—**6**) including a new compound (**1**) and phenylpropanoids (**7**—**9**). The structures of the isolated compounds were elucidated by spectroscopic methods such as ^{1}H-, ^{13}C-NMR and MS. The known compounds were characterized as (+)-pinoresinol (**2**),[7] (−)-pinoresinol β-D-glucopyranoside (**3**),[7] (−)-epipinoresinol (**4**),[8] (−)-lariciresinol (**5**),[9] (−)-*ent*-isolariciresinol (**6**),[10] coniferyl aldehyde (**7**),[11] cinnamic acid (**8**)[12] and *p*-coumaric acid (**9**),[13] by comparison of their spectral data with those in the literatures. From the *n*-BuOH-soluble fraction showing a weak activity, coniferin (**10**)[14] and β-sitosterol 3-*O*-β-D-glucopyranoside (**11**)[15] were also isolated by silica gel, Sephadex LH-20 and Cosmosil 75C_{18}-OPN CC together with lignans (**2**—**6**).

New compound (**1**) was obtained as a white amorphous solid showing $[\alpha]_D^{23}$ −66.9° (c=0.28, $CHCl_3$). The molecular formula of **1** was determined to be $C_{19}H_{20}O_5$ from high resolution (HR)-FAB-MS. IR spectrum of **1** showed an absorption at 3369 cm^{-1} for hydroxyl groups.

The ^{1}H-NMR spectrum of **1** showed signals of 1,3,4-trisubstituted phenyl protons at δ 6.89 (d, 1.5), 6.88 (d, 8.2) and 6.82 (dd, 8.2, 1.5), and 1,4-substituted phenyl protons at δ 7.21 (2H, d, 8.4) and 6.80 (2H, d, 8.4), and a signal due to a methoxyl protons at δ 3.90 (3H, s). The ^{13}C-NMR spectrum revealed 18 carbon signals typical to the lignan skeleton besides a methoxyl carbon signal at δ 55.9.

The methoxyl group was thought to be attached at C-3″ position by consideration of heteronuclear multiple bond connectivity (HMBC) correlations between the protons at δ 3.90 (3H, s) and the carbon at δ 146.7 (C-3″). In addition, the planar structure of **1** was also supported by other HMBC correlations, *i.e.*, between the hydroxyl proton at δ 5.28 (1H, s) and δ 155.3 (C-4′), 115.4 (C-3′, 5′), and also between the proton at δ 5.63 (1H, s) and δ 145.2 (C-4″), 114.3 (C-5″), 146.7 (C-3″).

Six carbon signals at δ 54.0 (C-1), 54.2 (C-5), 71.5 (C-8), 71.8 (C-4), 85.6 (C-2) and 85.9 (C-6) indicated the 3,7-dioxabicyclo[3.3.0]octane ring with two axial protons at 2 and 6 positions, which was seen in eudesmin and (−)-pinoresinol (**2**).[7,16] Futhermore, in the differential nuclear Overhauser effect (NOE) experiment, when two oxymethine protons (H-2 and H-6) were irradiated, differential NOEs were observed at H-4α (4%) and H-8α (4%), respectively.

By the comparison of circular dichroism (CD) spectrum of **1** ($[\theta]_{242}$ +228, $[\theta]_{280}$ −1038) with that of isolated **2** ($[\theta]_{244}$ +217, $[\theta]_{282}$ −2631), it is indicated that the absolute stereochemistry of **1** was as same as that of **2**. Thus, the structure of **1** was finally concluded to be the demethoxyl derivative of (−)-pinoresinol (**2**): (1*S*,2*R*,5*S*,6*R*)-2-(4-hydroxyphenyl)-6-

* To whom correspondence should be addressed. e-mail: ikegami@faculty.chiba-u.jp

Table 1. ^{1}H- and ^{13}C-NMR Data for **1** and **2** ($CDCl_3$)

