Available online at www.sciencedirect.com
SCIENCE DIRECT®
Phytochemistry 65 (2004) 2255–2260
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Monoamine oxidase inhibitors from *Gentiana lutea*

Hiroyuki Haraguchi [a,*], Yasumasa Tanaka [a], Amal Kabbash [b], Toshihiro Fujioka [c], Takashi Ishizu [b], Akira Yagi [b]

[a] *Faculty of Life Science and Biotechnology, Fukuyama University, Gakuen-cho, Fukuyama 729-0292, Japan*
[b] *Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, Gakuen-cho, Fukuyama 729-0292, Japan*
[c] *Faculty of Pharmaceutical Sciences, Fukuoka University, Fukuoka 814-0180, Japan*

Received in revised form 19 March 2004

Abstract

Three monoamine oxidase (MAO) inhibitors were isolated from *Gentiana lutea*. Their structures were elucidated to be 3-3″linked-(2′-hydroxy-4-*O*-isoprenylchalcone)-(2‴-hydroxy-4″-*O*-isoprenyldihydrochalcone) (**1**), 2-methoxy-3-(1,1′-dimethylallyl)-6a,10a-dihydrobenzo(1,2-*c*)chroman-6-one and 5-hydroxyflavanone. These compounds, and the hydrolysis product of **1**, displayed competitive inhibitory properties against MAO-B which was more effective than MAO-A.

Keywords: *Gentiana lutea*; Gentianaceae; Monoamine oxidase; 3-3″Linked-(2′-hydroxy-4-*O*-isoprenylchalcone)-(2‴-hydroxy-4″-*O*-isoprenyldihydrochalcone); 2-Methoxy-3-(1,1′-dimethylallyl)-6a,10a-dihydrobenzo(1,2-*c*)chroman-6-one; 5-Hydroxyflavanone

1. Introduction

Monoamine oxidase (EC 1.4.3.4, MAO) catalyzes the oxidative deamination of a variety of monoamines such as dopamine, norepinephrine and serotonin. MAO is a FAD-containing enzyme located in the outer membrane of mitochondria (Greenawalt, 1972). Two different forms, MAO-A and MAO-B, have been identified based on their substrate selectivity (Murphy, 1978), inhibitor sensitivity (Knoll and Magyar, 1972), and amino acid sequence (Bach et al., 1988). Both MAO-A and -B seem to be present in most areas of the brain (Benedetti and Dostert, 1992). They have attracted considerable interest due to their key roles in monoamine neurotransmitter metabolism and their involvement in many neuropsychiatric disorders (Strolin-Benedetti and Dostert, 1989). The MAO reaction yields aldehydes and hydrogen peroxide (H_2O_2), which induces apoptosis (Malorni et al., 1998). Inhibition of MAO and subsequent H_2O_2 generation effectively prevents depression and various oxidative stresses in the brain (Kalgutkar et al., 1995). However, classical MAO inhibitors have been shown to have side-effects (Power et al., 1995; Kohda et al., 1998). Studies on model compounds to develop synthetic MAO inhibitors have been carried out indirectly (Gao et al., 2001) due to the lack of reliable three-dimensional structural information on the active sites of MAO-A and -B (Gnerre et al., 2000). On the other hand, the presence of plant-derived MAO inhibitors suggests that such plant extracts could be useful as potential neuroprotectants in the treatment or prevention of depression (Sloley et al., 2000).

In the course of screening for bioactive compounds from botanical sources, it was found that the methanol extract of the dried bark of *Gentiana lutea* L. (Gentianaceae) potently inhibited rat brain MAO, while the roots are used to treat stomach-ache and gall and liver diseases (Nebojsa et al., 2000a). In this study, we identified

* Corresponding author. Tel.: +81-849-36-2111; fax: +81-849-36-2023.
E-mail address: haragu@bt.fubt.fukuyama-u.ac.jp (H. Haraguchi).

0031-9422/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2004.06.025

MAO inhibitors in *G. lutea* and characterized their inhibitory activities toward MAO-A and -B.

