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

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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 for an antiasmatic in Thailand.2) An earlier phytochemical study on Balanophora japonica Bl. (Balanophoraceae) was reported.10) Furthermore, in the differential nuclear Overhauser effect (NOE) experiment, 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 with that of isolated 2, 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): (1S,2R,5S,6R)-2-(4-hydroxyphenyl)-6-...
Since it has been reported that some lignans have the inhibitory activity on the LPS-induced iNOS expression, 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, 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 1H and 100 MHz for 13C) or a JEOL JNM-A500 spectrometer (500 MHz for 1H and 125 MHz for 13C). 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 F254 (Merck) and Cosmosil 75C18-OPN (Nacalai Tesque). TLC was performed on pre-coated silica gel 60 F254 (0.25 mm) (Merck) or RP-18 F254 (0.25 mm) (Merck), and spots were detected by UV (254 nm) or by 50% H2SO4 spraying reagent followed by heating.

**Plant Material** The whole plants of Balanopora abbreviata Bl. (Bal-
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 H₂O-n-hexane, and the remaining H₂O layer was successively extracted with EtOAc and n-BuOH. A portion of the EtOAc-soluble fraction (25.5 g) was subjected to silica gel CC (2.2 i.d. × 18 cm) of increasing polarity to provide 7 fractions. Fr. 1B was subjected to silica gel CC (2.2 i.d. × 45 cm) of increasing polarity and Sephadex LH-20 CC (0.9 i.d. × 45 cm: CHCl₃/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/CHCl₃) and then to aluminium oxide CC (0.9 i.d. × 17 cm: n-hexane/CHCl₃/MeOH and silica gel CC (0.9 i.d. × 21 cm: n-hexane/EtOAc) of increasing polarity to provide (−)-epipinoresinol (2, 53.7 mg) and (−)-epi-lariciresinol (4, 2.8 mg), respectively. Fr. 5A was repeatedly subjected to silica gel CC (2.2 i.d. × 17 cm: CHCl₃/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: CHCl₃/MeOH). Fr. 6A was also subjected to silica gel CC, Sephadex LH-20 and aluminium oxide CCs in the same manner to afford (−)-ent-lariciresinol (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 of the n-BuOH-soluble fraction (53.5 g) was separated by silica gel CC (3.6 i.d. × 50 cm) with CHCl₃/MeOH/H₂O 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 (−)-pinosylisin (2, 321.6 mg) and (−)-epi-pinosylisin (4, 31.0 mg). Fr. 2B was repeatedly subjected to silica gel CC (n-hexane/EtOAc, CHCl₃/MeOH) in the same manner to provide (−)-lariciresinol (5, 153.6 mg), (−)-ent-lariciresinol (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 Cernosíl 75C₂₅-OPN CC (2.6 i.d. × 17 cm: H₂O/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 coniferalin (10, 216.4 mg).

1S,2R,5S,6R)-2-(4-hydroxyphenyl)-6-(3-methoxy-4-hydroxyphenyl)-3,7-dioxabicyclo[3.3.0]octane (1): White amorphous solid. [ε]₀°$^{12}$ +66.9° (c=0.28, CHCl₃). UV λmax (EtOH) nm (log ε): 279 (1.58), 228 (5.55). CD (EtOH, c=0.006%)[θ]nm: +228 (242), −1030 (280). IR (KBr cm⁻¹: 3369. EI-MS m/z: 112 [M⁺]. HR-FAB-MS m/z: 128.3296 [M⁺]. [Calcd for C₇H₁₀O₂: 128.3286, 111.4-C-NMR data are shown in Table 1.]

Evaluation of the Inhibitory Effect of Each Compound on iNOS Expression

RAW 264.7 cells (2×10⁵ 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 incubation, 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 µl-Tris HCl, 150 µM-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 radish 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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