A New Neuroprotective Pinusolide Derivative from the Leaves of *Biota orientalis*

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A new pinusolide derivative, 15-methoxypinusolidic acid (1), and another new isopimarane diterpene, *ent*-isopimara-15-en-3α,8α-diol (2) with three known diterpenes, lambertianic acid (3), isopimara-8(9),15-dien-18-oic acid (4) and isopimara-7(8),15-dien-3β,18-diol (5) were isolated from the 90% MeOH fraction of *Biota orientalis* (L.) Endl. (Cupressaceae) leaves. Chemical structures of 1—5 were elucidated by analyses of their spectral data, including the two-dimensional (2D) NMR technique. Compound 1 showed significant protective activity against glutamate-induced neurotoxicity in primary cultures of rat cortical cells.

Key words *Biota orientalis*; 15-methoxypinusolidic acid; neuroprotective activity; *ent*-isopimara-15-en-3α,8α-diol; glutamate; Cupressaceae

During our search for potential natural products against glutamate-induced neurotoxicity, we have discovered that the 90% MeOH fraction of *Biota orientalis* leaves showed significant protective activity. Glutamate-induced neurotoxicity has been implicated in the neuronal cell death of such neurological disorders as ischemia,[1] trauma,[2] seizures,[3] and Alzheimer's disease.[4—6]

Dried leaves of *B. orientalis* (L.) Endl. (=*Thuja orientalis* L., *Platycladus orientalis* (L.) Franco) (Cupressaceae) have been used as a hemostatic, expectorant and hypotensor in Korean folk medicine.[7] A number of flavonoids,[8—10] terpenes[11—14] and phenolics[15,16] were previously reported as chemical constituents of this tree. However, to date, there is no report on the chemical constituent of *B. orientalis* possessing neuroprotective activity. Thus, we attempted to isolate neuroprotective compounds from the 90% MeOH fraction of *B. orientalis* leaves using primary cultures of rat cortical cells injured by excess glutamate as an *in vitro* assay system.[17—19]

In the present study, we isolated a new neuroprotective pinusolide derivative (**1**) against glutamate-induced neurotoxicity in primary cultures of rat cortical cells, and another new isopimarane diterpene (**2**) with three known diterpenes (**3**—**5**) from 90% MeOH extract of *B. orientalis* leaves using a bioactivity-guided fractionation technique. The chemical structure of three known compounds (**3**—**5**) were identified as lambertianic acid (**3**), isopimara-8(9),15-dien-18-oic acid (**4**) and isopimara-7(8),15-dien-3β,18-diol (**5**) by comparison of their spectral data with those reported in the literature, respectively.[20—22]

Compound **1** was obtained as yellowish viscous oil. The HR-EI-MS (highresolution electron impactmass spectra) showed a molecular ion at *m*/*z* 362.2088 (Calcd 362.2093) corresponding to the molecular formula $C_{21}H_{30}O_5$. The IR spectrum indicated the presence of a carboxylic group (3450, 1665 cm^{-1}) and an α,β-unsaturated lactone (1750 cm^{-1}). The ^{13}C (Table 1) and DEPT (distortionless enhancement by polarization transfer) NMR spectra indicated that **1** possessed a diterpene skeleton having a methoxyl and a carboxyl group. The ^{1}H (Table 1) and ^{1}H–^{1}H COSY (correlation spectroscopy) NMR spectral data showed that **1** had the characteristic pattern of a labdane-type diterpene with exo-methylene protons (δ 4.55, 4.91) and two tertiary methyl protons (δ 0.62, 1.26). Additionally, the ^{1}H-NMR signals at δ 5.78, 6.78, and ^{13}C-NMR signals at δ 102.0, 139.1, 141.7 and 171.0 demonstrated the presence of an α,β-unsaturated γ-lactone. All the spectral data resembled those of another pinusolide derivative, 15-hydroxypinusolidic acid, previously isolated from *B. orientalis*.[23] The complete structure of **1** was determined from HMBC (heteronuclear multiple bond correlation) and NOESY (nuclear Overhauser effect spectroscopy) spectral data. As shown in Fig. 2, the carboxyl carbon at δ 183.9 (C-19) showed correlations with Me-18, H-3 and H-5, and the quaternary carbon at δ 44.0 (C-4) showed correlations with Me-18, H-2 and H-3. Another quaternary carbon at δ 147.4 (C-8) showed correlations with H-6, H-7, H-9 and H-17, and the exo-methylene carbon at δ 106.7 (C-17) showed correlations with H-7 and H-9. The methine car-