2. Results and discussion

The methanol extract of the dried bark of *G. lutea* was partitioned between ethyl acetate and water and the ethyl acetate extract was found to potently inhibit MAO. Chromatographic purification of the ethyl acetate extract resulted in active compounds **1**, **3** and **4**, which were subjected to structural investigations. On positive FAB MS, **1** showed peaks due to parent and fragment ions at *m/z* 617, 311 and 309, whereas two phenol groups at peri-position to the carbonyl groups at 12.7 and 13.4 ppm was observed in the ^{1}H NMR spectrum, and a bathochromic shift was upon the addition of $AlCl_3$ reagent in the UV spectrum. ^{1}H and ^{13}C NMR and positive ion FAB MS spectral analyses indicated that **1** was composed of chalcone and dihydrochalcone with an isoprenyl group, respectively. On acid hydrolysis, **1** provided **2**, in which the isoprenyl groups were eliminated. Based on ^{1}H and ^{13}C NMR spectroscopic analyses, together with that of HMBC, HMQC, and positive ion FAB MS and HR FAB MS data, **2** was determined to be composed of chalcone and dihydrochalcone skeletons. The isoprenyl groups in **1** were determined to be located at C-4 and C-4″ in the chalcone and dihydrochalcone skeletons, respectively, by ^{1}H NMR, HMBC and HMQC spectral analyses. Linkage between the carbons at C-3 and C-3″ in **1** was established from the coupling constants of ABX type-proton signals at the B-ring in the chalcone and dihydrochalcone skeletons and HMBC examination (Fig. 1).

On positive ion HR FAB MS, **3** had a peak due to the parent ion at *m/z* 297 $[M+H]^{+}$, and a typical dimethylallyl group and a lactone ring as revealed by analyses of the ^{1}H NMR and IR spectra, respectively. In the ^{13}C NMR and UV spectral data of **3**, the absence of a double bond in the coumarin ring and conjugation with the benzene ring at position 6a and 10a, resulting in a dihydrobenzene ring, were suggested based on data in the literature (Zdero et al., 1990). The presence of a methoxy group at C-2 position in the dihydrobenzo(1,2-*c*)chroman-6-one ring of **3** was confirmed by NOE and HMBC experiments. Based on ^{1}H and ^{13}C NMR spectral data analyses, together with HMBC, HMQC and NOE experiments, the structure of **3** was established to be 2-methoxy-3-(1,1′-dimethylallyl)-6a,10a-dihydrobenzo(1,2-*c*)chroman-6-one (Fig. 1).

On positive ion HR FAB MS, **4** showed a peak due to the parent ion at *m/z* 241 $[M+H]^{+}$. Compound **4** showed a phenol group peri- to the carbonyl group in the flavanone A-ring at 12.7 ppm on ^{1}H NMR inspection of the spectrum as well as showing a bathochromic shift upon addition of $AlCl_3$ reagent in the UV spectrum. Based on ^{1}H and ^{13}C NMR spectral data analyses, **4** was determined to be 5-hydroxyflavanone (Kallay et al., 1969) (see Table 1).

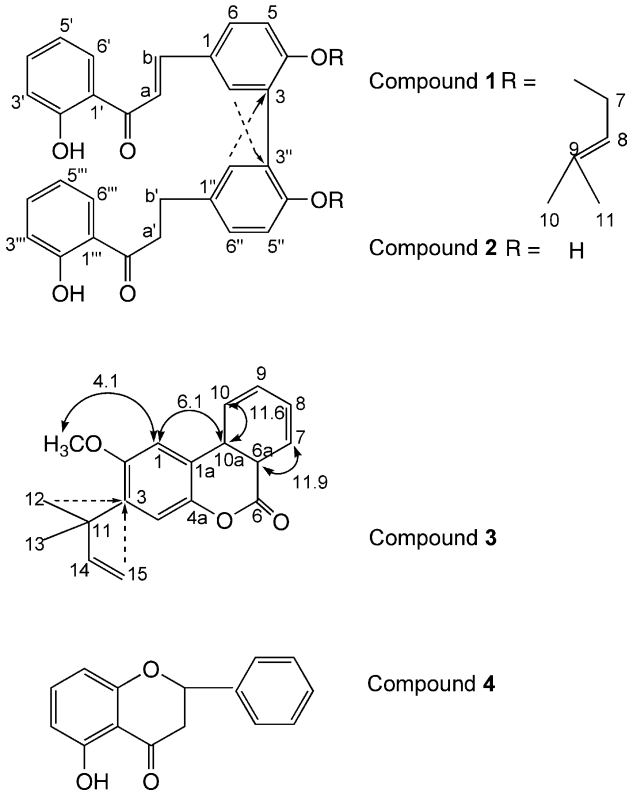

Fig. 1. Chemical structures of monoamine oxidase inhibitors isolated from *Gentiana lutea*. NOE (%), HMBC.

Monoamine oxidase activity in rat brain mitochondria was measured using the non-selective substrate kynuramine (Thull et al., 1998), and clorgyline (Naoi and Nagatsu, 1987) or pargyline (Sandri et al., 1990) was added to define MAO-A or MAO-B, respectively. As shown in Table 2, compound **1** exhibited 50% inhibition against MAO-B at 48.7 μM, while only 10% inhibition was observed against MAO-A at the same concentration. The hydrolyzed product (**2**) of compound **1** exhibited more potent inhibition against MAO-B, and also inhibited MAO-A. Compound **3** also selectively inhibited MAO-B, and complete inhibition was observed at 10 μM. Compound **4** showed more potent MAO inhibitory activity against MAO-B than against MAO-A.

