Stilbene Derivatives from *Pholidota chinensis* and Their Anti-inflammatory Activity[1)]

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Ethyl acetate extract of *Pholidota chinensis* L. showed strong NO production inhibitory activity in murine macrophage-like cell line, RAW 264.7, which was activated by a lipopolysaccharide (LPS) and interferon-γ (IFN-γ). Fractionation of the active extract led to the isolation of two new stilbene derivatives, 2,3′-dihydroxy-5-methoxy-3,4-methylenedioxydihydrostilbene (Pholidotol A) and 2-hydroxy-5-methoxy-3,4,3′,4′-dimethylenedioxydihydrostilbene (Pholidotol B) together with six known stilbene derivatives. Pholidotols A both B and inhibited Nitric oxide (NO) production with an IC_{50} value at 24.3 and 17.1 μM, respectively.

Key words *Pholidota chinensis*; Orchidaceae; stilbene; dihydrostilbene; nitic oxide (NO); 1,1-diphenyl-2-picrylhydrazil (DPPH)

Pholidota chinensis (Orchidaceae), commonly named as Shi-Xian-To (石仙桃), is a kind of traditional medicinal plant distributed in the southeast of China. The plant has been well used as remedy of acute or chronic bronchitis, toothache and duodenal ulcer.[2—4)] A report about obtained triterpenes, cyclopholidonol and cyclophlidone[5)] from this plant have been found. Macrophages play major roles in the immunity and inflammatory responses involved in host defense. Once activated, they initiate the production of cytokines, oxygen and nitrogen species, and eicosanoids. In macrophages, bacterial lipopolysaccharide (LPS), alone or in combination with recombinant mouse interferon-γ (IFN-γ), is the stimuli best known to induce the transcription of gene encoding pro-inflammatory proteins. Such stimulation results in cytokine release and the synthesis of enzymes such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). The nitric oxide (NO) radical is known to play a central role in inflammatory and immune reactions. However, excessive production of NO may cause tissue damage. In inflammatory diseases such as rheumatoid arthritis, excessive NO production by activated macrophages has been observed. During our studies on antiallergic activity of Chinese medicine, the 60% EtOH extract of *P. chinensis* inhibited NO production from activated macrophage (IC_{50}=33.6 μg/ml). And this is the first report of anti-inflammatory constituents study from this plant.

The aerial part of *P. chinensis* were extracted with 60% EtOH. The aqueous was then partitioned with hexane, ethyl acetate and *n*-butanol, respectively. As the ethyl acetate extract showed strong NO production inhibitory activity (89.2% at 30 μg/ml), further bioassay-guided fractionation was done and led to the isolation of two new stilben derivatives, pholidotols A (**1**) and B (**2**) together with known six stilben derivatives. Here, we describe the isolation, structure elucidation and biological evaluation of these compounds.

Results and Discussion

Pholidotol A (**1**), isolated as colorless needles, was assigned to possess a molecular formula of $C_{16}H_{16}O_5$ by high resolution electro impact (HR-EI) MS (*m/z* 288.1002, $[M]^+$). The UV spectrum displayed absorption maxima at 217, 258 and 281 nm. The phenolic nature was seen by its characteristic colour reactions with $FeCl_3$ (violet or intense blue was shown). The ^{13}C-NMR spectrum which showed 16 carbon signals, were classified as one methyl, three methylenes, five methines, and seven quaternary carbon signals by DEPT spectra. The ^{1}H-NMR spectrum showed signals for an aromatic methoxyl group at δ_H 3.69, methylenedioxy at δ_H 5.81 (2H, s), five aromatic methines at δ_H 6.67 (1H, br s, H-2′), δ_H 6.66 (1H, m, H-6′), δ_H 7.05 (1H, t, J=7.5 Hz, H-5′), δ_H 6.62 (1H, dd, J=7.5, 2.1 Hz, H-4′) and δ_H 6.19 (1H, s, H-6) and two aliphatic methylenes at δ_H 2.79 (2H, m) and 2.78 (2H, m) which were typical two methylene of dihydrostilbene.[5,6)] All of protonated carbon signals were assigned by the HMQC experiment. In the HMBC experiments, HMBC correlations from H-4′ to δ_C 115.3 (C-2′) and 121.6 (C-6′), from H-5′ to δ_C 143.7 (C-1′), δ_C 156.7 (C-3′), δ_C 112.6 (C-4′) and C-6′, from H-6′ to C-2′, C-4′ and δ_C 36.7 (C-α), from H-2′ to C-3′, C-4′ , C-α estimated the presence of 3′-hydroxyphenyl ring. The H-6 was correlated with δ_C 133.1 (C-2), δ_C 136.3 (C-4), δ_C 136.9 (C-5) and δ_C 32.4 (C-β), a methoxy signal (δ_H 3.69) was correlated with δ_C 136.9 (C-5) and a methylenedioxy signal (δ_H 5.81) was correlated with δ_C 134.6 (C-3) and δ_C 136.3 (C-4). These date suggested the presence of 2-hydroxy-3,4-methylenedioxy-5-methoxyphenyl moiety. In the difference nuclear Overhauser effect (NOE) experiment, NOEs were observed between δ_H 3.69 (OCH_3) and H-6; δ_H H-α H-β and H-6, H-2′ and H-6′. From the above results, the structure of **1** was determined to be 2,3′-dihydroxy-5-methoxy-3,4-methylenedioxydihydrostilbene.

