Flavonoids from the Heartwood of *Dalbergia odorifera* and Their Protective Effect on Glutamate-Induced Oxidative Injury in HT22 Cells

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Two flavonoids, 4,2′,5′-trihydroxy-4′-methoxychalcone (1) and (2*S*)-6,7,4′-trihydroxyflavan (2), along with fourteen known flavonoids and two other known arylbenzofurans were isolated from the heartwood of *Dalbergia odorifera*. The structure of compounds 1 and 2 were established by spectroscopic (NMR and MS) analyses. Of the isolates, eight compounds (1, 4, 7, 10, 12, 14, 15, 17) were found to have potent protective effect on glutamate-induced oxidative injury in HT22 cells.

Key words *Dalbergia odorifera*; Leguminosae; flavonoid; arylbenzofuran

Dalbergia odorifera T. Chen (Leguminosae) is a perennial tree that mainly grows in China. The heartwood of the plant is used to treat blood stagnation syndrome, ischemia, swelling, necrosis, and rheumatic pain in China and Korea.[1] Previous phytochemical studies on this plant have isolated flavonoids, quinones, and phenolic constituents.[2—4] We have isolated two new flavonoids, 4,2′,5′-trihydroxy-4′-methoxychalcone (**1**) and (2*S*)-6,7,4′-trihydroxyflavan (**2**) together with sixteen known compounds, including flavonoids and arylbenzofurans. In this paper, we report the structure elucidation of new compounds and the cytoprotective effects of isolated compounds on glutamate-induced oxidative injury in immortalized mouse hippocampal cells.

Results and Discussion

Repeated chromatography purification of the ethanol extracts of the heartwood of the *D. odorifera* gave two new compounds, **1** and **2**, along with the known compounds **3**—**18** (Fig. 1).

Compound **1** was obtained as a yellow amorphous solid. HR-FAB-MS showed an $[M+H]^+$ ion peak in accordance with the empirical molecular formula, $C_{16}H_{14}O_5$, which was supported by the ^{13}C-NMR spectrum and distortionless enhancement by polarization transfer (DEPT) data. The UV spectrum displayed bands assignable to a chalcone system at 355 and 207 nm.[5] The ^{1}H-NMR spectrum of **1** showed one pair of doublet *trans* protons [δ 7.77 (1H, d, J=15.6 Hz) and 7.50 (1H, d, J=15.6 Hz)], one A_2B_2-type doublet system [δ 7.60 (2H, d, J=8.7 Hz) and 6.84 (2H, d, J=8.7 Hz)], two singlet protons for the 2′,4′,5′-trisubstituted chalcone ring A [δ 7.40 (1H, s) and 6.48 (1H, s), respectively], and a methoxy group [δ 3.90 (3H, s, –OCH_3)].[6] The ^{13}C-NMR and DEPT experiments were consistent with these data, indicating the presence of eight methines, five non-protonated, and one methoxy carbon. The signals for each proton and carbon of this molecule were assigned unambiguously by the assistance of 2D-NMR (HMQC and HMBC). The two olefinic protons at δ 7.77 (H-β) and 7.50 (H-α) showed long-range correlations with the carbons at δ 192.2 (C=O), 130.5 (C-2, C-6), and 126.5 (C-1) (Fig. 2). The two aromatic protons at δ 7.40 (H-6′) and 6.48 (H-3′), showed long-range correlations with the carbons at δ 192.2 (C=O), 159.8 (C-2′), 155.6 (C-4′), 138.8 (C-5′), and 112.4 (C-1′). One methoxyl proton at δ 3.90 (4′-OCH_3) showed a long-range correlation with the carbon at δ 155.6 (C-4′). From the above data, the structure of the new natural product **1** was elucidated as 4,2′,5′-trihydroxy-4′-methoxychalcone.

Compound **2** was isolated as a yellow amorphous solid. HR-FAB-MS showed a molecular ion at *m*/*z* 258.0894 (M^+, Calcd 258.0892), indicating the molecular formula $C_{15}H_{14}O_4$. The ^{1}H- and ^{13}C-NMR spectral data of **2** were very similar to those of 6,4′-dihydroxy-7-methoxyflavan,[7] except for the ab-