Kinetic analyses for the inhibition of MAO-B by the products of *G. lutea* using Lineweaver–Burk plots are shown in Fig. 2. When the concentration of the substrate kynuramine was changed, the curves, obtained with the uninhibited enzyme and with the addition of compound **1** crossed at the ordinate. With compounds **2**, **3** and **4**, the reciprocal plot curves of enzyme activities and substrate concentration also intersected at the ordinate. These results indicate compounds in *G. lutea* that inhibited MAO exhibited competitive inhibition against

Table 1
^{13}C NMR chemical shifts for compounds **1**–**4** in $CDCl_3$ at 125.65 MHz

Carbon	**1**		**2**		Carbon	**3**	Carbon	**4**
1/1″	134.8	139.4	134.8	140.9	1	96.6		
2/2″	101.6	101.8	103.6[a]	103.7[a]	2	166.0	2	79.2
3/3″	113.3	114.0	113.8	114.5	3	110.4	3	44.3
4/4″	165.7	166.7	165.4	165.6	4	127.2	4	191.0
5/5″	128.4[a]	128.5[a]	129.0	129.0	4a	162.7	4a	114.9
6/6″	108.2[a]	108.4[a]	107.9[a]	107.8[a]	6	161.5	5	163.1
α/α′	120.4	30.3	120.4	30.3	6a	43.9	6	126.2
β/β′	144.3	39.7	144.3	39.7	7	128.5	7	128.9
1′/1‴	139.1[a]	139.2[a]	140.9	140.9	8	127.2	8	128.8
2′/2‴	165.6	165.6	166.7	165.4	9	127.5	8a	163.6
3′/3‴	131.2[a]	131.4[a]	113.8[a]	114.5[a]	10	127.5	1′	138.7
4′/4‴	129.0[a]	130.6[a]	129.1	129.1	10a	45.1	2′/6′	110.7
5′/5‴	129.0[a]	130.6[a]	128.6	128.6	1a	137.5	3′/5′	103.5
							4′	129.5
6′/6‴	131.2[a]	131.4[a]	132.2	129.9	11	39.7		
7/7′	65.2[a]	65.3[a]			12	27.6		
8/8′	118.7	118.7			13	27.6		
9/9′	126.3	128.4			14	148.5		
10/10′	18.2[a]	18.3[a]			15	107.8		
11/11′	25.8	25.9						
CO/CO	191.9	203.6	191.9	203.6	OCH_3	55.8	CO	191.0

[a] The carbon signals at 5/5″, 6/6″, 1′/1‴∼6′/6‴, 7/7′, 10/10′ in **1**; 2/2″, 6/6″, 3/3‴ in **2** can be interchanged.

Table 2
Activities of MAO inhibitors isolated from *Gentiana lutea* on brain mitochondrial enzyme

Compound	IC_{50}(μM)[a]	
	MAO-A	MAO-B
1	>100	48.7
2	12.5	6.2
3	>100	2.9
4	39.6	3.8

[a] Inhibitory activity was expressed as the mean of 50% inhibitory concentration (IC_{50}) of triplicate determinations, obtained by interpolation of concentration–inhibition curves.

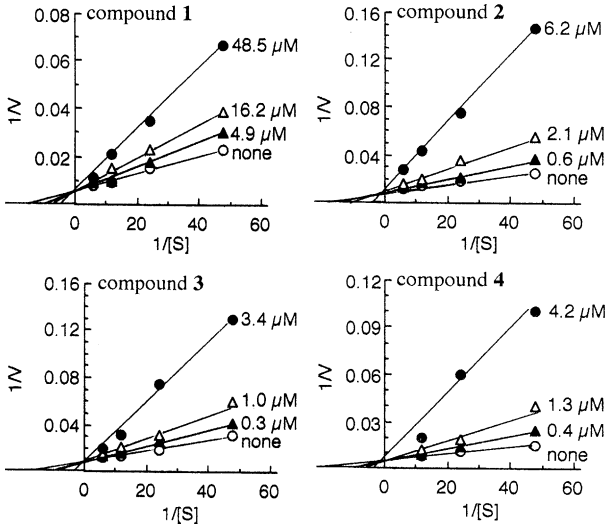

Fig. 2. Inhibitory effects of compounds **1**–**4** on MAO-B. Lineweaver–Burk plots in the absence and presence of each inhibitor are shown.

kynuramine. The K_i values of compounds **1**, **2**, **3** and **4** against MAO-B were 24.2, 1.2, 1.1 and 1.4 μM, respectively.