Pholidotol B (**2**) was obtained as colorless crystals and the molecular formula was determind to be $C_{17}H_{16}O_6$ by HR-EI-MS (*m/z* 316.0942 $[M]^+$). The ^{1}H- and ^{13}C-NMR spectra of **2** were very similar to those of **1**, except for the observation of a methylendioxy signal (δ_C 102.1 t, δ_H 5.90) in **2**. In addition, an aromatic quaternary carbon bearing oxgen signal at (δ_C 147.6 s, C-4′) was observed instead of an aromatic methine carbon signal (δ_C 112.6) in **1**. In the HMBC spectum, a methylenedioxy signal (δ_H 5.90) was correlated with δ_C 145.8 (C-3′) and δ_C 147.6 (C-4′). The above results suggested that the structure of **1** was determined to be in those of **2** to be 2-hydroxy-5-methoxy-3,4,3′,4′-dimethylenedioxydihydrostilbene.

The other six compounds were identified to be 3,4′-dihy-

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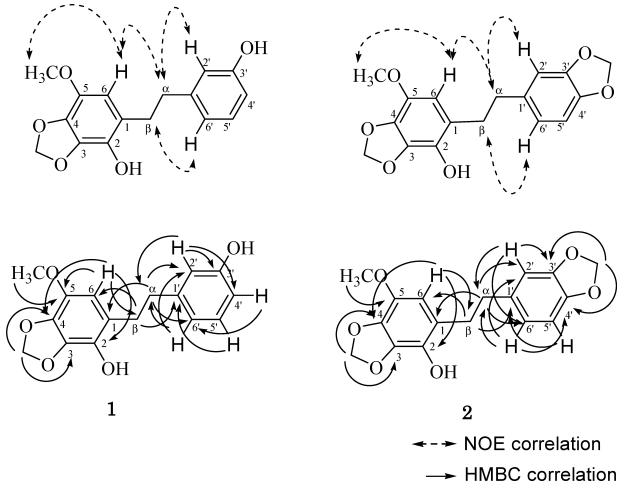
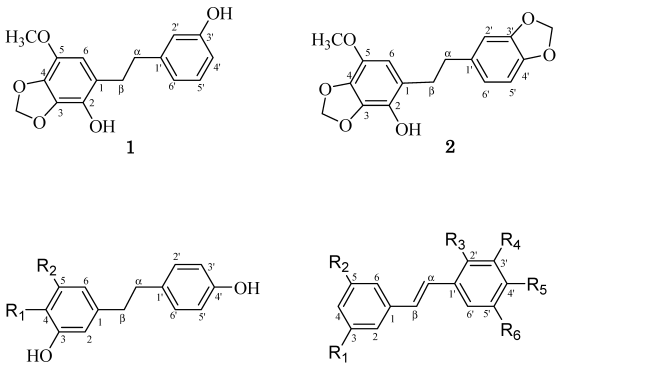


Fig. 1. HMBC and NOE Correlations of Pholidotols **1** and **2**



	R_1	R_2
3:	H	OCH_3
4:	OCH_3	H

	R_1	R_3	R_4	R_5	R_6	R_7
5:	OH	OCH_3	H	OH	H	H
6:	OH	OCH_3	OCH_3	OCH_3	H	H
7:	OH	OH	H	H	OH	H
8:	OH	OCH_3	OCH_3	OH	H	H

Chart 1. Structures of Stilbene Derivatives Isolated from *Pholidota chinensis*

droxy-5-methoxydihydrostilbene (**3**),[7] 3,4′-dihydroxy-4-methoxydihydrostilbene (**4**),[8] thunalbene (**5**),[9] *trans*-3-hydroxy-2′,3′,5-trimthoxystilbene (**6**),[10] resveratrol (**7**),[11] and *trans*-3-3′-dihydroxy-2′,5-dimthoxystilbene (**8**),[10] by comparison with literature spectral data.

