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Acetylcholinesterase Inhibitors from the Aerial Parts of *Corydalis speciosa*

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In a bioassay-guided search for acetylcholinesterase inhibitors from Korean natural resources, four isoquinoline alkaloids, corynoxidine (**1**), protopine (**2**), palmatine (**3**), and berberine (**4**) have been isolated from the methanolic extract of the aerial parts of *Corydalis speciosa*. Structures of these compounds were elucidated on the basis of spectroscopic techniques. These compounds inhibited acetylcholinesterase activity in a dose-dependent manner, and the IC_{50} values of compounds **1-4** were 89.0, 16.1, 5.8, and 3.3 μM, respectively.

Key words: *Corydalis speciosa*, Acetylcholinesterase, Isoquinoline alkaloids

INTRODUCTION

According to the cholinergic hypothesis of the pathogenesis of Alzheimers disease (AD), memory impairments in AD patients result from a deficit of cholinergic functions in the brain. An important therapeutic strategy for activating central cholinergic functions has been the use of inhibitors of acetylcholinesterase (AChE), which is responsible for the metabolic hydrolysis of the neurotransmitter acetylcholine (Kalauni *et al.*, 2002; Bartus *et al.*, 1982; Perry, 1986; Bartus, 2000). In an ongoing study into the discovery of AChE inhibitors from natural resources (Kim, 2002; Kim *et al.*, 2002; Kim *et al.*, 2003; Lee *et al.*, 2004), we found that a methanolic extract of the aerial parts of *Corydalis speciosa* Maximowicz (Papaveraceae) inhibited AChE in a dose-dependent manner. Subsequent activity-guided fractionation of the methanolic extract led to the isolation of four isoquinoline alkaloids as active components.

C. speciosa have been used in Korea and China as a folk medicine for its antipyretic, insectifuge, analgesic and diuretic properties (Ahn, 2001). Earlier investigations on the chemical constituents of *C. speciosa* dealt with the isolation of isoquinoline alkaloids such as corypalline, protopine, α-allocryptopine, capaurimine, capaurine and *dl*-tetrahydropalmatine (Tani *et al.*, 1975a).

Here we describe the isolation of four isoquinoline alkaloids from *C. speciosa* and their anti-AChE activity.

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MATERIALS AND METHODS

Plant materials

The aerial parts of *C. speciosa* were collected in May 2001 at Sunchang, Chonbuk, Korea. A voucher specimen is deposited in the herbarium of College of Pharmacy, Woosuk University (WSU-01-012).

General procedure

^{1}H- and ^{13}C-NMR spectra were determined on a JEOL JMN-EX 400 spectrometer. TLC was carried out on Merck precoated silica gel F_{254} plates and silica gel for column chromatography was Kiesel gel 60 (230-400 mesh, Merck). Sephadex LH-20 was used for column chromatography (Pharmacia, 25-100 μm). Column for LPLC was Lobar A (Merck Lichroprep Si 60, 240-10 mm). All of the chemicals and solvents used were analyticlal grade and used without further purification. Acetylthiocholine iodide (ASCh), 5,5-dithiobis-2-nitrobenzoic acid (DTNB) and AChE (Type V-S) were purchased from Sigma Chemical Co.

Extraction and isolation

The air-dried plant materials (600 g) were finely ground

and extracted at room temperature for 7 days with MeOH, and then extracted at 50°C (each two times). The resulting extract (120 g) was acidified with 1 N hydrochloric acid. The aqueous solution was then alkalized with 10% ammonium hydroxide, and extracted with chloroform. Evaporation of the organic layer left a crude extract weighing 13 g. This material was chromatographed on a silica gel column using a mixture of *n*-hexane-EtOAc with increasing polarity and yielded seven fractions (fr.1-fr.7). Among these fractions, fr.6 and 7 showed the most significant AChE inhibitory activity. Silica gel column chromatography of fr.6 with a solvent gradient of methanol in chloroform gave three subfraction (fr.61-fr.63). Subfraction fr.62 was further separated by Prep-TLC (Silica gel, $CHCl_3$-MeOH=5:1) and purified with Sephadex LH-20 column (MeOH) to give **1** (10 mg) and **2** (8 mg). Fraction fr.7 was separated by Lobar-A column (EtOAc:MeOH= 1:2) and purified by Sephadex LH 20 (MeOH) to yield **3** (10 mg) and **4** (7 mg).