	1		**2**	
	^{13}C	^{1}H	^{13}C	^{1}H
1	54.0[a)]	3.11 (1H, m)	54.1	3.10 (1H, m)
2	85.6	4.76 (1H, m)	85.8	4.74 (1H, d, 4.3)
4	71.8[b)]	3.87 (1H, m) ax	71.6	3.87 (1H, dd, 9.5, 5.5)
		4.26 (1H, m) eq		4.25 (1H, m)
5	54.2[a)]	3.11 (1H, m)	54.1	3.10 (1H, m)
6	85.9	4.76 (1H, m)	85.8	4.74 (1H, d, 4.3)
8	71.5[b)]	3.87 (1H, m) ax	71.6	3.87 (1H, dd, 9.5, 5.5)
		4.26 (1H, m) eq		4.25 (1H, m)
1′	133.0[c)]		132.8	
2′	127.6	7.21 (1H, d, 8.4)	108.6	6.89 (1H, d, 1.8)
3′	115.4	6.80 (1H, d, 8.4)	146.7	
4′	155.3		145.2	
5′	115.4	6.80 (1H, d, 8.4)	114.3	6.88 (1H, d, 8.2)
6′	127.6	7.21 (1H, d, 8.4)	118.9	6.82 (1H, dd, 8.2, 1.8)
1″	132.8[c)]		132.8	
2″	108.6	6.89 (1H, d, 1.5)	108.6	6.89 (1H, d, 1.8)
3″	146.7		146.7	
4″	145.2		145.2	
5″	114.3	6.88 (1H, d, 8.2)	114.3	6.88 (1H, d, 8.2)
6″	119.0	6.82 (1H, dd, 8.2, 1.5)	118.9	6.82 (1H, dd, 8.2, 1.8)
OCH_3	55.9	3.90 (3H, s)	55.9	3.90 (3H, s)
OCH_3			55.9	3.90 (3H, s)
C4′OH		5.28 (1H, s)		5.68 (1H, s)
C4″OH		5.63 (1H, s)		5.68 (1H, s)

a), *b*), *c*) Interchangeable.

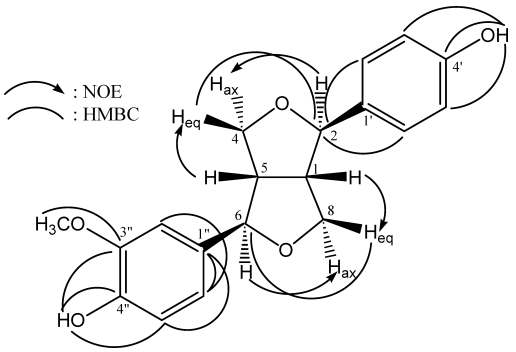

Fig. 1. HMBC Correlations and Observation of NOE Enhancement for **1**

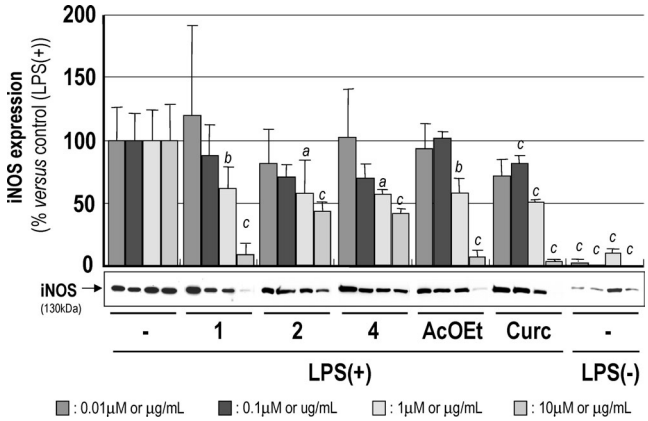

Fig. 2. Effect of Lignans and the EtOAc Fraction from *B. abbreviata* on iNOS Expression Induced by LPS in RAW264.7 Cells

Data represent mean±S.D. (% versus control) of the expressed iNOS as assessed by Western blot with rabbit anti-mouse iNOS antibody ($n=3$). EtOAc and Curc mean EtOAc fraction and curcumin, respectively. Significantly differences from control group are *a*) $p<0.05$, *b*) $p<0.01$ and *c*) $p<0.001$ determined by Dunnett test.

