Natural Product Sciences
11(3) : 145-149 (2005)

Monoamine Oxidase Inhibitor from *Uncaria rhynchophylla*

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Abstract – A methanol soluble extract from the dried hooks and stems of *Uncaria rhynchophylla* showed a strong inhibitory activity against monoamine oxidase in mouse brain. Using a bioassay-guided purification of this extract, a known β-carboline type alkaloid, harman (**1**), was obtained as an active constituent. In addition, five known indole alkaloids, isocorynoxeine (**2**), isorhynchophylline (**3**), corynoxeine (**4**), cadambine (**5**), and 3α-dihydrocadambine (**6**), were isolated and found to be weakly active or inactive.
Key words – *Uncaria rhynchophylla*, Rubiaceae, Harman, Monoamine oxidase inhibitor

Introduction

Uncaria rhynchophylla (Rubiaceae), mainly distributed in China and Japan, is a vine or shrub with characteristic peduncles that appear as curved hooks on the side shoots. The dried hooks and stems of this plant have been used as a traditional medicine for the treatment of headache and dizziness due to hypertension, and infantile convulsion and other nervous disorders (Jung and Shin, 1989). Previous phytochemical studies on *Uncaria* species have resulted in the isolation of various indole and oxindole alkaloids, which have been reported to have hypotensive and vasorelaxant effects, and Ca^{2+} channel blocking activity (Heitzman *et al.*, 2005; Laus, 2004; Park *et al.*, 1996; Tang and Eisenbrand, 1992; Yano *et al.*, 1991; Yuzurihara *et al.*, 2002). Recently, triterpene esters also have been isolated as inhibitors of phospholipase Cγ1 and cancer cell proliferation (Lee *et al.*, 2000).

In our ongoing search for monoamine oxidase (MAO) inhibitors from natural sources, it was found that an extract of *U. rhynchophylla* strongly inhibited the MAO activity. MAO plays a critical role in the regulation of monoamine neurotransmitters such as dopamine, norepinephrine, and serotonin. Two MAO isoenzymes, MAO-A and MAO-B, have been identified based on their substrate preference, specific inhibitor selectivity, and tissue distribution (Abell and Kwan, 2001; Murphy, 1978). Selective MAO-A inhibitors have been used clinically in the treatment depression and anxiety, while MAO-B inhibitors have been used coadjuvant agents in the treatment of Parkinson's and Alzheimer's diseases (Thomas, 2000; Yamada and Yasuhara, 2004; Youdim and Riederer, 2004).

Bioactivity-guided chromatographic fractionation led to the isolation of a known β-carboline alkaloid, harman (**1**), as an active compound along with five inactive or weakly active alkaloids, isocorynoxeine (**2**), isorhynchophylline (**3**), corynoxeine (**4**), cadambine (**5**), and 3α-dihydrocadambine (**6**). In the present study, the isolation and structure determination as well as inhibitory effects on mouse brain MAO are reported.

Experimental

Instruments and reagents – Melting points were measured on a Büchi model B-540 without correction. Optical rotations were determined on JASCO DIP-370 polarimeter at 25°C. IR spectra were taken on a JASCO FT/IR 300E spectrometer. UV spectra were obtained on a Milton Roy 3000 spectrometer. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded using a Bruker AMX 500 MHz NMR spectrometers using DMSO-d_6 or $CDCl_3$ as a solvent. EI-MS was measured on a Hewlett Packard 5989A mass spectrometer. Open column chromatography was performed using a silica gel (Kieselgel 60, 70-230 mesh, Merck), and thin layer chromatography (TLC) using a pre-coated silica gel 60

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F_{254} (0.25 mm, Merck). The fluorescence intensities were measured on a Perkin Elmer LS50B fluorescence spectrophotometer.

Kynuramine, 4-hydroxyquinoline, and iproniazid were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Plant material – The dried hooks and stems of *Uncaria rhynchophylla* were purchased from an herbal drug store at Cheongju, Korea, in September 2003 and identified by Emeritus Prof. Kyong Soon Lee, a plant taxonomist at Chungbuk National University. A voucher specimen (No.030902) has been deposited at the Herbarium of College of Pharmacy, Chungbuk National University, Korea.

