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Neuroprotective phenylpropanoid esters of rhamnose isolated from roots of *Scrophularia buergeriana*

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Abstract

Four phenylpropanoid esters of rhamnose, buergerisides A_1, B_1, B_2 and C_1 were isolated from roots of *Scrophularia buergeriana* MIQ. (Scrophulariaceae), and were characterized as 2-*O*-acetyl-3,4-di-*O*-(*E*)-*p*-methoxycinnamoyl-α-L-rhamnopyranoside, 2-*O*-acetyl-3-*O*-(*E*)-*p*-methoxycinnamoyl-α-L-rhamnopyranoside, 2-*O*-acetyl-3-*O*-(*Z*)-*p*-methoxycinnamoyl-α-L-rhamnopyranoside and 4-*O*-(*E*)-*p*-methoxycinnamoyl-α-L-rhamnopyranoside, respectively. In addition, six known phenylpropanoids were authenticated as: (*E*)-cinnamic acid, (*E*)-*p*-methoxycinnamic acid, (*E*)-*p*-methoxycinnamic acid methyl ester, (*E*)-*p*-coumaric acid, (*E*)-caffeic acid, (*E*)-ferulic acid and a phenylalcohol, 2-(3-hydroxy-4-methoxyphenyl)ethanol. These ten phenylpropanoids all attenuated glutamate-induced neurotoxicity when added to primary cultures of rat cortical cells in a dose-dependent manner. These results demonstrate that phenylpropanoids isolated from *S. buergeriana* may exert significant protective effects against glutamate-induced neurodegeneration in primary cultures of cortical neurons.

Keywords: *Scrophularia buergeriana*; Scrophulariaceae; Buergerisides; Phenylpropanoids; Primary cultures of rat cortical cells; L-glutamate; Neuroprotective activity; Excitotoxicity

1. Introduction

Glutamate is found in millimolar levels in the brain and plays a dominant role in central excitatory neurotransmission by acting upon *N*-methyl-D-aspartate (NMDA) receptors and on non-NMDA receptors (Monaghan et al., 1989). Several studies have indicated that such excitatory amino acids as glutamate are involved in neuronal survival, synaptogenesis, neuronal plasticity, learning, and memory processes (Kruk and Pycock, 1991; Monaghan et al., 1989). However, glutamate is also known to cause neuronal cell loss in the central nervous system (Choi, 1988) by two apparently distinct mechanisms, acute and delayed (Choi et al., 1987; Choi, 1985). For example, abnormalities in glutamate neurotransmitter systems may be involved in neurological disorders such as seizures (Zaczek and Coyle, 1982), Alzheimer's disease (Greenamyre and Young, 1989), and ischemia and spinal cord trauma (Albers et al., 1989; Kariman, 1985).

During our search for compounds from natural sources with protective effects against glutamate-induced injury on primary rat cortical cells in culture (Kim et al., 1998), we have discovered that a $CHCl_3$/MeOH extract from the roots of *Scrophularia buergeriana* MIQ. (Scrophulariaceae) exhibited significant neuroprotective activity. The genus *Scrophularia* is represented by over 300 species (Jiansu New Medical College, 1977). The dried roots of *Scrophularia* spp. have been used in Oriental medicine as a treatment for fever, swelling, constipation, pharyngitis, neuritis and laryngitis (Qian et al., 1992; Duck and Ayensu, 1985; Jiansu New Medical College, 1977). A number of iridoid glycosides, phenylpropanoids, terpenoids and fla-

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vonoids are reported from *Scrophularia* spp. (Li et al., 1999; Yamamoto et al., 1993; Kajimoto et al., 1989; Calis et al., 1987). To date, however, no precise correlation has been made between a particular constituent of these roots and an observed pharmacological activity except for effects on immunological activity. The bioactivity-guided fractionation of *S. buergeriana* roots resulted in the isolation of four new phenylpropanoid esters of rhamnose, **1–4**, six known cinnamate derivatives, **5–10** and a phenylalcohol, **11**. Among these compounds, phenylpropanoids, **1–10**, showed protective activity on primary cultures of rat cortical cells after exposure to the excitotoxin, glutamate.