Corynoxidine (1)

mp : 182-183°C; MS (EI, 70 eV, m/z) : 371 (M^+), 353, 338, 294, 149; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.01 (1H, d, *J*=8.4 Hz), 6.89 (1H, d, *J*=8.4 Hz), 6.70 (2H, s, H-1,4), 4.71 (1H, d, *J*=15.6 Hz), 4.51 (1H, d, *J*=15.6 Hz), 3.89 (6H, s, 2×OCH_3), 3.87 (3H, s, OCH_3), 3.86 (3H, s, OCH_3), 3.29 (1H, dd, *J*=16.0, 3.6 Hz), 2.72 (1H, dd, *J*=16.0, 3.2 Hz), ^{13}C-NMR (100 MHz, $CDCl_3$) δ: (Table I).

Protopine (2)

mp : 206-208°C; MS (EI, 70 eV, m/z) : 353 (M^+), 338, 336, 322, 281, 205, 148; ^{1}H-NMR (400 MHz, $CDCl_3$+CD_3OD= 2:1), δ: 6.89 (1H, s, H-1), 6.70 (1H, d, *J*=7.6, H-11), 6.67 (1H, d, *J*=7.6, H-12), 6.66 (1H, s, H-4), 5.96 (2H, s, OCH_2O), 5.94 (2H, s, OCH_2O), 3.73 (2H, s, H-13), 3.37 (2H, s, H-8), 1.91 (3H, s, N-CH_3), ^{13}C-NMR (100 MHz, $CDCl_3$+CD_3OD=2:1) δ: (Table I).

Palmatine (3)

^{1}H-NMR (400 MHz, CD_3OD) δ: 9.67 (1H, s, H-8), 8.71 (1H, s, H-13), 8.02 (1H, d, *J*=8.4 Hz, H-11), 7.92 (1H, d, *J*=8.4 Hz, H-12), 7.57 (1H, s, H-1), 6.96 (1H, s, H-4), 4.82 (2H, m, H-6), 4.11 (3H, s, 9-OCH_3), 4.01 (3H, s, 10-OCH_3), 3.89 (3H, s, 2-OCH_3), 3.84 (3H, s, 3-OCH_3), ^{13}C-NMR (100 MHz, $CDCl_3$) δ : (Table I).

Berberine (4)

^{1}H-NMR (400 MHz, $CDCl_3$+CD_3OD=1:1) δ: 9.80 (1H, s, H-8), 8.45 (1H, s, H-13), 7.96 (1H, d, *J*=9.2 Hz, H-11), 7.92 (1H, d, *J*=9.2 Hz, H-12), 7.48 (1H, s, H-1), 6.88 (1H, s, H-4), 6.12 (2H, s, OCH_2O), 4.96 (2H, m, H-6), 4.23 (3H, s, 9-OCH_3), 4.10 (3H, s, 10-OCH_3), 3.26 (2H, m, H-5), ^{13}C-NMR (100 MHz, $CDCl_3$+CD_3OD=1:1) δ: (Table I).

Table I. ^{13}C-NMR chemical shifts of compounds **1-4**

Carbon	1[a]	2[b]	3[c]	4[d]
1	109.0	108.5	109.9	105.5
2	148.1	146.4	150.9	148.9
3	148.5	148.3	151.9	151.2
4	111.9	110.6	112.2	108.7
4a	124.0*	133.0	135.3	130.3
5	24.5	31.9	27.8	27.6
6	64.9	57.6	57.4	56.3
8	67.9	50.8	146.4	145.3
8a	123.2	117.7	120.5	122.3
9	150.7	146.2	153.8	144.8
10	145.7	146.1	145.8	150.8
11	111.6	106.9	124.5	127.1
12	123.8	125.2	128.1	123.3
12a	124.8*	128.9	130.1	133.8
13	29.6	46.4	121.3	120.4
14	68.0	194.9	139.8	138.4
14a	125.6*	135.8	123.3	120.3
N-CH_3		41.2		
2-OCH_3	56.0		57.0	
3-OCH_3	55.9		57.6	
9-OCH_3	60.3		62.5	62.3
10-OCH_3	56.3		56.7	56.3
OCH_2O		101.4		102.5
		101.1		

[a]Recorded at 100 MHz in $CDCl_3$.
[b]Recorded at 100 MHz in $CDCl_3$+CD_3OD (2:1).
[c]Recorded at 100 MHz in CD_3OD.
[d]Recorded at 100 MHz in $CDCl_3$+CD_3OD (1:1).
*Assignments may be reversed.

Enzyme extraction

Male ICR mice were used in this experiment, and the procedures were performed in accordance with the animal care guidelines of the NIH. In order to extract AChE enzyme, the animals were put to death after total anesthesia by ether, then their brains were dissected. The forebrains were separated and homogenated with 5 volumes of a homogenation buffer [10 mM Tris-HCl (pH 7.2), containing 1 M NaCl, 50 mM $MgCl_2$, and 1% triton X-100] (Rieger *et al.*, 1980), then centrifuged at 10,000×g for 30 min. The resulting supernatant was used as an enzyme source. All extracting steps were carried out at 4°C. Protein concentration was determined using BCA kit (bicinchoninic acid, Sigma Co., USA) with bovine serum albumin (BSA) as the protein standard.