Animals – The ICR male 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 air-dried hooks and stems of *U. rhynchophylla* (3 Kg) were pulverized and extracted with MeOH at room temperature. After filtration and evaporation of the solvent under reduced pressure, the combined crude methanolic extract (760.2 g) was suspended in H_2O to yield an aqueous MeOH solution, which was then partitioned in turn with CH_2Cl_2, EtOAc and *n*-BuOH, to afforded dried CH_2Cl_2 (65.2 g), EtOAc (28.8 g), *n*-BuOH (83.6 g) and H_2O-soluble (67.5 g) extracts. The CH_2Cl_2 extract exhibiting 70.1% inhibition on MAO activity at 200 μg/ml was subjected to a vacuum liquid chromatography using a CH_2Cl_2-MeOH step gradient system (100 : 0, 50 : 1, 20 : 1, 5 : 1, 0 : 100, each 2 L) to give five to fractions (C1-C5). Fraction C4 (12.2 g) was further applied to column chromatography over silica gel (3×25 cm, 70-230 mesh) eluting with CH_2Cl_2-MeOH (50 : 1, 20 : 1, 10 : 1, 2 : 1) to yield eight fractions (C41-C48). The MAO inhibitory effects of these eight combined fractions were 8.4, 5.3, 4.7, 5.6, 5.4, 35.8, 81.5 and 74.5 % at the concentration of 50 μg/ml, respectively. Fraction C47 was subjected to chromatography over sephadex LH-20 eluting with $CHCl_3$-MeOH (1 : 1) to provide compound **1** (harman, 3.5 mg). The *n*-BuOH extract exhibiting 32.9 % inhibition on MAO activity at 200 μg/ml was subjected to chromatography over Diaion HP-20 using a H_2O-MeOH step gradient system to give five fractions (B1-B5). The MAO inhibitory effects of these five combined fractions were 2.5, 41.8, 66.6, 63.6 and 70.0% at the concentration of 150 μg/ml, respectively. Fraction B5 (9.0 g) was further applied to column chromatography over

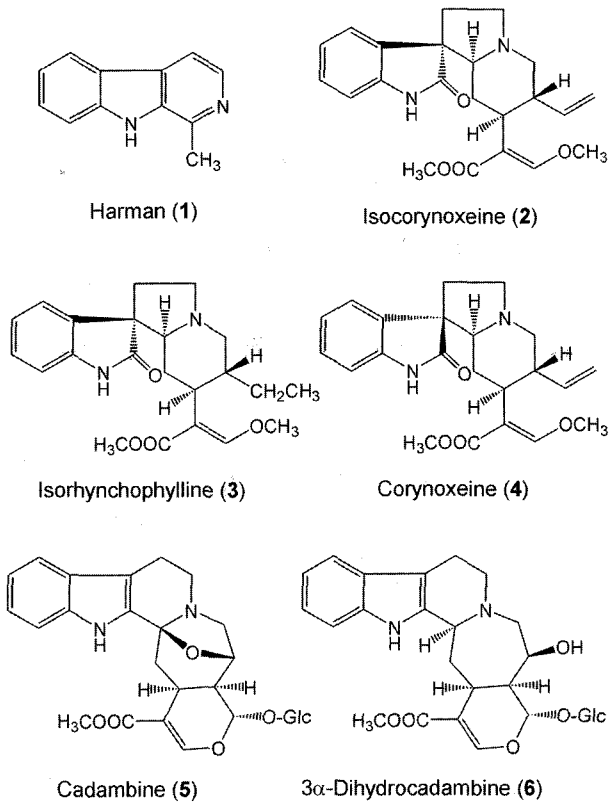

Fig. 1. Chemical structures of isolated compounds from *U. rhynchophylla*.

silica gel (2×15 cm, 70-230 mesh) eluting with EtOAc-MeOH-H_2O (100 : 1 : 0.2 → 10 : 1 : 0.2) to yield five fractions (B51-B55). Fractions B51-B55 were further purified by silica gel column chromatography (2×15 cm, 70-230 mesh) eluting with $CHCl_3$: MeOH (20 : 1) to yield compounds **2** (isocorynoxeine, 17.8 mg), **3** (isorhynchophylline, 16.5 mg), **4** (corynoxeine, 14.3 mg), **5** (cadambine, 15.7 mg), and **6** (3α-dihydrocadambine, 9.4 mg), respectively.