Recently, extracts of *G. lutea* were reported to have scavenging activity toward hydroxyl radical (Calliste et al., 2001). The reaction of MAO yields H_2O_2, which leads to the further generation of hydroxy radical, the most powerful oxidant in cellular systems. Furthermore, extracts of *G. lutea* have also been reported to have some effects on the central nervous system in mice (Ozturk et al., 2002). *G. lutea* contains secoiridoid glucosides and xanthones as pharmaceutically active constituents (Nebojsa et al., 2000b); the former are known to have antioxidative activities (Haraguchi, 2001) and the latter have been reported to inhibit MAO (Thull et al., 1993). In the present study, 3-3″linked-(2′-hydroxy-4-*O*-isoprenyl-chalcone)-(2‴-hydroxy-4″-*O*-isoprenyldihydro-chalcone) (**1**), 2-methoxy-3-(1,1′-dimethylallyl)-6a,10a-dihydro-benzo(1,2-*c*)chroman-6-one (**3**) and 5-hydroxyflavanone (**4**) were isolated from *G. lutea* as MAO inhibitors. A hydrolysis product (**2**) of **1** also inhibited MAO. The new dimeric chalcone derivative (**1**) and its hydrolysis product (**2**) showed more potent inhibitory activity toward MAO-B than toward MAO-A. 5-Hydroxyflavanone (**4**) was also effective against MAO-B. Isoliquiritigenin (chalcone) and liquiritigenin (flavanone) isolated from *Sinofranchetia chinensis* also inhibit MAO, however, these flavonoids inhibit MAO-A more strongly than MAO-B (Pan et al., 2000). Other flavones and flavonols (apigenin, chrysin, kaempferol, and quercetin) are also reported to inhibit MAO-A rather than MAO-B (Sloley et al., 2000). The new hydrophobic

dihydrocoumarin (**3**) was the most potent inhibitor against MAO-B among the components in *G. lutea*. Some naturally occurring (Jo et al., 2002; Hossain et al., 1996) and synthetic (Gnerre et al., 2000) coumarins inhibit MAO, compound **3** was interesting since it most strongly inhibited MAO-B. The inhibition of MAO-A is expected to be useful for the treatment of depression and anxiety, while that of MAO-B may be helpful in preventing Parkinson's disease (Silverman et al., 1993). Furthermore, MAO-B inhibitors have been suggested for use in the treatment of Alzheimer's disease (Boyd, 2000), since an increase in MAO-B activity increases brain levels of hydroxy radical. *G. lutea* which contains compounds with potent MAO-B inhibitory activity may be a possible source of pharmaceuticals for the treatment and prevention of depression and for protecting brain tissues against oxidative stress.

3. Experimental

3.1. General

Optical rotations were measured with a JASCO DIP-140, and positive ion FAB MS and HR FAB MA spectra were determined with a JEOL HX-110 using glycerol and polyethylene glycol as a matrix, respectively. UV and IR spectra were acquired on a UV 2400PC (Shimadzu) and FTIR (Shimadzu), respectively. NMR spectra were acquired on a JEOL Lambda 500 using ca. 5 mg of test sample dissolved in $CDCl_3$ in a 5-mm tube. ^1H and ^{13}C NMR spectral measurements were carried out at 500.00 and 125.65 MHz, respectively, at 27–28 °C. Chemical shifts are given in δ values in ppm with TMSi as an internal standard. The HMQC and HMBC spectra were obtained using the field-gradient method. HMQC and HMBC were recorded using a pulse sequence (Bax and Summers, 1986) involving a low-pass J-filter (3.8 ms) and a delay for long-range coupling of 70 ms. The spectra were acquired with a spectral width of 4480.3 Hz (HMBC) and 4553.7 Hz (HMQC) in 1 K data points using 96 (HMBC) and 32 (HMQC) scans for the 256 t_1 experiments. The data were processed in a power spectrum mode. Zero filling to 4 K data points in F_2 and 1 K data points in F_1 and multiplication with squared Blackman–Harris windows shifted by 90° in both dimensions were performed prior to double Fourier transformation. Analytical TLC was carried out on silica gel 60 F_{254} plates (Merck) using EtOAc–C_6H_6 (1:9). Compounds were visualized by spraying with H_2SO_4 after heating.

3.2. Chemicals

Kynuramine [3-(2-aminophenyl)-3-oxopropanamine] and clorgyline [*N*-methyl-*N*-propargyl-3-(2,4-dichlorophenoxy)propylamine] were purchased from Sigma Chemical Co. (St. Louis, MO). Whereas, pargyline (*N*-methyl-*N*-benzyl-2-propynylamine) was obtained from Nakalai Tesque Inc. (Kyoto, Japan). Percoll (colloidal silica coated with polyvinylpyrrolidone) was purchased from Pharmacia LKB (Uppsala, Sweden).

3.3. Plant material

Materials were obtained from a Mexican market and identified by Mitsui Chemical Co. Ltd., Japan. A voucher specimen (voucher No. MP-291) has been deposited at the herbarium of the herb garden at Fukuyama University.