(3-methoxy-4-hydroxyphenyl)-3,7-dioxabicyclo[3.3.0]octane, as shown in Fig. 1.

Since it has been reported that some lignans have the inhibitory activity on the LPS-induced iNOS expression,[17] the effects of isolated lignans (**1**—**6**) on the LPS-induced iNOS expression in RAW 264.7 cells were also investigated. A new lignan (**1**) showed a relatively potent inhibitory activity as same as curcumin,[18] a positive control, in a dose-dependent manner from 0.01 to 10 μM (Fig. 2). Other lignans, **2** and **4** exhibited a moderate activity. From these results, the effects of lignans in *B. abbreviata* were expected to constitute the anti-inflammatory effect of the extract by inhibition of iNOS expression.

Experimental

General UV spectra were recorded with a Hitachi U-3200 spectrophotometer. IR spectra were recorded with a JASCO FT/IR-230 spectrophotometer. Optical rotations were measured with a JASCO DIP-140 digital polarimeter. CD curves were recorded on a JASCO J-720WI. NMR spectra were recorded on a JEOL JNM-A400 spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C) or a JEOL JNM-A500 spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C). Chemical shifts are shown as δ values, using tetramethylsilane (TMS) as an internal reference. HMBC and differential NOE experiments were obtained with the usual pulse sequence, and data processing was performed with the standard JEOL software. The *J* value in HMBC experiments was 8 Hz. EI-MS were taken on a JEOL JMS-GCMATE mass spectrometer. FAB-MS were taken on a JEOL JMS-HX110A mass spectrometer in an *m*-nitrobenzylalcohol (NBA) matrix in the positive mode. Column chromatography was carried out on Kieselgel 60 (70—230 mesh, 230—400 mesh) (Merck), Sephadex LH-20 (Amersham Biosciences), Aluminium oxide 60 F_{254} (Merck) and Cosmosil 75C_{18}-OPN (Nacalai Tesque). TLC was performed on pre-coated silica gel 60 F_{254} (0.25 mm) (Merck) or RP-18 F_{254} (0.25 mm) (Merck), and spots were detected by UV (254 nm) or by 50% H_2SO_4 spraying reagent followed by heating.

Plant Material The whole plants of *Balanophora abbreviata* Bl. (Bal-

anophoraceae) were collected at Nakorn-Rachasima, Thailand in May 2002, and identified by Dr. N. Ruangrungsi. A voucher specimen (JH054003) is deposited in the herbarium, Graduate School of Pharmaceutical Sciences, Chiba University, Japan.