Compounds **1**—**8** were examined for their 1,1-diphenyl-2-picrylhydrazil (DPPH) radical scavenging activity and NO production inhibitory activity. Quercetin was used as positive control.[12,13] Compounds **1**—**8** inhibited NO production with IC_{50} values of 24.3, 17.1, 37.5, 31.4, 38.6, 87.1, 28.7, and 49.8 μM, respectively. Their activity was similar to quercetin (30.2 μM). These compounds exhibited no cytotoxicity at 30 μM. Compounds **1**—**8** were also tested for DPPH radical scavenging activity. Compounds **5**, **6**, **7** and **8** exhibited IC_{50} values 26.7, 29.2, 21.2 and 34.5 μM, respectively. These activities were similar IC_{50} value to antioxidant agent, quercetin (32.1 μM). But compounds **1**—**4** did not show scavenging activity at 100 μM. The above assay results suggested that the number of oxygen and the α, β bond of these compounds contributed a lot of anti-oxidation activity of the above compounds. Particularly, 4′-hydroxystilbene[13—15] derivatives showed more strong inhibitory activity of NO production and DPPH radical production than other stilbene and dihydrostilbene derivatives, the DPPH inhibitory activity reduced significantly while the NO inhibitory activity isn't affected much. This is a special finding for anti-oxidation activity of stilbene derivatives.

Experimental

General Experimental Procedures The UV spectrum was obtained on a Shimadzu UV-160 spectrophotometer, and the IR spectrum was recorded on a JASCO FT/300E spectrophtometer. The ^{1}H- and ^{13}C-NMR spectra were taken on a Varian Mercury-300Plus spectrometer, using tetramethylsilane (TMS) as an internal standard. The MS were obtained on a JEOL GC mate spectrometer.

Plant Material The whole plant of *Pholidota chinensis* was collected in Guangdong Province, People's Republic of China, in October 2004 and was identified by Professor Weichun Wu (Department of Medical Plants, Shenyang Pharmaceutical University, People's Republic of China). Voucher specimens have been deposited at the Department of Pharmacognosy, College of Pharmacy, Nihon University.

Extraction and Isolation The air-dried of *P. chinensis* (10 kg) were extracted twice with 60% ethanol. The 60% EtOH solution were combined and evaporated of dryness to give 789.6 g of the extract. The extract was suspended in water (2 l) and partitioned with hexane (3×2 l), ethyl acetate (3×2 l) and *n*-butanol (3×2 l), respectively. The amounted were hexane extract 36.1 g (44.7%), ethyl acetate extract 220.1 g (89.4%), butanol extract 149.8 g (68.7%), and yielded the aqueous extract 315.9 g (15.3%). The ethyl acetate extract was subjected to silica gel column chromatography (Wako gel C-300, Wako Pure Chemical Industry Ltd., 700 g, 12×17 cm), eluted with hexane and ethyl acetate in increased polarity. The fraction (200 ml each) were combined according to TLC monitoring into 9 portions. The fourth portion was then applied to a Sephadex LH-20 column (Pharmacia Fine Chemical Co., Ltd., 7.5×25 cm) eluted with $CHCl_3$–MeOH (50 : 50) to give fr. 1-4, fr. 2 was further purified by preparative HPLC (Senshu park PEGASIL ODS, 10×250 mm, 70% CH_3CN, 30% H_2O; flow rate 4 ml/min; UV detector at 254 nm) to give **3** (43 mg) and **4** (35 mg), respectively. The fifth portion was applied to a Sephadex LH-20 column (7.5×25 cm) eluted with $CHCl_3$–MeOH (50 : 50) to give fr. 1-5 and fr. 3 was further purified by preparative HPLC (Senshu pak PEGASIL ODS, 10×250 mm, 55% CH_3CN, 45% H_2O; flow rate 4 ml/min; UV detector at 254 nm) to give **7** (50 mg), **5** (33 mg), **1** (28 mg) and **2** (21 mg). The 7 portion was separated by silica gel column chromatography (3×21 cm, eluted with $CHCl_3$–MeOH 100 : 0—0 : 100) into fr. 1-7 and fr. 4 was further purified by HPLC [(Senshu pak PEGASIL ODS Al, 10×250 mm, MeOH–H_2O (70 : 30), flow rate 4 ml/min; UV detector at 254 nm)] to give **6** (75 mg) and **8** (13.4 mg).