3.4. Animals

Male Wistar rats (Japan SLC Inc.) were maintained in accordance with the guidelines for animal care of Fukuyama University.

3.5. Extraction and isolation

Dried powder of *G. lutea* wood (3 kg) was extracted three times with MeOH. The MeOH extract was evaporated under reduced pressure to give a residue (140 g) which was partitioned between EtOAc and water. The EtOAc extract (40 g) was subjected to a silica gel cc (70–230 mesh), (1.5×23 cm) using MeOH–C_6H_6 (1:49) at a flow rate of 2 ml/min, to yield crude compound **1** (elution volume 60–460 ml, 0.5 g) and a mixture of at least four compound (elution volume 0.5–2 l, 10.76 g). After recrystallization from MeOH, **1**, was obtained (0.4 g), R_f 0.85. The mixture containing for four compounds was recrystallized from MeOH to yield crude **3** (0.7 g) and the fraction (8.7 g) from the mother liquor was applied to a silica gel column (1.5×12 cm) using C_6H_6 as eluent to provide crude **3** (5 g). Recrystallization of combined fractions of crude **3** from MeOH gave pale yellow granules of **3** (0.1 g), (R_f 0.41). The combined fraction containing the type unknown compounds (9 g) was applied to a silica gel column (1.5×23 cm) using MeOH–C_6H_6 (1:49) to give a mixture (3.98 g), which was again subjected to a silica gel cc (1.5×6 cm) using hexane–C_6H_6 (1:41) to give **4** (1.7–2 l, 0.02 g) and unknown compounds which were not characterised further. The crude fraction of **4** was recrystallized from MeOH to give yellow needles (0.01 g) R_f 0.40.

3.6. 3-3″Linked-(2′-hydroxy-4-O-isoprenylchalcone)-(2‴-hydroxy-4″-O-isoprenyl dihydrochalcone) (*1*)

Yellow needles from MeOH, m.p. 86–9 °C; UV: λ_{max}^{MeOH} nm (log ε): 350 (sh), 314.8 (4.95), 280.8 (4.49); + $AlCl_3$: 405 (sh), 359.6, 310; + NaOAc: 318, 281.0. IR: γ_{max}^{KBr} cm^{-1}: 3300, 1631, 1612, 1572. ^1H

NMR (600 MHz, $CDCl_3$): δ 1.74 (3H×2, *s*, H-10/11 or H-10′/11′), 1.76 (3H×2, *s*, H-10/11 or H-10′/11′), 3.05 (2H, *t*, J=7 Hz, Hα′), 3.24 (2H, *t*, J=7 Hz, Hβ′), 4.53 (2H, *dd*, J=17.6 Hz, 8.2 Hz, H-7/7′), 4.57 (2H, *dd*, J=17.6 Hz, H-7/7′), 5.48 (2H, *m*, H-8/8′), 6.40 (1H, *dd*, J=8.2 Hz, H-6/6″), 6.42 (1H, *d*, J=2 Hz, H-2/2″), 6.48 (1H, *dd*, J=8, 2 Hz, H-6/6″), 6.50 (1H, *d*, J=2 Hz, H-2/2″), 7.21 (1H, *dd*, J=8, 2 Hz, H-3′/3‴), 7.24 (1H, *d*, J=8 Hz, H-5/5″), 7.29 (1H, *d*, J=8 Hz, H-5/5″), 7.42 (2H, *m*, H-5′/5‴), 7.44 (2H, *m*, H-4′/4‴), 7.58 (1H, *d*, J=15 Hz, Hα), 7.62 (1H, *d*, J=8.2 Hz, H-6′/6‴), 7.82 (1H, *dd*, J=8, 2 Hz, H-6′/6‴), 7.89 (1H, *d*, J=15 Hz, H*β*), 12.7 (1H, *s*, OH), 13.4 (1H, *s*, OH). Positive ion FAB MS: *m/z* 617 $(M+H)^+$, 311, 309 $(M/2+H)^+$, 243, 241 $(M/2-C_5H_9)^+$. HR-FAB MS: *m/z* observed. 309.1542, Calcd. for $C_{20}H_{21}O_3$: 309.1490.

