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Monoamine Oxidase Inhibitory Naphthoquinones from the Roots of *Lithospermum erythrorhizon*

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Activity-guided fractionation of a hexane-soluble extract of the roots of *Lithospermum erythrorhizon*, using a mouse brain monoamine oxidase (MAO) inhibition assay, led to the isolation of two known naphthoquinones, acetylshikonin and shikonin, and a furylhydroquinone, shikonofuran E. These compounds were shown to inhibit MAO with IC_{50} values of 10.0, 13.3, and 59.1 μM, respectively. Although no specificity for MAO-A and MAO-B was shown by acetylshikonin and shikonin, a Lineweaver-Burk plot analysis indicated that the inhibition was competitive for both MAO-A and MAO-B activity.

Key words: *Lithospermum erythrorhizon*, Boraginaceae, Naphthoquinone, Monoamine oxidase inhibitor

INTRODUCTION

Lithospermum erythrorhizon Sieb. et Zucc. (Boraginaceae) is a perennial herb found in Korea, China, and Japan. The dried roots of this plant have been used for antimicrobial, antitumor, immunostimulating, anti-inflammatory, and wound-healing purposes in oriental medicine (Sankawa *et al.*, 1977; Tanaka *et al.*, 1986; Tang and Eisenbrand, 1992). This plant contains a number of naphthoquinone pigments such as shikonin, acetylshikonin, β-hydroxyisovalerylshikonin, isovalerylshikonin, and isobutylshikonin, as well as the glycans lithosperman A, B, and C (Tang and Eisenbrand, 1992).

Monoamine oxidase (MAO), a flavin-containing enzyme localized in the outer membrane of neuronal and glial cells, among others, plays a critical role in the regulation of monoamine neurotransmitters such as serotonin, noradrenaline and dopamine. MAO isoenzymes are classified on the basis of their substrate preference, sensitivity towards specific inhibitors, and tissue distribution (Abell and Kwan, 2001; Johnston, 1968; Murphy, 1978). MAO-A preferentially deaminates serotonin, noradrenaline, and adrenaline and is selectively inhibited by clorgyline, whereas MAO-B preferentially deaminates dopamine, β-phenylethylamine, and benzylamine and is irreversibly inhibited by L-deprenyl (Bach *et al.*, 1988; Knoll and Magyar, 1972). Selective MAO-A inhibitors have been used clinically in the treatment of depression and anxiety, while MAO-B inhibitors have been used as coadjutant agents in the treatment of Parkinson's and Alzheimer's diseases (Foley *et al.*, 2000; Murphy *et al.*, 1984; Wouters, 1998). To date, many plant-derived and synthetic compounds such as isoquinoline (Thull *et al.*, 1995), xanthones (Nunez *et al.*, 2004; Suzuki *et al.*, 1981), stilbenoids (Han *et al.*, 1990), and coumarins (Jo *et al.*, 2002) have been identified as MAO inhibitors.

In our ongoing search for MAO inhibitors derived from natural sources, it was found that an extract of *L. erythrorhizon* strongly inhibited MAO activity. Bioactivity-guided fractionation of the *n*-hexane extracts of the roots of *L. erythrorhizon* followed by repeated column chromatography led to the isolation of two known naphthoquinones, acetylshikonin and shikonin and a furylhydroquinone, shikonofuran E. The inhibitory effects on mouse brain MAO of these three known isolates are described herein.

MATERIALS AND METHODS

General experimental procedures

Melting points were measured without correction on a

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Büchi model B-540. Optical rotations were determined using a JASCO DIP-370 polarimeter at 25°C. IR spectra were obtained using a JASCO FT/IR 300E spectrometer. UV spectra were obtained using a Milton Roy 3000 spectrometer. ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra were obtained using a Bruker AMX 500 MHz NMR spectrometer, using DMSO-d_6 or $CDCl_3$ as a solvent. ESI-MS and EI-MS were measured using a Finnigan Navigator and a Hewlett Packard 5989A mass spectrometer, respectively. Open column chromatography was performed using a silica gel (Kieselgel 60, 70-230 mesh and 230-400 mesh, Merck), and thin layer chromatography (TLC) was performed using a pre-coated silica gel 60 F_{254} (0.25 mm, Merck).