2. Results and discussion

Compound **1**, buergeriside A_1, was obtained as a yellowish needle. The molecular formula of **1** was determined to be $C_{28}H_{30}O_{10}$ by HR-FABMS. The positive FABMS of **1** exhibited significant fragment peaks at m/z 549 $[M + Na]^+$, 527 $[M]^+$ and 179 [methoxycinnamic acid + H]$^+$. The IR spectrum of **1** showed absorption bands for hydroxyl groups (3400 cm^{-1}, *br*), α, β-unsaturated esters ($\nu_{C=O}$ 1690, $\nu_{C=C}$ 1625, ν_{C-O} 1025 cm^{-1}), and aromatic moiety (1600, 1515 cm^{-1}). The ^1H NMR spectral data of **1** confirmed two (*E*)-*p*-methoxycinnamic acid moieties in the molecule, as represented by eight protons of two *para*-substituted aromatic rings at δ 7.41 (*d*, H-2′ and 6′), 7.38 (*d*, H-2″ and 6″), 6.84 (*d*, H-3′ and 5′), and 6.82 (*d*, H-3″ and 5″), four *trans* olefinic protons at δ 7.60 (*d*, H-β′), 7.55 (*d*, H-β″), 6.22 (*d*, H-α′), and 6.18 (*d*, H-α″), and six protons of two methoxy groups at δ 3.83 (*s*, OCH_3), and 3.82 (*s*, OCH_3). In addition, an acetyl singlet at δ 2.15 was observed in ^1H NMR spectrum. The ^1H NMR signals at δ 5.58 (1H, *dd*), 5.32 (1H, *dd*), 5.29 (1H, *t*), 5.20 (1H, *d*), 4.24 (1H, *dd*) and 1.25 (3H, *d*) suggested the presence of a sugar moiety; the ^{13}C NMR signals at δ 92.60, 71.52, 71.21, 69.46, 67.51 and 18.00 indicated that the sugar in **1** was α-L-rhamnopyranose, which was confirmed by GC analysis after alkali hydrolysis of **1**. In the ^1H NMR spectrum, the presence of diagnostic H-3, H-2 and H-4 resonances at δ 5.58, 5.32 and 5.29 proved that the hydroxyl groups at C-3, C-2 and C-4 were acylated. Furthermore, the hydroxyl group at C-1 was free because the ^{13}C NMR signal of C-1 was shifted upfield (δ 92.60) (Barakat et al., 1997). The esterification sites of two separate (*E*)-*p*-methoxycinnamoyl groups were con-

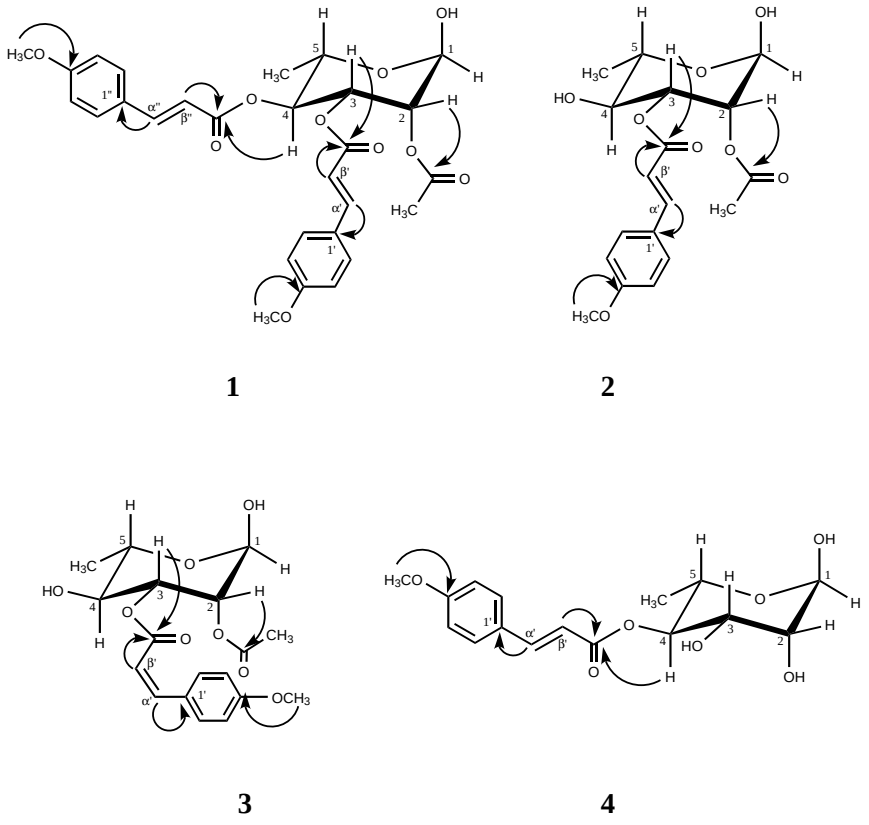

Fig. 1. Selected HMBC correlations for compounds **1–4**.