Extraction and Isolation The dried and crushed whole plant of *B. abbreviata* (950 g) was extracted with 75% EtOH at room temperature. The solvent was evaporated under reduced pressure to give the extract (258 g). The extract (254 g) was partitioned with H_2O-*n*-hexane, and the remaining H_2O layer was successively extracted with EtOAc and *n*-BuOH. A portion (10.5 g) of the EtOAc-soluble fraction (25.5 g) was subjected to silica gel column chromatography (CC) (3.6 i.d.×27 cm) with $CHCl_3$–MeOH of increasing polarity to give 10 fractions. Fr. 2A was applied to silica gel CC (2.2 i.d.×18 cm: *n*-hexane/$CHCl_3$) of increasing polarity and Sephadex LH-20 CC (0.9 i.d.×45 cm: $CHCl_3$/MeOH) to afford coniferyl aldehyde (**7**, 1.6 mg). Fr. 3A was subjected to silica gel CC (2.6 i.d.×18 cm: *n*-hexane/$CHCl_3$) and then to aluminium oxide CC (0.9 i.d.×17 cm: *n*-hexane/$CHCl_3$/MeOH) and silica gel CC (0.9 i.d.×21 cm: *n*-hexane/EtOAc) of increasing polarity to provide (−)-pinoresinol (**2**, 53.7 mg) and (−)-epipinoresinol (**4**, 2.8 mg), respectively. Fr. 5A was repeatedly subjected to silica gel CC (2.2 i.d.×17 cm: $CHCl_3$/MeOH, *n*-hexane/EtOAc) to provide (−)-lariciresinol (**5**, 145.1 mg), cinnamic acid (**8**, 1.0 mg) and **1**-rich fractions. Compound **1** (8.5 mg, 0.002%/g dried plant) was finally obtained from **1**-rich fractions by aluminium oxide CC (1.1 i.d.×10 cm: $CHCl_3$/MeOH). Fr. 6A was also subjected to silica gel, Sephadex LH-20 and aluminium oxide CCs in the same manner to afford (−)-*ent*-isolariciresinol (**6**, 10.6 mg) and *p*-coumaric acid (**9**, 2.6 mg). Compound **3** (155.4 mg) was provided from Fr. 8A by silica gel, Sephadex LH-20 and aluminium oxide CCs in the same manner.

A portion (20.7 g) of the *n*-BuOH-soluble fraction (53.5 g) was separated by silica gel CC (3.6 i.d.×50 cm) with $CHCl_3$/MeOH/H_2O of increasing polarity to give 7 fractions. Fr. 1B was subjected to silica gel CC (2.2 i.d.×15 cm: *n*-hexane/EtOAc) to provide (−)-pinoresinol (**2**, 321.6 mg) and (−)-epipinoresinol (**4**, 31.0 mg). Fr. 2B was repeatedly subjected to silica gel CC (*n*-hexane/EtOAc, $CHCl_3$/MeOH) in the same manner to provide (−)-lariciresinol (**5**, 153.6 mg), (−)-*ent*-isolariciresinol (**6**, 10.6 mg) and β-sitosterol 3-*O*-β-D-glucopyranoside (**11**, 4.3 mg), respectively. Fr. 3B was separated by silica gel CC (3.6 i.d.×15 cm: EtOAc/MeOH) and Cosmosil 75C_{18}-OPN CC (2.6 i.d.×17 cm: H_2O/MeOH) of decreasing polarity to give (−)-pinoresinol β-D-glucopyranoside (**3**, 260.6 mg), and fr. 4B was also separated in the same manner to afford coniferin (**10**, 216.4 mg).

(1*S*,2*R*,5*S*,6*R*)-2-(4-hydroxyphenyl)-6-(3-methoxy-4-hydroxyphenyl)-3,7-dioxabicyclo[3.3.0]octane (**1**): White amorphous solid. $[\alpha]_D^{23}$ −66.9° (c=0.28, $CHCl_3$). UV λ_{max} (EtOH) nm (log ε): 279 (1.58), 228 (5.55). CD (EtOH, c=0.006)[θ](nm): +228 (242), −1038 (280). IR (KBr) cm^{-1}: 3369. EI-MS *m/z*: 328 $[M]^+$. HR-FAB-MS *m/z*: 328.1296 $[M]^+$ (Calcd for $C_{19}H_{20}O_5$, 328.1311). ^{1}H- and ^{13}C-NMR data are shown in Table 1.