Kynuramine [3-(2-aminophenyl)-3-oxopropanamine], clorgyline [*N*-methyl-*N*-propargyl-3-(2,4-dichlorophenoxy) propylamine], L-depreyl [R(-)-*N*-a-dimethyl-*N*-(2-propynyl) phenethylamine], 4-hydroxyquinoline, and iproniazid were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Plant materials

The dried roots of *L. erythrorhizon* were purchased at an herbal drug store in Cheongju, Korea, in April 2003 and identified by Prof. Kyong Soon Lee, a plant taxonomist at Chungbuk National University. A voucher specimen (No.010405) was deposited in the Herbarium at the College of Pharmacy, Chungbuk National University, Korea.

Animals

The male ICR mice were purchased from Samyook Animal Center (Soowon, Korea) and maintained in accordance with the guidelines for animal care and use of laboratory animals, Chungbuk National University, Korea.

Extraction and activity-guided isolation

The dried roots of *L. erythrorhizon* (3 kg) were extracted using MeOH three times at room temperature. The MeOH extract (200 g) was obtained by evaporation of the solvent and partitioned with hexane and water. The dried hexane extract (20 g) exhibiting 60% inhibition on MAO activity at 100 μg/mL was subjected to a silica gel column (4.5×50 cm) chromatography using a hexane-EtOAc step gradient system (50:1, 30:1, 20:1, 10:1, 5:1, 2:1, 0:1, each 1.5 L) to provide seven fractions (A1-A7). The MAO inhibitory effects of the seven fractions (A1-A7) were 76.7, 69.5, 82.9, 92.8, 88.5, 93.7 and 20.9% at a concentration of 100 μg/mL, respectively. Acetylshikonin (500 mg) was isolated from fraction A3 by recrystallization in hexane. The other active fraction, A4 (2 g), was repeatedly chromatographed on a silica gel column (4×45 cm) with a hexane-acetone step gradient system (30:1, 20:1, 10:1, 5:1, 0:1, each 1.0 L) to produce shikonin (100 mg). Fraction A6 was further chromatographed over a silica gel column (4×45 cm), eluted with hexane-EtOAc (10:1 and 5:1), to produce shikonofuran E (50 mg).

Acetylshikonin

Dark brown needle crystal; mp 106-107°C; $[\alpha]_{546}^{25}$ +230 (*c* 0.1, isopropanol); UV (MeOH): λ_{max} nm (log ε) = 275 (3.8), 485 (3.6), 520 (3.8), 561 (3.6); IR (KBr): ν_{max} = 3470, 3080, 2995, 2920, 1745, 1625, 1595, 1470, 860, 800; ESI-MS: *m/z* 353 $[M+Na]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$), ^{13}C-NMR (75 MHz, $CDCl_3$), and DEPT spectra, and physical constants were identical to previous reports (Cho *et al.*, 1999; Inoue *et al.*, 1985).

Shikonin

Dark brown needle crystal; mp 145-147°C; $[\alpha]_{546}^{25}$ +295 (*c* 0.1, isopropanol); UV (MeOH): λ_{max} nm (log ε) = 275 (3.9), 485 (3.8), 518 (3.9), 556 (3.6); IR (KBr): ν_{max} = 3400, 3250, 2985, 2920, 1625, 1595, 1550, 1470, 1220, 1090, 790; ESI-MS: *m/z* 311 $[M+Na]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$), ^{13}C-NMR (75MHz, $CDCl_3$), and DEPT spectra, and physical constants were identical to previous reports (Cho *et al.*, 1999; Inoue *et al.*, 1985).

Shikonofuran E

Colorless oil; $[\alpha]_D^{25}$ -65° (*c* 0.1, MeOH); UV (MeOH): λ_{max} nm (log ε) = 214 (4.3), 264 (4.0), 269 (4.1), 281 (4.2), 323 (4.0) IR (film): ν_{max} = 3400, 1700, 1510, 1440, 1220, 1160, 870; EI-MS: *m/z* 356 $[M]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$), ^{13}C-NMR (75 MHz, $CDCl_3$), and DEPT spectra, and physical constants were identical to previous reports (Yazaki *et al.*, 1986).