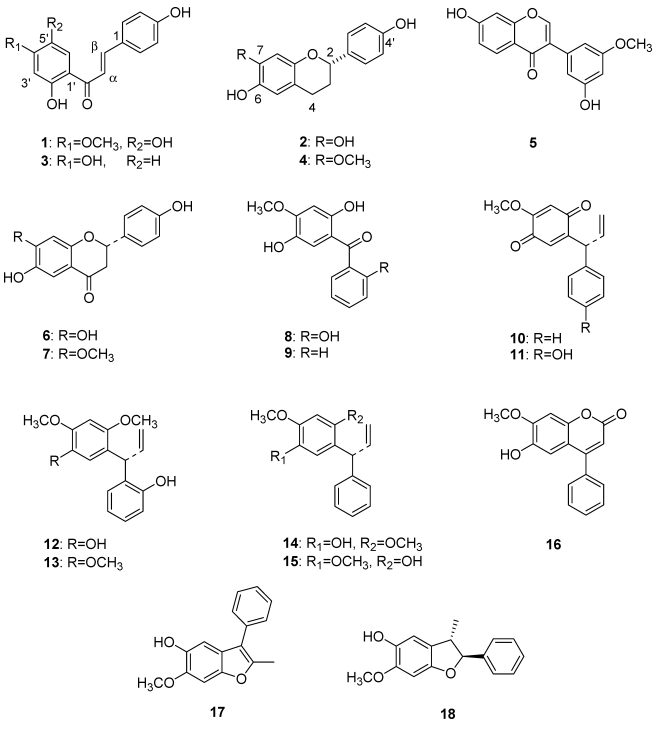

Fig. 1. Compounds Isolated from Heartwoods of *D. odorifera*

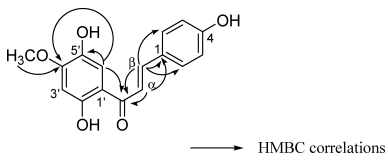

Fig. 2. Selected HMBC Correlations of Compound **1**

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Table 1. Protective Effects of Compounds **1**—**18** on Glutamate-Induced Oxidative Injury in HT22 Cells

Compound	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	Trolox[a]
EC_{50} (μM)	7.47	17.83	>50	2.85	21.68	>50	3.3	>50	>50	8.54	>50	5.82	25.79	6.54	8.14	NT[b]	3.09	>50	15.8

a) Trolox was used as a positive control. *b*) Not tested due to the lack of compound.

sence of the methoxy group. According to the literature,[8,9] a negative optical rotation, $[\alpha]_D$ value indicates that C-2 possesses an *S* configuration. **2** had a negative $[\alpha]_D^{26}$ value, indicating an *S* configuration at C-2. From the above data, the structure of the new natural product **2**, was elucidated as (2*S*)-6,7,4′-trihydroxyflavan.

In addition, we isolated and identified the known compounds: isoliquilitigenin (**3**),[10,11] (2*S*)-6,4′-dihydroxy-7-methoxyflavan (**4**) ($[\alpha]_D^{26}$ +2.2°, *c*=0.51, MeOH), 2,2′,5-trihydroxy-4-methoxybenzophenone (**8**) ($[\alpha]_D^{26}$ +4.0°, *c*=0.10, MeOH), 9-hydroxy-6,7-dimethoxydalbergiquinol (**15**) ($[\alpha]_D^{26}$ −28.0°, *c*=0.50, MeOH),[7] 7,3′-dihydroxy-5′-methoxyisoflavone (**5**),[12] 6,7,4′-trihydroxyflavanone (**6**), 6,4′-dihydroxy-7-methoxyflavanone (**7**),[13] *R*(+)-4-methoxydalbergione (**10**) ($[\alpha]_D^{26}$ +47.0°, *c*=0.17, $CHCl_3$),[14,15] 4′-hydroxy-4-methoxydalbergione (**11**) ($[\alpha]_D^{26}$ −30.6°, *c*=0.63, MeOH),[15,16] cearoin (**9**), *R*(−)-latifolin (**12**) ($[\alpha]_D^{26}$ −64.0°, *c*=0.14, MeOH), *R*(−)-5-*O*-methylatifolin (**13**) ($[\alpha]_D^{26}$ −58.0°, *c*=0.20, MeOH), *R*(+)-dalbergiphenol (**14**) ($[\alpha]_D^{26}$ +50.0°, *c*=0.58, MeOH),[14] dalbergin (**16**),[17] isoparvifuran (**17**),[18] and (2*R*,3*R*)-obtusafuran (**18**) ($[\alpha]_D^{26}$ +27.6°, *c*=0.50, MeOH).[19]

Flavonoids and related plant-derived phenolic compounds are well-known to have a wide range of biological activities. Accordingly, in this study we investigated whether isolated compounds affect cytoprotection of HT22 cells by antioxidative effect. Glutamate cytotoxicity contributes to neuronal degeneration in central nervous system (CNS) diseases such as epilepsy and ischemia. Glutamate cytotoxicity is mediated by receptor-initiated excitotoxicity and non-receptor-mediated oxidative stress.[20] Excitotoxicity refers to an excessive activation of neuronal amino acid receptors, and glutamate-induced excitotoxicity mediates neuronal death in many disorders.[21] The immortalized mouse hippocampal cell line, HT22, phenotypically resembles neuronal precursor cells but lacks functional ionotropic glutamate receptors, thus excluding excitotoxicity as a cause for glutamate triggered cell death.[22] Therefore, HT22 cells are a useful model for studying oxidative glutamate toxicity.