Acetylcholinesterase inhibition assay

The AChE assay was performed by the method of Ellman *et al.* with minor modifications, using acetylthiocholine iodide as a substrate (Ellman *et al.*, 1961). Ellmans reaction mixture was made from a combination of 0.5 mM acetylthiocholine iodide and 1 mM 5,5'-dithio-bis-(2-nitrobenzoic acid) in a 50 mM sodium phosphate buffer (pH

8.0). The rates of hydrolysis by AChE were monitored spectrophotomatically using a 96-well microtiter plate reader (Mortensen *et al.*, 1996). Each extract (or compound, 10 μL) and 50 mM sodium phosphate buffer (30 μL) were mixed with the enzyme solution (10 μL). Ellmans reaction mixture (50 μL) was further added to give a final volume of 100 μL, and the mixture was incubated at 37°C for 30 min. Absorbance at 450 nm was measured immediately after adding the Ellmans reaction mixture. Measurement was repeated for 10 min at 2 min intervals to verify that the reaction occurred linearly. Blank reaction was measured by substituting saline for the enzyme (Chung *et al.*, 2001; Park *et al.*, 1996).

Statistical analysis

The IC_{50} values were calculated with the SPSS program (Version 8.0).

RESULTS AND DISCUSSION

The methanolic extract of the aerial parts of *C. speciosa* was found to exhibit significant anti-AChE activity. To isolate the AChE inhibitory constituents from *C. speciosa*, the crude alkaloids was chromatographed as described in extraction and isolation section. Four compounds (**1**-**4**) were separated as active constituents and identified as corynoxidine, protopine, palmatine and berberine, respectively, from physicochemical and spectral data in comparison with those of published literatures (Fig. 1).

Compounds **1**-**4** have similar patterns in their NMR spectra, and showed positive results in Dragendorff's reagent. The EI-MS of **1** gave a molecular ion at *m/z* 371 [M^+]. In the ^{1}H-NMR spectrum of **1**, signals of four methoxyl groups (δ 3.89, 2×OCH_3; 3.87; 3.86), and four aromatic protons at δ 7.01 (1H, d, *J*=8.4 Hz), 6.89 (1H, d, *J*=8.4 Hz), and 6.70 (2H, s) were observed. The ^{13}C-NMR spectrum of **1** exhibited signals of twelve aromatic carbons (δ 150.7, 148.5, 148.1, 145.7, 125.6, 124.8, 124.0, 123.8, 123.2, 111.9, 111.6, 109.0), three nitrogen-bearing carbons (δ 68.0, 67.9, 64.9), four methoxyl groups (δ 60.3, 56.3, 56.0, 55.9), and two aliphatic carbons (δ 29.6, 24.5). From these results, compound **1** indicated to be a tetrahydroprotoberberine type alkaloid. In addition to these evidences, comparison of spectral data with those published in the literature established the structure of **1** to be corynoxidine, previously isolated from *C. koidzumiana* (Tani *et al.*, 1975b).

The EI-MS of **2** gave a molecular ion at *m/z* 353 [M^+]. In the ^{1}H-NMR spectrum of **2**, signals of the *N*-methyl group (3H, δ 1.91, *s*), two methylenedioxide groups (δ 5.96, 5.94, each 2H, s), and the four aromatic protons at δ 6.89 (1H, s), 6.70 (1H, d, *J*=7.6 Hz), 6.67 (1H, d, *J*=7.6 Hz),