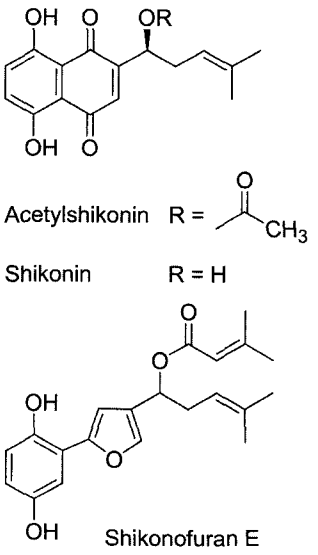

Fig. 1. Chemical structures of isolated compounds from *L. erythrorhizon*

MAO preparation from mouse brain

The crude MAO was prepared using Naoi's method with minor modifications (Naoi and Nagatsu, 1978; Ro *et al.*, 2001). In brief, the male ICR mice (20-25 g) were decapitated, and the brains were homogenized in an ice-cold 20 ml of 0.25 M sucrose solution containing a 10 mM potassium phosphate buffer (pH 7.4) and centrifuged at 1,200 g for 5 min at 4°C. The supernatant was collected and further centrifuged at 16,000 g for 20 min. The crude mitochondrial pellet was washed using a 10 mM sodium phosphate buffer (pH 7.4) and suspended in the same buffer. The Lowry method was used to determine protein amounts using bovine serum albumin as the standard (Lowry *et al.*, 1951).

Assay for MAO activity

The MAO activity, with kynuramine as a substrate, was assayed using a modification of Kraml's fluorometric method (Kraml, 1965; Ro *et al.*, 2001). Fluorescence of 4-hydroxyquinoline, which was formed from kynuramine by MAO, was measured at 380 nm with excitation at 315 nm. MAO-A and MAO-B activity in the mouse brain were measured in the presence of 1 μM L-deprenyl (MAO-B inhibitor) and clorgyline (MAO-A inhibitor), respectively.

Kinetic analyses of MAO inhibition were performed in incubation at five substrate concentrations, with or without inhibitors. The reciprocal values of reaction velocities were then presented as a function of the reciprocal substrate concentration.

Table I. Inhibitory effects of acetylshikonin, shikonin, and shikonofuran E from *L. erythrorhizon* on mouse brain monoamine oxidase (MAO) activity

	Concentration (μM)	MAO activity (% of control) (nmol/min/mg protein)	IC_{50} (μM)
Control		2.305 ± 0.012 (100.0)	
Iproniazid	10	1.328 ± 0.020 (57.6)	12.9
Acetylshikonin	2	2.259 ± 0.018 (98.0)	10.0
	7	1.503 ± 0.007 (65.2)**	
	21	0.710 ± 0.005 (30.8)***	
	35	0.341 ± 0.009 (14.8)***	
Shikonin	2	2.164 ± 0.010 (93.9)	13.3
	7	1.168 ± 0.007 (73.2)*	
	21	0.906 ± 0.005 (39.3)***	
	35	0.544 ± 0.004 (23.6)***	
Shikonofuran E	20	1.565 ± 0.005 (67.9)	59.1
	45	1.337 ± 0.005 (58.0)**	
	90	0.987 ± 0.003 (42.8)**	
	180	0.583 ± 0.006 (25.3)***	

The data represents the mean ± S.E.M. of three independent experiments performed in triplicate. Significantly different from the control value: * P<0.05; ** P<0.01; *** P<0.001 (Student's *t*-test).

RESULTS AND DISCUSSION

In the search for MAO inhibitors derived from plants, we found that the *n*-hexane extract of the roots of *L. erythrorhizon* displayed potent inhibitory effects on mouse brain MAO. Three known quinone derivatives, acetylshikonin, shikonin, and shikonofuran E were isolated and the structures were identified by comparison of their physical properties, including optical rotation values and spectral data (mp, UV, IR, $[\alpha]_D$, MS, ^{1}H-, ^{13}C-NMR and DEPT), with values found in literature (Cho *et al.*, 1999; Inoue *et al.*, 1985; Yazaki *et al.*, 1986).