Compounds **1**, **2**, **4**, **5**, **7**, **10**, **12**—**15**, **17** exhibited protective effects with EC_{50} values of 7.47, 17.83, 2.85, 21.68, 3.3, 8.54, 5.82, 25.79, 6.54, 8.14, and 3.09 μM, respectively, as shown in Table 1. Furthermore, compounds **1**, **4**, **7**, **10**, **12**, **14**, **15**, **17** were more potent than the positive control, Trolox® which demonstrated an EC_{50} value of 15.8 μM. From these results, we have demonstrated that above eight compounds may possess the neuroprotective activity against oxidative cellular injuries, and it would be also need further evaluation as potential neuroprotective agents.

Experimental

Melting points were determined with a Yanaco MP-S3 micro melting point and are not corrected. Optical rotations were measured on a JASCO P-1020 polarimeter. UV spectra were obtained on a Shimadzu UV-1601 UV–visible spectrometer. The NMR spectra were recorded on a JEOL Eclipse-500 MHz spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C), and chemical shifts are quoted *versus* tetramethylsilane. ESI-MS and HR-FAB-MS were obtained on a Quattro LC-MS (Micromass) and JMS-HX 110/110A Tandem Mass spectrometer (JEOL), respectively.

Plant Material The heartwood of *D. odorifera* was purchased from the herbal medicine co-operative association of Jeonbuk Province, Korea, in October 2001. A voucher specimen (No. WP02-008) has been deposited in the Herbarium of the College of Pharmacy, Wonkwang University, Korea.

Extraction and Isolation Dried heartwoods of *D. odorifera* (593 g) were extracted twice with EtOH (1 l) under ultrasonic conditions for 1 h. After evaporation of the solvent *in vacuo*, the extract (58 g) was treated with EtOAc to give EtOAc-soluble and EtOAc-insoluble portions. The EtOAc-soluble fraction (50 g) was separated on a silica gel column using a *n*-hexane–EtOAc (gradient) to obtain five fractions (Fr. A—E). Fr. A (7 g) was further separated on a silica gel column with *n*-hexane : CH_2Cl_2 (1 : 3) to give **10** (3 g) together with two subfractions (Fr. A1, A2). Fr. A1 (513 mg) was subjected to Sephadex LH-20 (*n*-hexane–$CHCl_3$–MeOH, 4 : 3 : 1) chromatography to give **14** (23 mg) and **15** (32 mg). Fr. A2 (550 mg) was separated over a silica gel column with *n*-hexane–EtOAc (3 : 1—1 : 1) to yield **4** (50 mg), **5** (19 mg), and **7** (68 mg). Fraction B (21 g) was separated by Sephadex LH-20 column chromatography (eluent: $CHCl_3$) to yield three fractions (Fr. B1—B3). Fr. B1 (2 g) was subjected to Sephadex LH-20 (*n*-hexane–$CHCl_3$–MeOH, 4 : 3 : 0.5) chromatography and purified by a silica gel column eluting with *n*-hexane–EtOAc (2 : 1) to give **17** (17 mg) and **18** (12 mg). **12** (2 g) was crystallized from fraction B2 (*n*-hexane) as white crystals. Fr. B3 (125 mg) was subjected to silica gel column (*n*-hexane–EtOAc, 2 : 1) chromatography and purified by Sephadex LH-20 eluting with *n*-hexane–$CHCl_3$–MeOH (2 : 3 : 0.3) to yield **9** (3 mg) and **13** (13 mg). Fraction C (6 g) was separated by chromatography on a Sephadex LH-20 column eluted with *n*-hexane–$CHCl_3$–MeOH (3 : 3 : 1) to yield five fractions (Fr. C1—C5). Fr. C1 (1 g) was purified by Sephadex LH-20 column chromatography using $CHCl_3$–MeOH (10 : 1) to give **8** (7 mg). Fr. C2 (428 mg) was further separated on a silica gel using *n*-hexane–EtOAc (2 : 1) to yield **11** (6 mg). Fr. C3 (2 g) was also purified by silica gel column chromatography using *n*-hexane–acetone (2 : 1) to give **16** (2 mg). Fraction D (6 g) was subjected to Sephadex LH-20 column chromatography with MeOH–H_2O (9 : 1) to give four subfractions (Fr. D1—D4). Fr. D2 (928 mg) was further separated on a silica gel column with $CHCl_3$–MeOH (10 : 1) to give **1** (17 mg) and **3** (38 mg). Fr. D3 (2 g) was subjected to Sephadex LH-20 column chromatography with EtOAc–MeOH (4 : 1) and further purified with a silica gel column using *n*-hexane : EtOAc (1 : 1) to give **2** (3 mg) and **6** (6 mg).