Evaluation of the Inhibitory Effect of Each Compound on iNOS Expression RAW 264.7 cells (2×10^4 cells/well) were cultured in 24 well plate until approximately 80% confluence with 10% fetal bovine serum (FBS)-contained RPMI 1642 medium (Sigma). Then, the cells were starved for 6 h with serum free (SF) RPMI 1642 medium. Cells were stimulated to induce inducible nitric oxide synthase (iNOS) expression by the addition of lipopolysaccharide (LPS, 100 ng/ml) in SF-RPMI 1642 medium together with each compound for 18 h. Curcumin was added as a positive inhibitor of iNOS expression. After drug challenge, cells were washed with cold phosphate buffered saline (PBS), and frozen in liquid nitrogen and thawed at room temperature three times. Cells were collected in homogenate buffer (50 μl), 50 mM-Tris HCl, 150 mM-NaCl, 5 mM-EDTA, 1%-Triton X-100 and 1 mM-PMSF, with cell scraper and centrifuged at 13000 ***g*** for 20 min at 4 °C. Supernatants were used as the total cell lysate for Western blotting as follows: Samples (20 μg of protein) were applied on SDS-PAGE (8% acrylamide gel) and blotted to the nitrocellulose filters. Rabbit polyclonal NOS II (iNOS) (Sigma) and mouse monoclonal anti-β-actin antibodies (Sigma) were used as primary antibodies. Horse raddish peroxidase (HRP)-conjugated IgG (anti-rabbit and anti-mouse) (Santa Cruz) was used as secondary antibodies. iNOS and β-actin were detected by enhanced chemiluminescent reaction.

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References

1) Shumei H., Murata J., *Flora of China*, **5**, 272—276 (2003).
2) Podimuang V., Mongkolsuk S., Yoshihara K., Natori S., *Chem. Pharm. Bull.*, **19**, 207—208 (1971).
3) Jiang Su, Xin Yi, Xue Yuan, "Dictionary of Chinese Traditional Medicines (Zhong Yao Da Ci Dian)," eds. by Shanghai Science and Technology Publishing House, China, 1998, pp. 564, 3497.
4) Yadagiri B., Raj K., Subba Rao G. S. R., *J. Nat. Prod.*, **47**, 182—191 (1984).
5) Sekine T., Inagaki M., Ikegami F., Fujii Y., Ruangrungsi N., *Phytochemistry*, **52**, 87—94 (1999).
6) Sumino M., Sekine T., Ruangrungsi N., Igarashi K., Ikegami F., *Chem. Pharm. Bull.*, **50**, 1484—1487 (2002).
7) Inouye H., Takeda Y., Nishimura H., *Yakugaku Zasshi*, **93**, 44—46 (1973).
8) Ishii H., Isikawa T., Mihara M., Akaike M., *Yakugaku Zasshi*, **103**, 279—292 (1983).
9) Zhuang L.-G., Seligmann O., Jurcis K., Wagner H., *Planta Med.*, **45**, 172—176 (1982).
10) Urones J. G., Teresa J. de P., Marcos I. S., Martin D. D., *Phytochemistry*, **26**, 1540—1541 (1987).
11) Haruna M., Koube T., Ito K., Murata H., *Chem. Pharm. Bull.*, **30**, 1525—1527 (1982).
12) "The Aldrich Library of ^{13}C and ^{1}H FT-NMR Spectra **1** (2)," ed. by Pouchert C. J., Behnke J., 1043C (1993).
13) "The Aldrich Library of ^{13}C and ^{1}H FT-NMR Spectra **1** (2)," ed. by Pouchert C. J., Behnke J., 1051C (1993).
14) Sugiyama M., Nagayama E., Kikuchi M., *Phytochemistry*, **33**, 1215—1219 (1993).
15) Sekine T., Kurihara H., Waku M., Ikegami F., Ruangrungsi N., *Chem. Pharm. Bull.*, **50**, 645—648 (2002).
16) Pelter A., Ward R. S., *Tetrahedron*, **32**, 2783—2788 (1976).
17) Lee A. K., Sung S. H., Kim Y. C., Kim S. G., *Br. J. Pharmacol.*, **139**, 11—20 (2003).
18) Pan M.-H., Lin-Shiau S.-Y., Lin J.-K., *Biochem. Pharmacol.*, **60**, 1665—1676 (2000).