firmed as the C-3 and C-4 positions of the rhamnose unit by the long-range (HMBC) correlations (Fig. 1) observed between the signals at δ_C 166.82 (carbonyl of (*E*)-*p*-methoxycinnamoyl group) and δ_H 5.58 (H-3), δ_C 166.97 (another carbonyl of (*E*)-*p*-methoxycinnamoyl group) and δ_H 5.29 (H-4), respectively. The acylation site by an acetyl group was determined to be the C-2 position of rhamnose by the HMBC correlation observed between the signals at δ_C 170.78 (carbonyl of acetyl group) and δ_H 5.32 (H-2). Thus, the structure of **1** was assigned as 2-*O*-acetyl-3,4-di-*O*-(*E*)-*p*-methoxycinnamoyl-α-L-rhamnopyranoside.

Compound **2**, buergeriside B_1, was obtained as a white amorphous powder. The molecular formula of **2** was determined to be $C_{18}H_{22}O_8$ by HR-FABMS. The positive FABMS of **2** exhibited significant fragment peaks at *m*/*z* 389 $[M + Na]^+$, 366 $[M]^+$ and 179 [methoxycinnamic acid + H]$^+$. The IR spectrum of **2** showed absorption bands similar to those in compound **1**. The ^{1}H NMR spectral data of **2** showed the presence of an (*E*)-*p*-methoxycinnamoyl group [δ 7.64 (*d*, H-β′), 7.54 (*d*, H-2′ and 6′), 6.95 (*d*, H-3′ and 5′), 6.35 (*d*, H-α′), 3.62, (*s*, OCH_3)], an acetyl group [δ 2.12, *s*] and a sugar moiety [δ 5.26 (1H), 5.22 (1H), 5.01 (1H), 4.04 (1H), 3.69 (1H) and 1.29 (3H)]. The ^{1}H and ^{13}C NMR spectral data indicated that the sugar of **2** was in the α-L-rhamnopyranose form. This L-rhamnopyranose of **2** was confirmed by GC analysis after alkali hydrolysis of **2**. The signals of H-2 and H-3 of rhamnose were shifted 1.4 ppm downfield (δ 5.22 and 5.26, respectively), indicating acylation at these locations. The upfield shifts of H-4 (δ 3.69) also proved the acylation of the hydroxyl groups at C-2 and C-3. The acylations by the acetyl group and the (*E*)-*p*-methoxycinnamoyl group were determined to be at the C-2 and C-3 positions of the rhamnose based on HMBC correlations (Fig. 1) observed between the signals at δ_C 171.61 (carbonyl of acetyl group) and δ_H 5.22 (H-2), δ_C 167.63 (carbonyl of (*E*)-*p*-methoxycinnamic acid) and δ_H 5.26 (H-3), respectively. Thus, the structure of **2** was assigned as 2-*O*-acetyl-3-*O*-(*E*)-*p*-methoxycinnamoyl-α-L-rhamnopyranoside.

Compound **3**, buergeriside B_2, was also obtained as a white amorphous powder. The spectroscopic data of **3** were similar to those of **2** except for two olefinic proton signals at δ 6.89 (*d*, J = 12.66 Hz, H-β′) and 5.79 (*d*, J = 12.66 Hz, H-α′). Besides these olefinic protons, four aromatic protons and three protons of a methoxyl group were attributed to (*Z*)-*p*-methoxycinnamic acid. The esterification sites were determined to be the hydroxyl groups of C-2 and C-3 from acylation-induced shifts at δ 5.18 and 5.23, respectively. The acylation sites were confirmed from the HMBC correlations (Fig. 1) observed between the signals at δ_C 170.36 (carbonyl of acetyl group) and δ_H 5.18 (H-2), δ_C 166.56 (carbonyl of (*Z*)-*p*-methoxycinnamic acid) and δ_H 5.23 (H-3), respectively. Thus, the structure of **3** was assigned as 2-*O*-acetyl-3-*O*-(*Z*)-*p*-methoxycinnamoyl-α-L-rhamnopyranoside.