3.7. Compound **2**

Hydrolysis of **1** (50 mg) was carried out with 2 N HCl in EtOH (2 ml) at 100 °C for 1 h. The hydrolysate was subjected to a silica gel cc (0.5×4.5 cm) to provide **2** (19 mg), which was recrystallized from MeOH, m.p. 107–9 °C; R_f 0.40, UV: λ_{max}^{MeOH} nm (log ε): 318.6 (4.58), 280.5 (4.56); + $AlCl_3$: 352.8, 305.8; + NaOAc: 320.5, 278.2. IR: γ_{max}^{KBr} cm^{-1}: 3207, 1633.6, 1600.8, 1558.4. ^1H NMR (600 MHz, $CDCl_3$): δ 3.05 (2H, *t*, J=7 Hz, Hα), 3.24 (2H, *t*, J=7 Hz, H *β*), 6.30 (2H, *dd*, J=8, 2 Hz, H-6/6″), 6.37 (1H, *brs*, H-2), 6.47 (1H, *brs*, H-2″), 7.22 (2H, *m*, H-4/4‴), 7.25 (2H, *m*, H-5′/5‴), 7.29 (2H, *dd*, J=8, 2 Hz, H-6′), 7.62 (1H, *d*, J=8 Hz, H-6′), 7.63 (2H, *d*, J=8.2 Hz, H-3′/3‴), 7.84 (1H, *d*, J=8 Hz, H-6‴), 12.7 (1H, OH), 13.4 (1H, OH). Positive ion FAB MS: *m/z* 481, 243, 241. HR-FAB MS: *m/z* observed. 241.0866, Calcd. for $C_{15}H_{13}O_3$: 241.0866.

3.8. Methoxy-3-(1,1′-dimethylallyl)-6a,10a-dihydrobenzo(1,2-c)chroman-6-one (**3**)

M.p. 183–4 °C; $[\alpha]_D^{18}$ +58.0° (c 0.5 $CHCl_3$); UV: λ_{max}^{MeOH} nm (log ε): 308.6 (4.25); + $AlCl_3$: no shift. IR: γ_{max}^{KBr} cm^{-1}: 1720, 1689. ^1H NMR (600 MHz, $CDCl_3$): δ 1.34 (3H×2, *s*, H-12/13), 3.61 (3H, *s*, CH_3O), 4.28 (1H, *dd*, J=9.8, 7.4 Hz, H-10a), 4.47 (1H, *dd*, J=9.8, 7.7 Hz, H-6a), 4.73 (1H, *dd*, J=17, 0.9 Hz, H-15), 4.75 (1H, *dd*, J=10, 0.9 Hz, H-15′), 5.81 (1H, *s*, H-1), 6.00 (2H, *dd*, J=17, 10 Hz, H-14), 7.20 (1H, *dd*, J=8.0–8.5 Hz, H-10), 7.26 (1H, *brs*, H-9), 7.28 (1H, *brs*, H-4), 7.29 (1H, *brs*, H-8), 7.30 (1H, *dd*, J=8.0–8.5 Hz, H-7). Positive ion HR-FAB MS: *m/z* observed. 297.1486 $(M+H)^+$, Calcd. for $C_{19}H_{21}O_3$: 297.1489.

3.9. Hydroxyflavanone (**4**)

Pale yellow needles from MeOH, m.p. 124 °C; $[\alpha]_D^{18}$ –11.0° (c 0.1 $CHCl_3$); UV: λ_{max}^{MeOH} nm (log ε): 310 (sh.), 271.0 (4.02), 231.8 (3.97); + $AlCl_3$: 360 (sh.), 309.4, 274.2. IR: γ_{max}^{KBr} cm^{-1}: 3000, 1737.7, 1606.6. ^1H NMR (600 MHz, $CDCl_3$): δ 2.85 (1H, *dd*, J=15, 3 Hz, H-3), 3.05 (1H, *dd*, J=15, 13 Hz, H-3), 5.47 (1H, *dd*, J=13, 3 Hz, H-2), 6.47 (1H, *m*, H-5′), 6.56 (1H, *dd*, J=8, 2 Hz, H-2′/6′), 7.38 (1H, *m*, H-7), 7.43 (1H, *dd*, J=7, 2 Hz, H-6/8), 7.4–7.5 (2H, *m*, H-3′/4′), 7.46 (1H, *dd*, J=7, 2 Hz, H-6/8), 7.86 (1H, *dd*, J=8, 2 Hz, H-2′/6′), 12.7 (1H, OH). Positive ion HR-FAB MS: *m/z* observed. 241.0859 $(M+H)^+$, Calcd. for $C_{15}H_{12}O_3$: 241.0864.

3.10. Preparation of brain mitochondria

Cerebral hemispheres of Wistar male rats weighing 100–150 g were removed quickly and soaked in ice-cold 0.5 mM Tris–HCl buffer (pH 7.4) containing 0.25 M sucrose and 0.5 mM EDTA (isolation medium), and gently homogenized by up-and-down strokes with a glass pestle using a Digital Homogenizer HOM (Iuchi Co. Ltd., Japan). The total homogenate was centrifuged at 2000*g* for 10 min at 4 °C, and the supernatant was then centrifuged at 12,500*g* for 10 min. The crude mitochondrial pellet was suspended in a solution consisting of 3% Ficoll, 0.12 M mannitol, 0.03 M sucrose and 25 μM EDTA (pH 7.4). The suspension was overlayered onto a solution containing 6% Ficoll, 0.24 M mannitol, 0.06 M sucrose and 50 μM EDTA (pH 7.4) and centrifuged at 11,500*g* for 30 min. The mitochondrial pellet was resuspended in isolation medium, recentrifuged at 12,500*g* for 10 min, and finally resuspended in isolation medium (Clark and Nicklas, 1970).