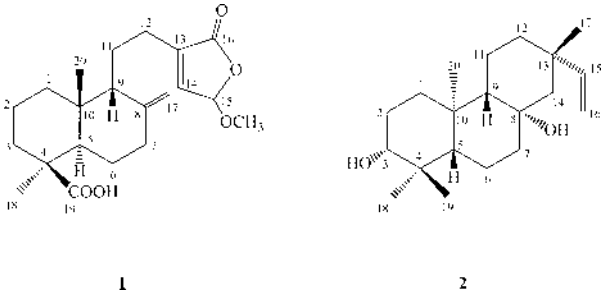

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Fig. 1. Compounds Isolated from *B. orientalis*

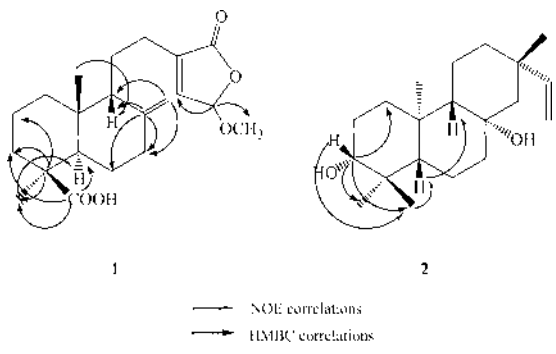

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Fig. 2. HMBC and NOESY Correlations of Compounds **1** and **2**

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

Position	**1**		**2**	
	δ ^{1}H *a)*	δ ^{13}C *b)*	δ ^{1}H *a)*	δ ^{13}C *b)*
1	1.11 m, 1.85 m	39.0	1.75 m	37.7
2	1.52 m, 1.56 m	19.7	1.7 m	27.1
3	1.06 m, 2.29 m	37.8	3.17 dd (10, 6)	78.9
4		44.0		38.9
5	1.34 m	56.3	0.82 m	55.5
6	1.95 m	25.9	1.62 m	17.5
7	1.92 m, 2.45 m	38.4	1.72 m	43.5
8		147.4		72.3
9	1.65 br s	55.6	0.83 m	56.7
10		40.4		36.9
11	1.64 m, 1.80 m	21.7	1.51 m	17.1
12	2.12 m, 2.50 m	24.5	1.30 m	38.0
13		139.1		36.5
14	6.78 s	141.7	1.36 m	51.4
15	5.78 s	102.0	5.69 dd (10.5, 17.5)	151.5
16		171.0	4.78 dd (10.5, 1.5)	108.6
16′			4.84 dd (17.5, 1.5)	
17	4.55 br s	106.7	1.15 s	24.2
17′	4.91 br s			
18	1.26 s	28.8	0.95 s	28.2
19		183.9	0.78 s	15.5
20	0.62 s	12.8	0.95 s	15.6
OCH_3	3.55 s	57.9		

a) Values were recorded at 400 MHz, δ in ppm, J (in parentheses) in Hz; assignments from ^{1}H–^{1}H COSY, HMQC, HMBC and NOESY data. *b*) Values were recorded at 100 MHz, δ in ppm; assignments from DEPT, HMQC, and HMBC experiments.

Table 2. Neuroprotective Activity of Compound **1** in Primary Cultures of Rat Cortical Cells Insulted by Glutamate

Compound	Cell viability (%)		
	0.1 μM	1.0 μM	10 μM
Control	100.0±0.1	100.0±0.1	100.0±0.1
Glutamate-treated *a)*	0.1±0.1	0.1±0.1	0.1±0.1
15-Methoxypinusolidic acid (**1**)	35.3±3.5*	48.3±3.9**	72.7±4.7***

Compound **1** was pretreated with the indicated concentration 1 h before glutamate insult. Cell viability was quantified 24 h after glutamate insult. Control is the value for cultures not exposed to glutamate. Values are the mean±S.D. of three separate cultures. Optical density (OD) of control and glutamate-treated cultures was 0.95±0.12 and 0.67±0.09, respectively. The neuroprotective activity of MK-801 *b)* used as a positive control was 91.7±3.6*** at the concentration of 10 μM. Mean value is significantly different (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) from the value of the glutamate-treated cultures. *a*) Glutamate-treated values differ from the control at a level of $p<0.001$. *b*) MK-801: dizocipline maleate, a non-competitive antagonist of the NMDA receptor.

bon at δ 102.0 (C-15) of the lactone moiety showed correlations with the 15-methoxyl group and H-14. From the above results, **1** was identified as 15-methoxy-8(17),13-labdadien-16,15-olide-19-oic acid and was named 15-methoxypinusolidic acid.