Compound **4**, buergeriside C_1, was obtained as a pale yellow amorphous powder. The molecular formula of **4** was determined to be $C_{16}H_{20}O_7$ by HR-FABMS. The positive FABMS of **4** exhibited significant fragment peaks at *m*/*z* 347 $[M + Na]^+$, 325 $[M + H]^+$ and 179 [methoxycinnamic acid + H]$^+$. The IR spectrum of **4** also showed absorption bands similar to **1**. The ^{1}H NMR spectral data indicated that **4** contained an (*E*)-*p*-methoxycinnamic acid and a sugar moiety. The ^{1}H and ^{13}C NMR signals indicated that the sugar moiety in **4** was α-L-rhamnopyranose, which was confirmed by GC analysis after alkali hydrolysis of **4**. In the ^{1}H NMR spectrum of **4**, the presence of the diagnostic H-4 resonances at δ 4.76 and upfield shifts of H-3 and H-5 proved that the hydroxyl group of C-4 was acylated. In the ^{13}C NMR spectrum, upfield shift of C-1 (δ 93.93) revealed the free hydroxyl group of C-1 (Barakat et al., 1997). The site of esterification by the (*E*)-*p*-methoxycinnamoyl group was determined to be C-4 of the rhamnopyranose, which was confirmed from the HMBC correlation (Fig. 1) observed between the signals at δ_C 167.51 (carbonyl of (*E*)-*p*-methoxycinnamoyl group) and δ_H 4.76 (H-4′). Thus, the structure of **4** was determined as 4-*O*-(*E*)-*p*-methoxycinnamoyl-α-L-rhamnopyranoside.

The neuroprotective activity of the isolated compounds (**1–11**) was quantified by measuring the release of LDH into the culture media from primary cultures of rat cortical cells injured with glutamate (Table 1). At concentrations of 0.1–10.0 μM, compounds **1–4** blocked the release of LDH from glutamate-insulted primary cultures of rat cortical cells significantly and also preserved the cell survival rate (data not shown). Compounds **5–10** also reduced the release of LDH and showed some improvement in the cell survival rate in a dose-dependent manner. However, phenylalcohol (**11**) did not show significant neuroprotective activity. At higher concentration (above 10 μM), **1** and **4** showed no improvement in the cell survival rate due to inherent cytotoxicity. For example, when all isolated compounds except **1** and **4** were used at concentrations of 0.1–50.0 μM, the percentage cell viability was not affected. By contrast, when compounds **1** and **4** were used at 10.0 μM, the percentage cell viability was significantly lowered (**1**: 85%, $p < 0.05$ and **4**: 79%, $p < 0.01$ of non-treated normal control, respectively).

On the basis of these results, phenylpropanoids bearing a cinnamoyl moiety, including the four newly-isolated phenylpropanoid esters of rhamnose, exerted significant neuroprotective effects on primary cultures of rat cortical cells injured by glutamate.

3. Experimental

3.1. General

^{1}H NMR and ^{13}C NMR spectra were recorded using a JEOL GSX 400 spectrometer operating at 400 and 100 MHz, respectively, with TMS as internal standard. FT-IR spectra were recorded on a Perkin Elmer 1710 spectrophotometer. UV spectra were recorded on a Shimadzu UV-2100 spectrophotometer. EIMS was obtained on a VG Trio 2 spectrometer. FABMS and HR-FABMS spectra were obtained on a VG 70-VSEQ mass spectrometer with direct inlet system using PEG 600/glycerol as a matrix. TLC was carried out on silica gel precoated plates (No. 5715, Merck).

3.2. Plant material

The roots of *S. buergeriana* were purchased from Kyongdong Oriental Herbal Market, Korea in 1996 and identified by Dr. Dae S. Han, Professor Emeritus, College of Pharmacy, Seoul National University. Voucher specimens (SNUPH-0303) documenting this purchase have been deposited in the Herbarium of the Medicinal Plant Garden, College of Pharmacy, Seoul National University.

3.3. Extraction and isolation

Dried roots of *S. buergeriana* (20 kg) were extracted for 3 h with $CHCl_3$–MeOH (1:1) (3 ×, each 10 l) using a reflux apparatus that yielded an extract (1.5 kg) upon removal of the solvent in vacuo. This extract was suspended in water and extracted with CH_2Cl_2. The CH_2Cl_2 extract was evaporated to dryness in vacuo and the resultant CH_2Cl_2 fraction (327.3 g) was suspended in 90% MeOH and extracted with *n*-hexane. The residual 90% MeOH suspension was dried in vacuo to yield 250 g of 90% MeOH fraction. The 90% MeOH fraction, which showed significant neuroprotective activity (67.4% protection against glutamate-induced neurotoxicity at 10 μg/ml, $p < 0.001$) was subjected to chromatography using a silica gel column (2.5 kg, 230–400 mesh, column size 12 × 130 cm) eluted with a step-wise gradient from $CHCl_3$–MeOH (100:1) to MeOH to give 250 fractions (1 l each). Fractions were combined to give eight subfractions after monitoring their TLC (silica gel) pattern using a mix-