Two main naphthoquinones, acetylshikonin and shikonin, inhibited mouse brain MAO activity in a concentration-dependent manner and their IC_{50} values were 10.0 and 13.3 μM, respectively. However, shikonofuran E, a furyl-hydroquinone derivative, exhibited a weaker MAO inhibi-

Table II. Inhibitory effects of acetylshikonin and shikonin on MAO-A and MAO-B in mouse brain

	Concentration (μM)	MAO activity (% of control) (nmol/min/mg protein)	IC_{50} (μM)
Control		2.305 ± 0.080	
MAO-A (Deprenyl-treated)			
Control + Deprenyl		0.863 ± 0.010 (100.0)	
Acetylshikonin	10	0.632 ± 0.007 (73.3)*	16.9
	17	0.429 ± 0.004 (49.7)**	
	23	0.301 ± 0.012 (34.8)***	
	30	0.226 ± 0.002 (26.2)***	
Shikonin	10	0.557 ± 0.007 (64.6)*	16.4
	17	0.395 ± 0.007 (45.8)**	
	31	0.255 ± 0.015 (29.6)***	
	42	0.233 ± 0.002 (27.1)***	
MAO-B (Clorgyline-treated)			
Control + Clorgyline		1.155 ± 0.009 (100.0)	
Acetylshikonin	3	0.958 ± 0.009 (83.0)	10.1
	7	0.793 ± 0.006 (68.7)*	
	17	0.356 ± 0.003 (30.9)***	
	23	0.270 ± 0.007 (23.4)***	
Shikonin	7	0.863 ± 0.008 (74.7)	13.6
	14	0.532 ± 0.009 (46.1)**	
	24	0.350 ± 0.005 (30.4)***	
	35	0.223 ± 0.002 (19.3)***	

MAO-A and MAO-B activity in mouse brain were measured in the presence of 1 μM L-deprenyl or clorgyline, respectively. The data represents the mean ± S.E.M. of three independent experiments performed in triplicate. Significantly different from the control value: *P<0.05; **P<0.01; ***P<0.001 (Student's *t*-test).

tion with an IC_{50} value of 59.1 μM. In this assay iproniazid was used as a positive control and exhibited an IC_{50} value on the enzyme activity at the concentration of 12.9 μM (Table I).

We further investigated the inhibitory potency and selectivity of two naphthoquinone derivatives, acetylshikonin and shikonin, against MAO-A and MAO-B activity. A deprenyl-treated MAO preparation was used to measure MAO-A activity, and a clorgyline-treated preparation was used to measure MAO-B activity. Acetylshikonin and shikonin inhibited MAO-A activity with IC_{50} values of 16.9 and 16.4 μM, respectively. In the case of MAO-B, the IC_{50} values were 10.1 and 13.6 μM, respectively (Table II). This data indicates that acetylshikonin and shikonin have a non-selective effect on MAO-A and B activity.