Harman (1) – white amorphous powder; mp 237-238 °C; UV λ_{max} MeOH nm (log ε): 350 (4.0), 289 (4.3), 250sh (4.3), 238 (4.7); IR ν_{max} cm^{-1}: 1660, 1605, 1570, 1500; EI-MS *m/z*: 182 $[M]^+$; ^{1}H-NMR (500 MHz, DMSO-d_6) δ : 11.6 (1H, br s, NH), 8.21 (1H, d, *J* = 5.2 Hz, H-3), 8.19 (1H, d, *J* = 7.7 Hz, H-5), 7.92 (1H, d, *J* = 5.2 Hz, H-4), 7.59 (1H, d, *J* = 7.7 Hz, H-8), 7.53 (1H, t, *J* = 7.7 Hz, H-7), 7.25 (1H, t, *J* = 7.7 Hz, H-6), 2.78 (3H, s, CH_3); ^{13}C-NMR (125 MHz, DMSO-d_6) δ : 142.1 (C-1), 140.4 (C-8a), 137.5 (C-3), 134.5 (C-9a), 127.7 (C-7), 126.9 (C-4a), 121.7 (C-5), 121.1 (C-4b), 119.1 (C-6), 112.6 (C-4), 111.9 (C-8), 20.5 (C-1').

Isocorynoxeine (2) – yellow amorphous powder; $[\alpha]^{25}_D$ +7.6° (c = 0.1, $CHCl_3$); IR ν_{max} cm^{-1}: 1710, 1640, 1620;

EI-MS *m/z*: 382 $[M]^+$; ^{1}H-NMR (500 MHz, $CDCl_3$), ^{13}C-NMR (125 MHz, $CDCl_3$) and physical constants were identical with the previous report (Kitajima *et al.*, 2001; Mimaki *et al.*, 1997; Park *et al.*, 1993).

Isorynchophylline (3) – pale yellow amorphous powder; $[\alpha]^{25}_D$ +7.5° (c = 0.1, $CHCl_3$); IR ν_{max} cm^{-1}: 1705, 1645, 1630; EI-MS *m/z*: 384 $[M]^+$; ^{1}H-NMR (500 MHz, $CDCl_3$), ^{13}C-NMR (125 MHz, $CDCl_3$) and physical constants were identical with the previous report (Park *et al.*, 1993; Wagner *et al.*, 1985).

Corynoxeine (4) – yellow amorphous powder; $[\alpha]^{25}_D$ –35.5° (c = 0.1, $CHCl_3$); IR ν_{max} cm^{-1}: 1710, 1650, 1610; EI-MS *m/z*: 382 $[M]^+$; ^{1}H-NMR (500 MHz, $CDCl_3$), ^{13}C-NMR (125 MHz, $CDCl_3$) and physical constants were identical with the previous report (Nozoye *et al.*, 1975; Park *et al.*, 1993).

Cadambine (5) – colourless prism; $[\alpha]^{25}_D$ –150° (c = 0.1, MeOH); IR ν_{max} cm^{-1}: 3400, 1695, 1620; EI-MS *m/z*: 544 $[M]^+$; ^{1}H-NMR (500 MHz, $CDCl_3$), ^{13}C-NMR (125 MHz, $CDCl_3$) and physical constants were identical with the previous report (Endo *et al.*, 1983; Handa *et al.*, 1983).