Table 1
Neuroprotectant activities of compounds **1**–**10** on primary cultures of rat cortical cells injured by glutamate[a]

Compounds	Cell viability (%)		
	0.1 μM	1.0 μM	10.0 μM
Control	100.0 ± 4.2	100.0 ± 4.2	100.0 ± 4.2
Glutamate-treated[b, c]	0.0 ± 1.3	0.0 ± 1.3	0.0 ± 1.3
Buergeriside A_1 (**1**)	71.6 ± 3.9***	76.4 ± 2.7***	48.9 ± 1.7**
Buergeriside B_1 (**2**)	65.2 ± 2.9**	70.3 ± 1.8**	53.6 ± 2.5**
Buergeriside B_2 (**3**)	35.9 ± 2.3*	48.5 ± 3.3**	45.8 ± 5.2**
Buergeriside C_1 (**4**)	48.3 ± 3.3**	77.6 ± 3.6***	36.6 ± 2.2*
(*E*)-cinnamic acid (**5**)	28.2 ± 2.9	38.5 ± 1.5*	24.7 ± 3.0
(*E*)-*p*-methoxycinnamic acid (**6**)	66.4 ± 2.6***	78.8 ± 3.9***	72.5 ± 1.1***
(*E*)-*p*-methoxycinnamic acid methyl ester (**7**)	25.4 ± 0.8	45.2 ± 3.1**	36.3 ± 2.4*
(*E*)-*p*-coumaric acid (**8**)	10.1 ± 1.7	31.3 ± 2.2*	48.5 ± 3.3**
(*E*)-cafferic acid (**9**)	9.6 ± 2.3	11.7 ± 1.4	33.1 ± 0.5*
(*E*)-ferulic acid (**10**)	54.8 ± 1.1**	66.8 ± 2.8***	57.5 ± 4.0**
MK-801[d]	31.8 ± 7.1*	61.5 ± 2.7***	83.6 ± 4.2***
APV[e]	5.7 ± 1.9	27.8 ± 4.4*	43.6 ± 3.2**
CNQX[f]	28.1 ± 5.6*	47.3 ± 3.6**	61.6 ± 2.7***

[a] Cortical cells cultures were washed with DMEM and incubated with test compounds for 1 h. The cultures were then exposed to 50 μM glutamate + test compound for 30 min and washed. After 24 h incubation in DMEM in the presence of the test compounds, the cultures were assessed for the extent of neuronal damage (*treatment throughout*). The values shown are the mean ± SEM of three experiments.

[b] LDH released from control and glutamate-treated cultures were 110.9 ± 8.3 and 197.6 ± 10.2 mU/ml, respectively. Cell viability was calculated as 100 × (LDH released from glutamate-treated — LDH released from glutamate + test compound)/(LDH released from glutamate-treated — LDH released from control).

[c] Glutamate-treated value differs significantly from the untreated, control at a level of $p < 0.001$.

[d] MK 801: dizocipline maleate, a non-competitive antagonist of the NMDA receptor.

[e] APV: DL-2-amino-5-phosphonovaleric acid, a competitive antagonist of the NMDA receptor.

[f] CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione, non-NMDA receptor antagonist.

Results differ significantly from glutamate-treated, * $p < 0.05$, ** $p < 0.01$, ***, $p < 0.001$.

ture of $CHCl_3$–Me_2CO–MeOH (100:1:1 → 2:1:1) as developing solvent systems. Subfractions 2 and 5 showed significant neuroprotective activity against glutamate-induced neurotoxicity (56.2% and 74.0% protections at 10 μg/ml, respectively, $p < 0.001$). Subfraction 2 (20 g) was applied to on a silica gel column (600 g, 230–400 mesh, column size 4.5 × 100 cm) eluted with a step-wise gradient using *n*-hexane–EtOAc (100:1) to EtOAc, with 125 fractions (500 ml each) to give seven fractions (2-i–2-vii). Compounds **1** (200 mg), **2** (15 mg) and **3** (10 mg) were obtained after the separation of subfraction 2-ii by Sephadex LH-20 with *n*-hexane–CH_2Cl_2–MeOH (5:1:1) and subsequent silica gel column chromatography with $CHCl_3$–Me_2CO (100:1 → 10:1). Crystallization of subfraction 5 from MeOH yielded compound **4** (642 mg) after recrystallization with *n*-hexane–CH_2Cl_2 (1:4). Crystallization of subfraction 2-ii from MeOH yielded compounds **5** (780 mg) and **6** (2 g) after repeated crystallization with *n*-hexane–CH_2Cl_2–MeOH (5:1:1). Compound **7** (150 mg) was obtained as a colorless crystal by crystallization of fraction 2-i with EtOH. Crystallization of fraction 2-iii from MeOH yielded **8** (86 mg) and **10** (120 mg) after repeated crystallization from H_2O, respectively. Compounds **9** (274 mg) and **11** (30 mg) were obtained as a yellow crystal and a white amorphous powder, respectively, from fraction 2-v.