Protein concentrations of the suspensions were determined by the method of Lowry et al. (1951).

3.11. Measurement of MAO activity

Rat brain mitochondria were incubated at 37 °C in 3 ml of 50 mM KH_2PO_4–Na_2HPO_4 (pH 7.4). The suspension was preincubated with either clorgyline or pargyline at 1 μM for 5 min to measure MAO-A or MAO-B activity, respectively. The reaction was initiated by the addition of 0.167 mM kynuramine. After the reaction mixture was shaken for 5 min, the reaction was terminated by the addition of 2 ml of 10% $ZnSO_4$ and 100 μl of 1 N NaOH. The solution was heated for 2 min in a boiling water bath. After the mixture was cooled, the flocculent precipitate was removed by centrifugation, and 1.2 ml of 1 N NaOH was added to the supernatant (Satoh and Yamazaki, 1989). The fluorescence of 4-hydroxyquinoline, which was formed from kynuramine by MAO, was measured at an excitation wavelength of 315 nm and an emission wavelength of 380 nm using a Hitachi 650-10S fluorescence spectrometer.

Kinetic studies on MAO inhibition were performed with incubation at four substrate concentrations, with

or without inhibitors. The reciprocal values of reaction velocities were then presented as a function of the reciprocal substrate concentration.

Acknowledgements

The authors thank to Miss Y. Yamamoto for her technical assistance.

References

Bach, A.W.J., Lan, N.C., Johanson, D.L., Abell, C.W., Bembenek, M.E., Kwan, S.W., Seeburg, P.H., Shih, J.C., 1988. cDNA cloning of human monoamine oxidase A and B: molecular basis of differences in enzymatic properties. Proc. Natl. Acad. Sci. USA 85, 4934–4938.

Bax, A., Summers, M.S., 1986. ^{1}H and ^{13}C-assignments from sensitivity-enhanced detection of heteronuclear multiple-bond connectivity by 2D multiple quantum NMR. J. Am. Chem. Soc. 108, 2093–2094.

Benedetti, M.S., Dostert, P., 1992. Monoamine oxidase: from physiology and pathophysiology to the design and clinical application of reversible inhibitors. In: Testa, B. (Ed.), Advances in Drug Research, vol. 23. Academic Press, New York, pp. 65–125.

Boyd, B., 2000. Ongoing progress in Alzheimer's disease arena. Drug News Perspect. 13, 425–438.

Calliste, C.A., Trouillas, P., Allais, D.P., Simon, A., Duroux, J.L., 2001. Free radical scavenging activities measured by electron spin resonance spectroscopy and B16 cell antiproliferative behaviors of seven plants. J. Agr. Food Chem. 49, 3321–3327.

Clark, J.B., Nicklas, W.J., 1970. The metabolism of rat brain mitochondria. J. Biol. Chem. 245, 4724–4731.

Gao, G.Y., Li, D.J., Keung, W.M., 2001. Synthesis of potential antidipsotropic isoflavones: inhibitors of the mitochondrial monoamine oxidase–aldehyde dehydrogenase pathway. J. Med. Chem. 44, 3320–3328.

Gnerre, C., Catto, M., Leonetti, F., Weber, P., Carrupt, P.-A., Altomare, C., Carotti, A., Testa, B., 2000. Inhibition of monoamine oxidases by functionalized coumarin derivatives: biological activities, QSARs, and 3D-QSARs. J. Med. Chem. 43, 4747–4758.

Greenawalt, J.W., 1972. Localization of monoamine oxidase in rat liver mitochondria. Adv. Biochem. Psychopharmacol. 5, 207–226.

Haraguchi, H., 2001. Antioxidative plant constituents. In: Tringali, C. (Ed.), Bioactive Compounds from Natural Sources. Isolation, Characterization and Biological Properties. Taylor and Francis, London, pp. 337–377.

Hossain, C.F., Okuyama, E., Yamazaki, M., 1996. A new series of coumarine derivatives having monoamine oxidase inhibitory activity from *Monascus anka*. Chem. Pharm. Bull. 44, 1535–1539.

Jo, Y.S., Huong, D.T., Bae, K., Lee, M.K., Kim, Y.H., 2002. Monoamine oxidase inhibitory coumarin from *Zanthoxylum schinifolium*. Planta Med. 68, 84–85.

Kalgutkar, A.S., Castagnoli Jr., N., Testa, B., 1995. Selective inhibitors of monoamine oxidase as probes of its catalytic site and mechanism. Med. Res. Rev. 4, 325–388.