3α-Dihydrocadambine (6) – colourless amorphous solid; $[\alpha]^{25}_D$ –91° (c = 0.1, MeOH); IR ν_{max} cm^{-1}: 3400, 1700, 1625; EI-MS *m/z*: 546 $[M]^+$; ^{1}H-NMR (500 MHz, $CDCl_3$), ^{13}C-NMR (125 MHz, $CDCl_3$) and physical constants were identical with the previous report (Endo *et al.*, 1983).

MAO preparation and assay for MAO activity – The crude MAO was prepared by Naoi's method with minor modification (Naoi and Nagatsu, 1987; Ro *et al.*, 2001). The MAO activity with kynuramine as a substrate was assayed by a modification of the fluorometric method of Kraml (Kraml, 1965; Ro *et al.*, 2001). The samples (50 μl) were added to 0.2 M potassium phosphate buffer (750 μl, pH 7.4), which contained 30 μl of mouse brain mitochondrial suspension. The reaction was initiated by the addition of 200 μl of 500 mM kynuramine. After incubation of 37°C for 30 min, the reaction was terminated by the addition of 250 μl of 10% $ZnSO_4$ and 50 μl of 1 N NaOH, and the reaction mixture was centrifuged at 3,000 x *g* for 5 min. 1.4 ml of 1 N NaOH was added in 700 μl of assay mixture taken from the supernatant, then the mixture was transferred into a fluoro 96-well plate. Fluorescence intensity of 4-hydroxyquinoline, which was formed from kynuramine by MAO, was measured at 380 nm with excitation at 315 nm.

Results and Discussion

The MeOH extract of the hooks and stems of *U. rhynchophylla* showed potent inhibitory effects on mouse brain MAO activity (Table 1). Bioassay-directed fractionation of the CH_2Cl_2 soluble fraction resulted in the isolation of an active β-carboline alkaloid, harman (**1**), along with five weakly active or inactive alkaloids, isocorynoxeine (**2**), isorhynchophylline (**3**), corynoxeine (**4**), cadambine (**5**), and 3α-dihydrocadambine (**6**).

Table 1. Inhibitory effects of the solvent extracts from the *U. rhynchophylla* on MAO in mouse brain

Sample	Concentration (μg/ml)	MAO activity (% of control) (nmol/min/mg protein)	
Control		0.854 ± 0.021	(100.0)
MeOH extract	250	0.296 ± 0.020	(55.3)**
CH_2Cl_2 extract	200	0.160 ± 0.018	(29.9)**
EtOAc extract	200	0.381 ± 0.007	(71.2)
BuOH extract	200	0.359 ± 0.005	(67.1)*
H_2O extract	200	0.737 ± 0.009	(86.3)

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

Compound **1**, the most active alkaloid, was obtained as white amorphous powder and gave a molecular ion $[M]^+$ at *m/z* 182 by EI-MS, consistent with an elemental formula of $C_{12}H_{10}N_2$. The UV spectrum displayed maxima at 238, 250 sh, 289, and 350 nm characteristic of a β-carboline chromophore. In the ^{1}H-NMR spectrum, four vicinal aromatic protons at δ 8.19 (1H, d, J = 7.7 Hz), 7.59 (1H, d, J = 7.7 Hz), 7.53 (1H, t, J = 7.7 Hz), 7.25 (1H, t, J = 7.7 Hz), and two protons as an AB system at δ 8.21 (1H, d, J = 5.2 Hz) and 7.92 (1H, d, J = 5.2 Hz), indicated the presence of only one substituent at C-1 of the β-carboline skeleton. The remaining proton signal at δ 2.78 (3H, s) was assigned to the methyl group attached at C-1 position. The ^{13}C-NMR and DEPT spectra of **1** indicated the presence of one methyl carbon, six methine carbons, and five quaternary carbons. Therefore, the structure of compound **1** was determined to be harman and was confirmed by comparison to previously reported data (Seki *et al.*, 1993; 2000). The β-carboline alkaloids distributed in many plants and exhibited a wide spectrum of pharmacological and neuroactive actions (Allen and Holmstedt, 1980; Adell *et al.*, 1996). Recently, it has been reported that harman co-occur in *Uncaria tomentosa* accompanied with β-carboline type monoterpenoid glucoindole alkaloids (Kitajima *et al.*, 2001).