3.3.1. Buergeriside A_1 (**1**)

Yellowish needle, R_f 0.59, ($CHCl_3$–MeOH = 50:1); HR-FABMS: m/z = 526.1843 $[M]^+$, $C_{28}H_{30}O_{10}$ requires 526.1845, FABMS: m/z = 549 $[M + Na]^+$, 526 $[M]^+$, 179 [methoxycinnamic acid + H]$^+$; UV λ_{max}^{MeOH} nm (log ε): 231.6 (2.543), 289.3 (1.825), 311.4 (1.223); IR ν_{max}^{KBr} cm^{-1}: 3400, 1690, 1625, 1600, 1515, 1100–1000; ^1H NMR spectral data (400 MHz, $CDCl_3$): δ 7.60 (1H, *d*, *J* = 15.84 Hz, H-β′), 7.55 (1H, *d*, *J* = 15.84 Hz, H-β″), 7.41 (2H, *d*, *J* = 8.79 Hz, H-2′ and 6′), 7.38 (2H, *d*, *J* = 8.79 Hz, H-2″ and 6″), 6.84 (2H, *d*, *J* = 8.79 Hz, H-3′ and 5′), 6.82 (2H, *d*, *J* = 8.79 Hz, H-3″ and 5″), 6.22 (1H, *d*, *J* = 15.84 Hz, H-α′), 6.18 (1H, *d*, *J* = 15.84 Hz, H-α″), 5.58 (1H, *dd*, *J* = 3.32, 9.99 Hz, H-3), 5.32 (1H, *dd*, *J* = 1.71, 3.32 Hz, H-2), 5.29 (1H, *br t*, *J* = 9.99 Hz, H-4), 5.20 (1H, *d*, *J* = 1.71, H-1), 4.24 (1H, *dq*, *J* = 6.20, 9.99 Hz, H-5), 3.83 (3H, *s*, OCH_3), 3.82 (3H, *s*, OCH_3), 2.15 (3H, *s*, $COOCH_3$) and 1.25 (3H, *d*, *J* = 6.20 Hz, H-6); ^{13}C NMR spectral data (100 MHz, $CDCl_3$): δ 170.78 ($COOCH_3$), 166.97 (COO′), 166.82 (COO″), 161.97 (C-4′), 161.89 (C-4″), 146.29 (C-β′), 146.05 (C-β″), 130.92 (C-2′ and 6′), 130.82 (C-2″ and 6″), 127.36 (C-1′), 127.27 (C-1″), 115.30 (C-α′), 115.19 (C-α″), 114.37 (C-3′ and 5′), 114.26 (C-3″ and 5″), 92.60 (C-1), 71.52 (C-2), 71.21 (C-3), 69.46 (C-4), 67.51 (C-5), 56.15 (OCH_3′), 55.40 (OCH_3″), 21.78 ($COOCH_3$), 18.00 (C-6).