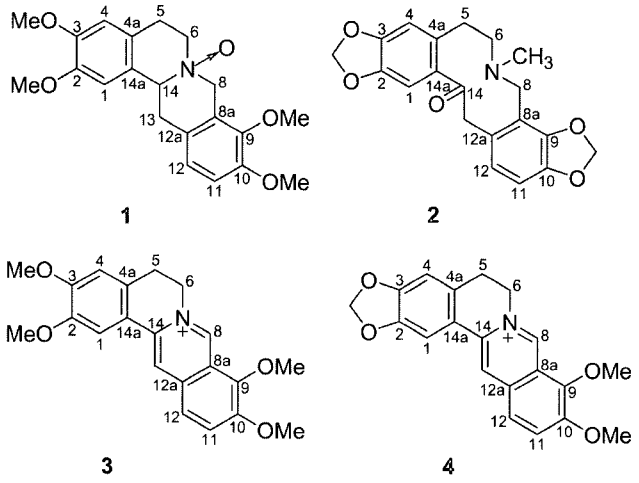

Fig. 1. Structures of compounds **1**-**4**

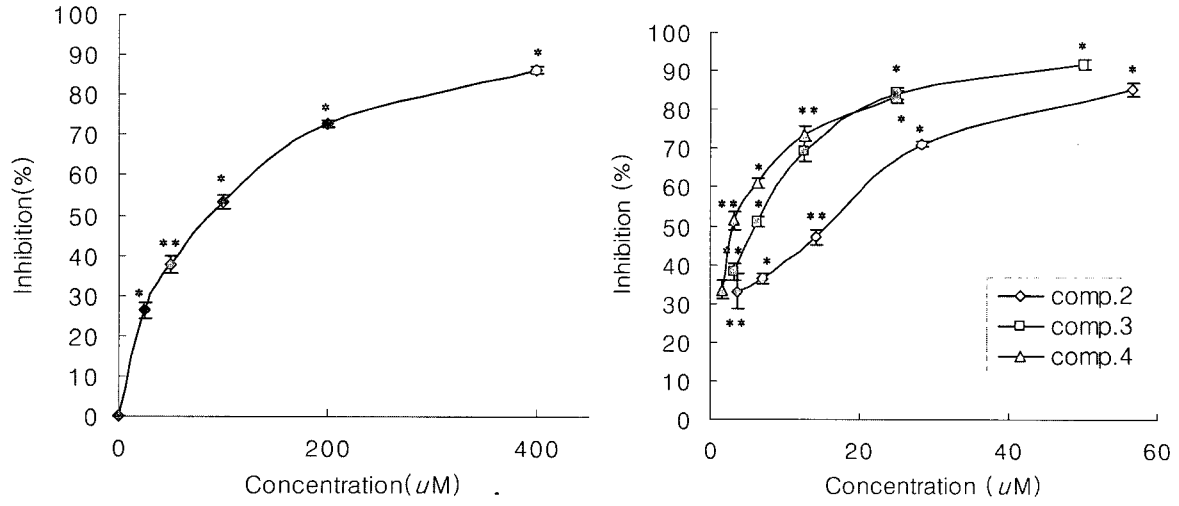

Fig. 2. The inhibitory activity of compounds **1** (left), **2**-**4** (right) on AChE. The asterisks indicate values significantly from the control at $p<0.05$ (*) or $p<0.01$(**).

and 6.66 (1H, s) were observed. The ^{13}C-NMR spectrum of **2** exhibited signals of twelve aromatic carbons, two nitrogen-bearing carbons (δ 57.6, 50.8), and two aliphatic carbons (δ 46.4, 31.9). These results suggested that the structure of **2** was a tetrahydroproberberine type alkaloid. Comparison of its spectral data with those published in the literatures established the structure of **2** to be protopine (Jewers *et al.*, 1972; Tani *et al.*, 1975a).

Compound **3** *was obtained as an amorphous yellow* powder from MeOH. The ^{1}H-NMR spectrum of **3** showed four methoxyl groups at δ 4.11, 4.01, 3.89, and 3.84, four aromatic protons as singlet at δ 9.67, 8.71, 7.57, and 6.96, and two aromatic protons as doublet δ 8.02 (1H, d, *J*=8.4 Hz) and 7.92 (1H, d, *J*=8.4 Hz). The ^{13}C-NMR spectrum of **3** exhibited fifteen aromatic carbons, and four methoxyl groups (δ 62.5, 57.6, 57.0, 56.7). From these results, compound **3** indicated to be a protoberberine type alkaloid. In addition to these evidences, comparison of spectral data with those published in the literatures establshed the structure of **3** to be palmatine (Jewers *et al.*, 1972; hussain *et al.*, 1989).

Compound **4** was obtained as an amorphous yellow powder from MeOH. The NMR spectrum of **4** was similar to that of **3**, suggesting it has the similar carbon skeleton. The main difference was the presence δ 102.5 ppm resonance instead of δ 570.0 and 57.6 ppm of **3** in the ^{13}C-NMR spectrum which can be assigned to a carbon of methylenedioxide group. On the basis of the these evidences, the structure of **4** was determined to be berberine, together with a comparison of the these data with those published in the literature (Jewers *et al.*, 1972).

Among compounds **1-4**, corynoxidine (**1**), palmatin (**3**) and berberine (**4**) were isolated for the first time from this plant. Compounds **1-4** inhibited AChE activity in a dose-dependent manner (Fig. 2). The IC_{50} (50% AChE inhibitory effect) values were determined to be 89.0, 16.1, 5.8, and 3.3 μM for **1-4**, respectively, while the IC_{50} value of a positive control, tacrine, was 0.2 μM.

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