The structures of five known indole and oxindole alkaloids were identified by comparison with their physical properties including optical rotation values and spectral data (mp, UV, IR, $[\alpha]_D$, MS, ^{1}H-, ^{13}C-NMR and DEPT) with literature values (Endo *et al.*, 1983; Handa *et*

Table 2. Inhibitory effects of harman (**1**) on MAO in mouse brain

	Concentration (μM)	MAO activity (% of control) (nmol/min/mg protein)		IC_{50} (μM)
Control		0.854 ± 0.011	(100.0)	
Harman (**1**)	50	0.320 ± 0.007	(37.5)**	11.1
	1	0.607 ± 0.002	(71.1)*	
	0.1	0.721 ± 0.005	(84.3)	
	0.05	0.803 ± 0.001	(94.0)	
Isocorynoxeine (**2**)	200	0.614 ± 0.010	(71.9)	>100
	100	0.755 ± 0.021	(88.5)	
Isorhynchophylline (**3**)	200	0.685 ± 0.042	(80.2)	>100
	100	0.793 ± 0.033	(92.9)	
Corynoxeine (**4**)	200	0.746 ± 0.028	(87.4)	>100
	100	0.803 ± 0.035	(94.0)	
Cadambine (**5**)	200	0.847 ± 0.016	(99.2)	>100
	100	0.865 ± 0.052	(101.3)	
3α-Dihydrocadambine (**6**)	200	0.875 ± 0.053	(102.5)	>100
	100	0.899 ± 0.026	(105.3)	

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

al., 1983; Kitajima *et al.*, 2001; Mimaki *et al.*, 1997; Park *et al.*, 1993; Nozoye *et al.*, 1975; Wagner *et al.*, 1985).

The MAO inhibitory effects of all of the isolates from *U. rhynchophylla* were measured using the non-selective substrate kynuramine. Among the six known compounds, only harman (**1**) was found to significantly inhibit the mouse brain MAO activity, with an IC_{50} value of 11.1 μM (Table 2). In this assay, iproniazid as a positive control exhibited an IC_{50} value on the enzyme activity at the concentration of 12.9 μM. Five known indole alkaloids, isocorynoxeine (**2**), isorhynchophylline (**3**), corynoxeine (**4**), cadambine (**5**), and 3α-dihydrocadambine (**6**), were found to be weakly active or inactive (IC_{50}: >100 μM).

Previous studies have reported that the 50% aqueous methanol extract of *U. rhynchophylla* showed a selective inhibitory effect against MAO-B (IC_{50}: 30 μg/ml) than MAO-A (IC_{50}: 190 μg/ml). Bioassay-guided isolation of *U. rhynchophylla* yielded two known compounds, (+)-catechin and (−)-epicatechin, which showed MAO-B inhibitory effect with the IC_{50} values of 25.7 μg/ml (88.6 μM) and 17.1 μg/ml (58.9 μM), respectively. (Hou *et al.*, 2005; Lin *et al.*, 2003).

In the present study, a known β-carboline alkaloid, harman, was isolated here for the first time as an active constituent from *U. rhynchophylla.* However, harman is already known as a potent inhibitor of monoamine oxidase A based on a result of its binding to the active site of the enzyme. (May *et al.*, 1991; Rommelspacher *et al.*, 1994) Moreover, it has been shown that β-carboline alkaloids act as potent and reversible inhibitors of MAO (Kim *et al.*, 1997).

Our findings indicate that harman, a β-carboline alkaloid, could be a main MAO inhibitory principle, though indole and oxindole alkaloids are the main constituents of *U. rhynchophylla.*

Acknowledgment

This work was supported by the research grant of the Chungbuk National University in 2003. We are grateful to Korea Basic Science Institute for ^{1}H- and ^{13}C-NMR spectral measurements.

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(Accepted August 10, 2005)