3.3.2. Buergeriside B_1 (**2**)

White amorphous powder, R_f 0.37, ($CHCl_3$–Me_2CO = 5:1); HR-FABMS: m/z = 366.3678 $[M]^+$, $C_{18}H_{22}O_8$ requires 366.3682; FABMS: m/z = 389 $[M + Na]^+$, 366 $[M]^+$, 179 [methoxycinnamic acid + H]$^+$; UV λ_{max}^{MeOH} nm (log ε): 231.4 (2.524), 278.9 (2.353), 311.3 (1.057); IR ν_{max}^{KBr} cm^{-1} : 3398, 1687, 1624, 1598, 1515, 1100–1000; ^1H NMR spectral data (400 MHz, CD_3OD): δ 7.64 (1H, *d*, *J* = 16.00 Hz, H-β′), 7.54 (2H, *d*, *J* = 8.79 Hz, H-2′ and 6′), 6.95 (2H, *d*, *J* = 8.79 Hz, H-3′ and 5′), 6.35 (1H, *d*, *J* = 15.84 Hz, H-α′), 5.26 (1H, *dd*, *J* = 3.40, 9.75 Hz, H-3), 5.22 (1H, *dd*, *J* = 1.71, 3.40 Hz, H-2), 5.01 (1H, *d*, *J* = 1.71 Hz, H-1), 4.04 (1H, *dq*, *J* = 9.75, 6.33 Hz, H-5), 3.69 (1H, *br t*, *J* = 9.75 Hz, H-4), 3.82 (3H, *s*, OCH_3), 2.12 (3H, *s*, $COOCH_3$), 1.29 (3H, *d*, *J* = 6.33 Hz, H-6); ^{13}C NMR spectral data (100 MHz, CD_3OD): δ 172.42 ($COOCH_3$), 168.54 (COO′), 164.37 (C-4′), 147.15 (C-β′), 132.00 (C-2′ and 6′), 129.71 (C-1′), 116.06 (C-α′), 115.27 (C-3′ and 5′), 93.98 (C-1), 71.56 (C-3), 71.23 (C-2), 70.88 (C-4), 67.86 (C-5), 54.41 (OCH_3′), 21.53 ($COOCH_3$), 16.67 (C-6).

3.3.3. Buergeriside B_2 (**3**)

White amorphous powder, R_f 0.42, ($CHCl_3$–Me_2CO = 5:1); HR-FABMS: m/z = 336.3677 $[M]^+$, $C_{18}H_{22}O_8$ requires 366.3682; FABMS: m/z = 389 $[M + Na]^+$, 366 $[M]^+$, 179 [methylcinnamic acid + H]$^+$; UV λ_{max}^{MeOH} nm (log ε): 231.4 (2.520), 277.9 (2.340), 311.3 (1.036); IR ν_{max}^{KBr} cm^{-1}: 3398, 1687, 1624, 1598, 1515, 1100–1000; ^1H NMR spectral data (400 MHz, CD_3OD): δ 7.68 (2H, *d*, *J* = 8.79 Hz, H-2′ and 6′), 6.89 (1H, *d*, *J* = 12.66 Hz, H-β′), 6.88 (2H, *d*, *J* = 8.79 Hz, H-3′ and 5′), 5.79 (1H, *d*, *J* = 12.66 Hz, H-α′), 5.23 (1H, *dd*, *J* = 3.40, 9.75 Hz, H-3), 5.18 (1H, *dd*, *J* = 1.71, 3.40 Hz, H-2), 4.99 (1H, *d*, *J* = 1.71 Hz, H-1), 4.02 (1H, *dq*, *J* = 9.75, 6.33 Hz, H-5), 3.68 (1H, *br t*, *J* = 9.75 Hz, H-4), 3.81 (3H, *s*, OCH_3), 2.00 (3H, *s*, $COOCH_3$), 1.26 (3H, *d*, *J* = 6.33 Hz, H-6); ^{13}C NMR spectral data (100 MHz, CD_3OD): δ 171.56 ($COOCH_3$), 168.23 (COO′), 163.26 (C-4′), 145.34 (C-β′), 134.06 (C-2′ and 6′), 129.74 (C-1′), 117.26 (C-α′), 114.28 (C-3′ and 5′), 93.93 (C-1), 71.43 (C-3), 71.00 (C-2), 70.30 (C-4), 67.90 (C-5), 54.31 (OCH_3′), 21.47 ($COOCH_3$), 16.47 (C-6).

3.3.4. Buergeriside C_1 (**4**)

Pale yellow powder, R_f 0.47, ($CHCl_3$–Me_2CO–MeOH = 5:1:1); HR-FABMS: m/z = 325.1299 $[M + H]^+$, $C_{16}H_{21}O_7$ requires 325.1321; FABMS: m/z = 347 $[M + Na]^+$, 325 $[M + H]^+$, 179 [methoxycinnamic acid + H]$^+$, UV λ_{max}^{MeOH} nm (log ε): 231.6 (2.543), 311.4 (1.223); IR ν_{max}^{KBr} cm^{-1}: 3400, 1690, 1625, 1600, 1515, 1100–1000; ^1H NMR spectral data (400 MHz, $CDCl_3$–CD_3OD = 2:1): δ 7.44 (1H, *d*, *J* = 15.84 Hz, H-β′), 7.28 (2H, *d*, *J* = 8.79 Hz, H-2′ and