Kallay, F., Janzso, G., Koczor, I., Radics, L., 1969. 5-Hydroxyflavone. Ind. J. Chem. 7, 524–526.

Knoll, J., Magyar, K., 1972. Some puzzling pharmacological effects of monoamine oxidase inhibitors. Adv. Biochem. Psychopharmacol. 5, 393–417.

Kohda, K., Noda, Y., Aoyama, S., Umeda, M., Sumino, T., Kaiya, T., 1998. Cytotoxicity of 1-amino-4-phenyl-1,2,3,6-tetrahydropyridine and 1-amino-4-phenylpyridium ion, 1-amino analogues of MPTP and MPP^{+}, to clonal pheochromocytoma PC12 cells. Chem. Res. Toxicol. 11, 1249–1253.

Lowry, O.H., Rosebrough, J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Malorni, W., Giammarioli, A.M., Matarrese, P., Pietrangeli, P., Agostinelli, E., Ciaccio, A., Grassilli, E., Mondovi, B., 1998. Protection against apoptosis by monoamine oxidase A inhibitors. FEBS Lett. 462, 155–159.

Murphy, D.L., 1978. Substrate-selective monoamine oxidases. Biochem. Pharmacol. 27, 1889–1893.

Naoi, M., Nagatsu, T., 1987. Quinoline and quininaldine as naturally occurring inhibitors specific for type A monoamine oxidase. Life Sci. 40, 1075–1082.

Nebojsa, M., Katarina, S.-F., Katica, S., 2000a. Chemical composition and seasonal variations in the amount of secondary compounds in *Gentiana lutea* leaves and flowers. Planta Med. 66, 178–180.

Nebojsa, M., Katarina, S.-F., Momcilovic, I., Grubisic, D., 2000b. Quantitative determination of secoiridoid and -pyrone compounds in *Gentiana lutea* cultured *in vitro*. Planta Med. 66, 96–98.

Ozturk, N., Can Baser, K.H., Aydin, S., Ozturk, Y., Calis, I., 2002. Effects of *Gentiana lutea* ssp. *symphyandra* on the central nervous system in mice. Phytother. Res. 16, 627–631.

Pan, X., Kong, L.D., Zhang, Y., Cheng, C.H., Tan, R.X., 2000. In vitro inhibition of rat monoamine oxidase by liquiritigenin and isoliquiritigenin isolated from *Sinofranchetia chinensis*. Acta Pharmacol. Sin. 21, 949–953.

Power, M.B., Hackett, L.P., Dusci, L.J., Ilett, K.F., 1995. Antidepressant toxicity and the need for identification and concentration monitoring in overdose. Clin. Pharmacokin. 29, 154–171.

Sandri, G., Panfili, E., Ernster, L., 1990. Hydrogen peroxide production by monoamine oxidase in isolated rat brain mitochondria: its effect on glutathione levels and Ca^{2+} efflux. Biochim. Biophys. Acta 1035, 300–305.

Satoh, Y., Yamazaki, M., 1989. Studies on the monoamine oxidase inhibitory potency of TL-1, isolated from a fungus, *Talaromyces luteus*. Chem. Pharm. Bull. 37, 206–207.

Silverman, R.B., Ding, C.Z., Gates, K.S., 1993. Design and mechanism of monoamine oxidase inactivators from an organic chemical perspective. In: Testa, B., Kyburz, E., Fuhrer, W., Giger, R. (Eds.), Perspective in Medicinal Chemistry. Verlag Helvetica Chemica Acta, Basel, pp. 73–86.

Sloley, B.D., Urichuk, L.J., Morley, P., Durkin, J., Shan, J.J., Pang, P.K.T., Coutts, R.T., 2000. Identification of kaempferol as a monoamine oxidase inhibitor and potential neuroprotectant in extracts of *Ginkgo biloba* leaves. J. Pharm. Pharmacol. 52, 451–459.

Strolin-Benedetti, M., Dostert, P., 1989. Monoamine oxidase, brain aging and degenerative diseases. Biochem. Pharmacol. 38, 555–561.

Thull, U., Kneubühler, S., Testa, B., Borges, M.F.M., Pinto, M.M.M., 1993. Substituted xanthones as selective and reversible monoamine oxidase A (MAO-A) inhibitors. Pharm. Res. 10, 1187–1190.

Thull, U., Carrupt, P.-A., Testa, B., 1998. Pargyline analogues as potent, non-selective monoamine oxidase inhibitors. Pharm. Pharmacol. Commun. 4, 579–581.

Zdero, C., Bohlmann, F., Niemeyer, H.M., 1990. Diterpenes and umbelliferone derivatives from *Haplopappus deserticola*. Phytochemistry 29, 326–329.