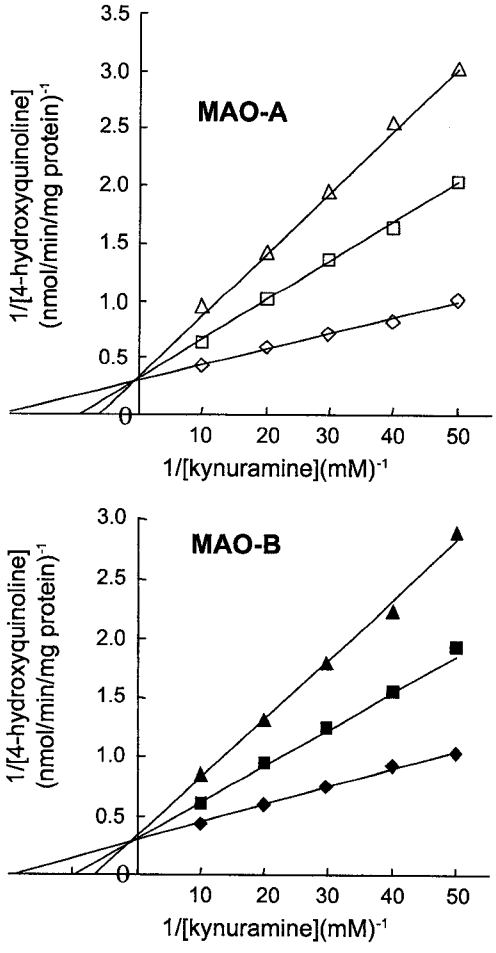

Fig. 2. Lineweaver-Burk plot for MAO-A and MAO-B by acetylshikonin. An MAO assay was performed as described in the materials and methods section at different concentrations of the substrate kynuramine. The reciprocals of MAO-A and MAO-B activity were plotted against the reciprocals of substrate concentration. The values are expressed as the average of the triplicates. The concentrations of acetylshikonin used were (μM): MAO-A, 0 (△); 17 (□); 27 (◇); MAO-B, 0 (▲); 7 (■); 12 (◆).

To examine the inhibition mode of the active compounds, we investigated the kinetic assays of MAO-A and MAO-B inhibition with different concentrations of substrates. As shown in Fig. 2, kinetic analysis using Lineweaver-Burk reciprocal plots indicated that acetylshikonin was a competitive inhibitor for both MAO-A and MAO-B with K_i values of 10.5 and 6.3 μM, respectively. Similarly, shikonin was also a competitive inhibitor of both MAO-A and MAO-B oxidation of kynuramine with K_i values of 12.8 and 13.0 μM, respectively (Fig. 3).

In conclusion, the present study demonstrated that naphthoquinones derived from *L. erythrorhizon* inhibit mouse brain MAO-A and MAO-B activity in a competitive manner. These results suggest that *L. erythrorhizon,* which contains naphthoquinones as major component, may be considered as a possible source of MAO-A and MAO-B inhibitors used in the treatment of depression and

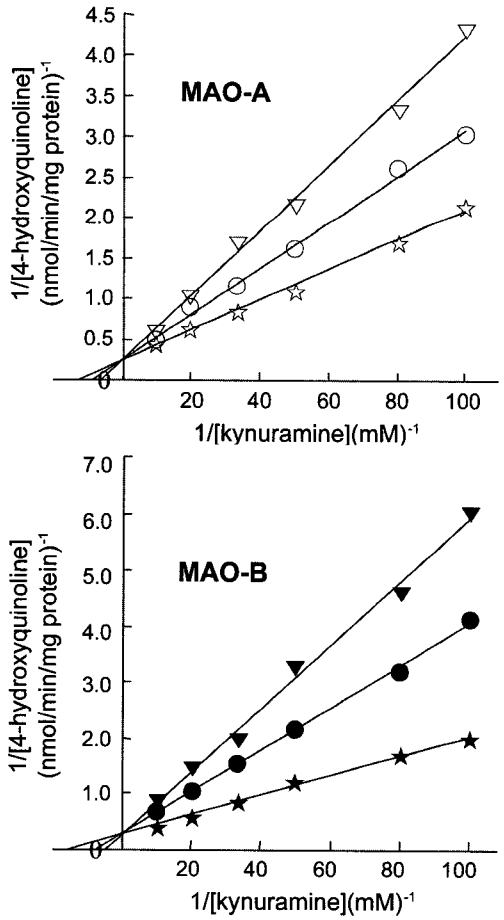

Fig. 3. Lineweaver-Burk plot for MAO-A and MAO-B by shikonin. An MAO assay was performed as described in the materials and methods section at different concentrations of the substrate kynuramine. The reciprocals of MAO-A and MAO-B activity were plotted against the reciprocals of substrate concentration. The values are expressed as the average of the triplicates. The concentrations of shikonin used were (μM): MAO-A, 0 (▽); 7 (○); 14 (☆); MAO-B, 0 (▼); 7 (●); 12 (★).

Alzheimer's disease, respectively. However, further pharmacological studies must be conducted in order to determine whether these compounds exhibit *in vivo* activity or not.

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