4,2′,5′-Trihydroxy-4′-methoxychalcone (**1**): Yellow amorphous solid. mp 188—190 °C. $[\alpha]_D^{26}$ −76.9° (*c*=0.52, MeOH). UV λ_{max}^{MeOH} nm (log ε): 355 (4.04), 207 (4.30). (−)-ESI-MS *m*/*z*: 285 $[M-H]^-$. HR-FAB-MS *m*/*z*: 287.0917 $[M+H]^+$ (Calcd for $C_{16}H_{14}O_5$+H: 287.0919). ^{1}H-NMR (500 MHz, CD_3OD) δ: 7.77 (1H, d, *J*=15.6 Hz, H-β), 7.60 (2H, d, *J*=8.7 Hz, H-2, H-6), 7.50 (1H, d, *J*=15.6 Hz, H-α), 7.40 (1H, s, H-6′), 6.84 (2H, d, *J*=8.7 Hz, H-3, H-5), 6.48 (1H, s, H-3′), 3.90 (3H, s, –OCH_3). ^{13}C-NMR (125 MHz, CD_3OD) δ: 192.2 (C=O), 160.3 (C-4), 159.8 (C-2′), 155.6 (C-4′), 144.5 (C-β), 138.8 (C-5′), 130.5 (C-2, C-6), 126.5 (C-1), 117.0 (C-α), 115.6 (C-3, C-5), 113.7 (C-6′), 112.4 (C-1′), 99.7 (C-3′), 55.2 (–OCH_3).

(2*S*)-6,7,4′-Trihydroxyflavan (**2**): Yellow amorphous solid. mp 192—194 °C. $[\alpha]_D^{26}$ −95.0° (*c*=0.25, MeOH). UV λ_{max}^{MeOH} nm (log ε): 279 (3.28), 225 (3.80), 203 (4.02). (−)-ESI-MS *m*/*z*: 257 $[M-H]^-$. HR-FAB-MS *m*/*z*: 258.0894 $[M]^+$ (Calcd for $C_{15}H_{14}O_4$ 258.0892). ^{1}H-NMR (500 MHz, CD_3OD) δ: 7.21 (2H, d, *J*=8.7 Hz, H-2′, H-6′), 6.76 (2H, d, *J*=8.7 Hz, H-3′, H-5′), 6.46 (1H, s, H-5), 6.26 (1H, s, H-8), 4.82 (1H, m, H-2), 2.83 (1H, m, H-4a), 2.60 (1H, m, H-4b), 2.06 (1H, m, H-3a), 1.96 (1H, m, H-3b). ^{13}C-NMR (125 MHz, CD_3OD) δ: 156.8 (C-4′), 148.3 (C-7), 144.1 (C-9), 138.8 (C-6), 133.1 (C-1′), 127.1 (C-2′, C-6′), 115.0 (C-5), 114.7 (C-3′, C-5′), 112.2 (C-10), 103.4 (C-8), 77.5 (C-2), 30.1 (C-3), 24.4 (C-4).[7—9]

Cell Culture Mouse hippocampal HT22 cells, a subclone of the HT4 hippocampal cell line, were obtained from Prof. Inhee-Mook (Seoul National University, Seoul, Korea). The cells were maintained at 5×10^5 cells/ml in DMEM medium supplemented with 10% heat-inactivated FBS,

penicillin G (100 units/ml), streptomycin (100 mg/ml), and L-glutamine (2 mM), and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air.

Cell Viability HT22 cells were initially pretreated with compounds or Trolox, positive control, for 1 h. The cells were then exposed to glutamate (5 mM) for 12 h followed by an assessment of cell viability. Four concentrations (1, 10, 20, 50 μM) were tested for each sample, and each experiment was triplicated. Cell viability was determined using the MTT assay. Briefly, cells were incubated with MTT (0.5 mg/ml) for 2 h at 37 °C, medium was discarded, acidic isopropanol (0.04 N HCl) was added, and after incubating for 30 min, absorbance was measured at 595 nm using a microplate reader (BIO-RAD, Hercules, CA, U.S.A.). The results expressed the EC_{50} values as the percentage of viability *vs.* control. The data were statistically assessed by using a linear regression model.

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