Pholidotol A (**1**): Colorless needles; mp 127—128 °C; UV λ_{max} (MeOH) nm (log ε): 281 (3.92), 258 (3.50), 217 (3.92); IR (KBr) cm^{-1}: 3279, 2924, 2858, 1600, 1520, 1458, 1350, 922. ^{1}H-NMR (300 MHz, CD_3OD) δ_H: 7.05 (1H, t, J=7.5 Hz, H-5′), 6.67 (1H, br s, H-2′), 6.66 (1H, m, H-6′), 6.62 (1H, dd, J=7.5, 2.1 Hz, H-4′), 6.19 (1H, s, H-6), 5.81 (2H, s, OCH_2O), 3.69 (3H, s, OCH_3), 2.79 (2H, m, H-β), 2.78 (2H, m, H-α); ^{13}C-NMR (75 MHz, CD_3O) δ_C: 156.7 (C-3′), 143.7 (C-1′), 136.9 (C-5), 136.3 (C-4), 134.6 (C-3), 133.1 (C-2), 129.1 (C-5′), 124.3 (C-1), 121.6 (C-6′), 115.3 (C-2′), 112.6 (C-4′), 109.5 (C-6), 101.4 (OCH_2O), 56.9 (OCH_3), 36.7 (C-α), 32.4 (C-β); HR-EI-MS *m/z*: 288.1002 (Calcd for $C_{16}H_{16}O_5$, 288.0998).

Pholidotol B (**2**): Colorless crystals; mp 140—141 °C; UV λ_{max} (MeOH) nm (log ε): 306 (3.95), 255 (3.25), 218 (3.96); IR (KBr) cm^{-1}: 3236, 2946, 2903, 1874, 1525, 1050, 954, 926. ^{1}H-NMR (300 MHz, CD_3OD) δ_H: 6.74 (1H, dd, J=7.8, 1.8 Hz, H-5′), 6.68 (1H, d, J=1.8 Hz, H-2′), 6.62 (1H, d, J=7.8 Hz, H-4′), 6.21 (1H, s, H-6), 5.95 (2H, s, OCH_2O), 5.90 (2H, s, OCH_2O), 3.80 (3H, s, OCH_3), 2.79 (2H, m, H-β), 2.78 (2H, m, H-α); ^{13}C-NMR (75 MHz, CD_3OD) δ_C: 147.6 (C-4′), 145.8 (C-3′), 137.7 (C-5), 135.8 (C-1′), 134.5 (C-4), 135.8 (C-3), 132.3 (C-2), 123.4 (C-1), 121.5 (C-6′), 109.3 (C-2′), 109.1 (C-6), 108.4 (C-5′), 101.1 (OCH_2O), 102.1 (OCH_2O), 56.9 (OCH_3), 36.9 (C-α), 32.9 (C-β); HR-EI-MS *m/z*: 316.0942 (Calcd for $C_{17}H_{16}O_6$, 316.0947).

DPPH Radical Scavenging Activity Radical scavenging activity was dertermined according to Biois's method.[16] The assay mixture contained DPPH radical solution (0.5 mM), 99% ethanol, 0.2 M acetic acid buffer and sample solution. The solution was rapidly mixed and the scavenging capacity was measured erectrophotometrically by monitoring the decrease in absorbance at 517 nm determined after 30 min and the scavenging activity calculated as a percentage of the radical reduction. Quercetin was used as a

positive control.

Inhibitory Activity on NO production from Activated Macrophages-Like Cell Line, RAW 264.7 The cells were seeded at 1.2×10^6 cells/ml into 96-well flat-bottomed plate (Sumitomo Bakelite, #8096R, Tokyo) and then incubated at 37 °C for 2 h. Samples were tested at various concentrations of 100, 30, 10 and 3 μM in (n=4) duplicate. Sample solutions were added to the culture simultaneously with both *Escherichia coli* LPS. (100 ng/ml) and recombinant mouse IFN-γ (0.33 ng/ml). Then cells were incubated at 37 °C for approximately 16 h and subsequently chilled on ice. One hundred microliters of the culture supernatant was placed in duplicate in the wells of 96-well flat-bottomed plates. A standard solution of $NaNO_2$ was placed in alternate wells on the same plate. To quantify nitrite, 50 μl of Griess reagent (1% sulfanilamide in 5% H_3PO_4 and 0.1% *N*-1-naphthyletylenediamidedihydrochloride) was added to each well. After 10 min the reaction products were colorimetrically quantified at 550 nm using a Model 3550 Microplate Reader (BIO-RAD) and the background absorbance (630 nm) was subtracted. The results were expressed as mean for duplicate independent experiments. Cytotoxicity was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay method.

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