6′), 6.69 (2H, *d*, *J* = 8.79 Hz, H-3′ and 5′), 6.13 (1H, *d*, *J* = 15.84 Hz, H-α′), 4.88 (1H, *d*, *J* = 1.71 Hz, H-1), 4.76 (1H, *br t*, *J* = 9.75 Hz, H-4), 3.86 (1H, *dq*, *J* = 6.33, 9.75 Hz, H-5), 3.75 (1H, *dd*, *J* = 9.75, 3.42 Hz, H-3), 3.67 (1H, *dd*, *J* = 1.71, 3.42 Hz, H-2), 3.62 (3H, *s*, OCH_3), 1.20 (3H, *d*, *J* = 6.33 Hz, H-6); ^{13}C NMR spectral data (100 MHz, $CDCl_3$–CD_3OD = 2:1): δ 167.51 (COO′), 161.26 (C-4′), 145.05 (C-α′), 129.49 (C-2′ and 6′), 126.54 (C-1′), 114.55 (C-α′), 113.96 (C-3′ and 5′), 93.83 (C-1), 74.34 (C-4), 71.28 (C-2), 68.94 (C-3), 65.52 (C-5), 54.86 (OCH_3′), 16.95 (C-6).

3.3.5. Identification of compounds **5–11**

The structures of the known compounds were identified as (*E*)-cinnamic acid (**5**), (*E*)-*p*-methoxycinnamic acid (**6**), (*E*)-*p*-methoxycinnamic acid methyl ester (**7**), (*E*)-*p*-coumaric acid (**8**), (*E*)-caffeic acid (**9**), (*E*)-ferulic acid (**10**) and 2-(3-hydroxy-4-methoxyphenyl)ethanol (**11**) by IR, MS, ^1H and ^{13}C NMR spectral data. These structures were confirmed by comparison with literature data (Helfrich and Rimpler, 1999; Luger et al., 1996; Kajimoto et al., 1989; Otsuka et al., 1989; Calis et al., 1988a, 1988b).

3.4. Alkali hydrolysis and GC analysis of **1–4**

Compounds **1–4** (4 mg each) were dissolved separately in 2 ml of 0.5 M KOH in a 10 ml round bottomed flask and heated at 60°C for 1 h. To isolate the sugar and the aglycone for further analysis, the reaction mixtures from **1–4** were evaporated to half their volumes to remove MeOH, and then were extracted several times with $CHCl_3$ by shaking vigorously in a test tube. In each case, the aglycone separated into the $CHCl_3$ fraction and the sugar into H_2O. For the analysis of sugar by GC, equal portions of sugar and of silylating agent (Tri-Sil/BSA in DMF, Pierce Chemicals, Rockford, IL) were vortexed and reacted for 1 h at 73°C. A GC 353 (GL Science, Japan) system equipped with a FID and DB-1 capillary column (0.25 mm × 30 m, i.d., film thickness 0.25 mm) was used. The GC conditions were as follows: injector temperature of 250°C; column temperature of 240°C; detector temperature of 250°C; a flow rate of 2.5 ml/min using He as a carrier gas; R_t (min): 9.61 (rhamnose).

3.5. Cell culture

Primary cultures of mixed cortical cells containing both neuronal and glial cells were prepared from late fetal (17–19-day gestation in utero) Sprague-Dawley rats as described previously (Kim et al., 1998). The cortical cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% heat-inactivated fetal calf serum (Gibco) with penicillin (100 IU/ml; Sigma) and streptomycin (10 μg/ml; Sigma) at 37°C in a humidified atmosphere of 95% air–5% CO_2. After 3 days in culture, non-neuronal cell division was halted by adding 5-fluorodeoxyuridine (50 μM; Sigma). Cultures were allowed to mature for 2 weeks.

3.6. Neurotoxicity

All tested compounds were dissolved in DMSO (final culture concentration, 0.1%). Preliminary studies indicated that the solvent had no effect on cell viability at the concentration used. Cortical cell cultures were washed with DMEM and incubated with test compounds for 1 h. The cultures were then exposed to 50 μM glutamate + test compound for 30 min and washed. After 24 h incubation in DMEM in the presence of the test compounds, the cultures were assessed for the extent of neuronal damage.

3.7. Assessment of neurotoxicity

Neuronal viability was quantified by measuring dehydrogenase activity retained in living cells using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Neuronal integrity was assessed by spectrophotometric measurement of the efflux of lactate dehydrogenase (LDH) into the culture medium by the methods described in our previous report (Kim et al., 1998).

3.8. Statistical analysis

The evaluation of statistical significance was determined by the 'ANOVA' test with a value of $p < 0.05$ considered to be statistically significant.

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