Compound **2** was obtained as a white amorphous powder. The HR-EI-MS showed a molecular ion at *m/z* 306.2568 (Calcd 306.2559) corresponding to the molecular formula $C_{20}H_{34}O_2$. The IR spectrum indicated the presence of a hydroxyl group (3438, 1023 cm^{-1}). The ^{13}C, ^{1}H (Table 1) and DEPT NMR spectral data indicated that **2** was a pimarane diterpene possessing one oxygenated methine carbon and one oxygenated quaternary carbon. In the ^{13}C-NMR spectral data, significant β-effects (downfield shifts) were observed for δ 27.1 (C-2) and δ 38.9 (C-4), while γ-effects (upfield shifts) occur for δ 37.7 (C-1) and δ 28.2 (C-18). A proton at δ 3.17 (H-3) appears as a doublet doublet with J_{ax-ax} 10 Hz and J_{ax-eq} 6 Hz, indicating to equatorial disposition of the OH group at C-3, and this was confirmed by NOESY spectral data. Also, chemical shifts of C-7, C-9 and C-14 exhibited downfield shifts compared to that of the corresponding carbons on isopimarane diterpene without an OH group at C-8. All signals of ^{1}H- and ^{13}C-NMR spectra were assigned based on HMQC (^{1}H-detected heteronuclear multiple quantum coherence) and HMBC spectral data. From the above spectral data and those of related compound, **2** was identified as *ent*-isopimara-15-en-3α,8α-diol.[24,25]

The neuroprotective activities of the five diterpenes were evaluated by the MTT assay measuring the viability in primary cultured rat cortical cells after glutamate insult. Among these five diterpenes, only 15-methoxypinusolidic acid (**1**) significantly attenuated neuronal cell death induced by glutamate in cultures. It was found to exhibit the most potent neuroprotective activity at a concentration of 10 μM (Table 2).

Experimental

General Experimental Procedures For column chromatography, silica gel 60 (Merck Art. 9385) and Sephadex LH-20 (Pharmacia) were used. The IR spectrum was obtained on a Perkin Elmer 1710 spectrophotometer. LR (low resolution)- and HR-MS were measured on a JEOL JMS AX 505WA spectrometer, and optical rotations on a JASCO DIP-100 polarimeter. The ^{1}H- and ^{13}C-NMR spectra were taken in a JEOL GSX 300 spectrometer operating at 400 and 100 NMR with tetramethylsilane (TMS) as an internal standard, respectively. Standard pulse sequences were used for ^{1}H–^{1}H COSY (PO=45 or 90°), NOESY (mixing time varying between 0.5 and 1.2 s) and HMBC [1/2J=70 ms for $J_{C,H}$=7 Hz]. The experiments were carried out at 300 K. An internal lock was applied and the reference was set to the solvent peak ($CDCl_3$, 7.24 for ^{1}H and 77.2 for ^{13}C). Heat-inactivated fetal calf serum was obtained from Hyclone Laboratories (Logan, UT, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), glutamate, MK 801, penicillin, streptomycin, cytosine-β-D-arabinofuranoside, and MTT were all purchased from Sigma Chemical Co. (St, Louis, MO, U.S.A.).

Plant Material The leaves of *B. orientalis* were purchased from Kyungdong Market, Seoul, Korea, and identified by Dr. Dae Suk Han, an emeritus professor of the College of Pharmacy, Seoul National University. A voucher specimen has been deposited in the Herbarium of the Medicinal Plant Garden, College of Pharmacy, Seoul National University.

Extraction and Isolation The dried leaves of *B. orientalis* (9 kg) were extracted three times with 80% MeOH in an ultrasonic apparatus. Removal of the solvent *in vacuo* yielded an 80% MeOH extract (830 g). The 80% MeOH extract was suspended in distilled water and then partitioned with CH_2Cl_2. The CH_2Cl_2 layer was suspended in 90% MeOH and then partitioned with *n*-hexane. Column chromatography of the 90% MeOH fraction, showing neuroprotective activity (48.6% at 50 μg/ml), over silica gel using an *n*-hexane–EtOAc–MeOH mixture with increasing polarity, yielded 30 fractions (fr. 1—fr. 30). Among these fractions, fr. 10 showed the most potent neuroprotective activity (54.8% at 50 μg/ml) against glutamate insult. Consequently, fr. 10 was subjected to column chromatography over silica gel to yield 15 fractions (fr. 10-1—fr. 10-15) using an *n*-hexane–EtOAc mixture with increasing polarity. Column chromatography of fr. 10-8 over C18 resin resulted in 10 fractions (fr. 10-8-1—fr. 10-8-10). Among the above ten fractions, compound **1** (65 mg) was obtained by an additional purification step on the RP-HPLC from fr. 10-8-7. The HPLC (Hitachi L-6200, Japan) system equipped with a UV visible detector and a Microsorb C18 80-299-C5 semi-preparative column (Rainin Inst. Co.) was used for purification. The mobile phase for HPLC was a mixture of H_2O, MeOH and AcCN (45 : 20 : 35) and, was detected at 220 nm. Compound **2** (24 mg) was isolated from fr. 10-14 by column chromatography over silica gel using a mixture of *n*-hexane–EtOAc as an eluting solvent. Compound **3** was isolated from fr. 10-8-3 by column chromatography on Sephadex LH-20 using MeOH. Compounds **4** and **5** were isolated from fr. 10-8-5 and fr. 10-8-6 by column chromatography on silica gel using a mixture of *n*-hexane–EtOAc as an eluting solvent.

15-Methoxy-8(17),13-labdadien-16,15-olide-19-oic acid (**1**): Yellowish oil. $[\alpha]_D^{25}$ +43° (c=0.83, $CHCl_3$). IR (KBr) ν_{max}: 3450, 2923, 1750, 1665,

1110 cm^{-1}. ^1H- ($CDCl_3$) and ^{13}C-NMR ($CDCl_3$): see Table 1. EI-MS (70 eV) *m/z* (rel. int.): 362 $[M]^+$ (3), 344 $[M-H_2O]^+$ (6), 317 $[M-COOH]^+$ (13), 285 (13), 235 (22), 189 (40), 128 (100), 96 (59), 81 (28). HR-EI-MS *m/z* $[M]^+$: 362.2088 (Calcd for $C_{21}H_{30}O_5$, 362.2093).

ent-Isopimara-15-en-3α,8α-diol (**2**): White amorphous powder. $[\alpha]_D^{25}$ +3° (*c*=1.0, $CHCl_3$). IR (KBr) ν_{max}: 3438, 2917, 1594, 1383, 1023 cm^{-1}. ^1H- ($CDCl_3$) and ^{13}C-NMR ($CDCl_3$): see Table 1. EI-MS (70 eV) *m/z* (rel. int.): 306 $[M]^+$ (45), 292 (100), 288 $[M-H_2O]^+$ (78), 270 (49), 237 (35), 189 (28), 148 (50), 136 (61), 105 (51), 81 (77), 69 (65). HR-EI-MS *m/z* $[M]^+$: 306.2568 (Calcd for $C_{20}H_{34}O_2$, 306.2559).

Cell Culture Primary cultures of rat cortical cells containing both neurons and non-neuronal cells such as astrocytes and glia were prepared from 17—19-d-old fetal rats (Sprague-Dawley), as previously described.[17—19] Cortical cells were seeded onto a collagen-coated 48-well-plate at a density of 1×10^6 cells/ml. The cultures were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum (Gibco) with 100 IU/ml penicillin and 10 μg/ml streptomycin at 37 °C in a humidified atmosphere of 95% air–5% CO_2. Cytosine-β-D-arabinofuranoside (1 μM) was added to the culture medium 3 d after plating to inhibit the proliferation of non-neuronal cells. Cultures were allowed to mature for 14, 15 d before being used for experiments.

Glutamate-Induced 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. For evaluation the neuroprotective activity of samples, cortical cell cultures were rinsed once with HBSS (Hanks' balanced salt solution) and then changed to a serum-free DMEM. Cortical cell cultures were pre-treated with test compounds for 1 h and then exposed to 50 μM glutamate. After incubation for an additional 24 h, neuronal viability of the cultures was assessed.

Assessment of Neurotoxicity Neuronal viability was assessed by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, which reflects the mitochondrial enzyme function of cells. Cell viability was calculated as 100×[(OD of test compound+glutamate-treated culture)−OD of glutamate-treated culture/OD of control culture−OD of glutamate-treated culture].

Statistical Analysis Data were evaluated for statistical significance using an analysis of variance (ANOVA) with a computerized statistical package. The data were considered to be statistically significant if the probability value was <0.05.

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