(+)-α-Viniferin, a Stilbene *Trimer* from *Caragana chamlague*,[1] Inhibits Acetylcholinesterase

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In the course of screening natural products for anti-acetylcholinesterase (AChE) activity, we found that a total methanolic extract of the underground parts of *Caragana chamlague* (Leguminosae) had significant inhibition towards AChE. Bioactivity-guided fractionation of the total methanolic extract resulted in the isolation and identification of two active stilbene oligomers, (+)-α-viniferin (1) and kobophenol A (2). Both 1 and 2 inhibited AChE activity in a dose-dependent manner, and the IC_{50} values of 1 and 2 were 2.0 and 115.8 μM, respectively. The AChE inhibitory activity of 1 was specific, reversible and noncompetitive.

Key words *Caragana chamlague*; Leguminosae; AChE inhibitors; (+)-α-viniferin; kobophenol A

Alzheimer's disease (AD) is the most common cause of senile dementia in later life. Whereas several neurotransmitter systems are known to be involved and depleted in AD, the cholinergic system still receives the greatest attention by far. This is particularly true with regards to pharmacotherapy research and development[2] due to the involvement of the cholinergic system in learning and memory processing.[3] One promising therapeutic strategy for re-activating central cholinergic function has been the use of inhibitors of acetylcholinesterase (AChE), the enzyme responsible for the metabolic hydrolysis of ACh. Hypothetically, AChE inhibitors should increase the efficiency of cholinergic transmission by preventing the hydrolysis of released ACh, thus making more ACh available at the cholinergic synapse.[4—6] Such AChE inhibitors as physostigmine or tacrine are known to have limitations for clinical use due to their short half-lives and/or untoward side effects.

Thus, we have tried to search for more efficient AChE inhibitors with lower side effects from natural resources. During screening for AChE inhibitors from natural resources, we found that a total methanolic extract of the underground parts of *Caragana chamlague* LAMRK (Leguminosae) showed significant inhibition towards AChE. Subsequent activity-guided fractionation of the total methanolic extract led to the identification and isolation of two anti-AChE stilbenes, a resveratrol trimer, (+)-α-viniferin (**1**), and a resveratrol tetramer, kobophenol A (**2**).

The underground parts of *C. chamlague* have been used in Korea and China as a folk medicine purportedly effective against neuralgia, rheumatism, and arthritis.[7] Previous chemical studies of *C. chamlague* reported the isolation of saponins and oligomeric stilbenes such as (+)-α-viniferin, kobophenol A, caraganaphenol A and miyabenol C.[8—10] Among them, the resveratrol trimer, (+)-α-viniferin, was reported to have inhibitory effects on tyrosinase,[11] and on prostaglandin H-2 synthase.[12] (+)-α-Viniferin was also reported to be an anti-inflammatory principle of this plant.[9] By contrast, a resveratrol tetramer, kobophenol A, exhibited antimicrobial activity on *Staphylococcus aureus*.[8] However, there is no report of anti-AChE activity from *C. chamlague* or its constituents. Here, we describe anti-AChE activity of stilbene oligomers from *C. chamlague*.

MATERIALS AND METHODS

General Experimental Methods IR spectra were run in KBR on a Perkin Elmer 1710 spectrophotometer. All NMR experiments were performed on JEOL GSX 400 and 300 spectrometers equipped with 5 mm ^1H and ^{13}C probes operating at 400 and 75 MHz, respectively. The sample was run in CD_3OD or acetone-d_6 with tetramethylsilane (TMS) as an internal standard. Matrix assisted laser desorption ionization (MALDI) MS was obtained on a Voyagr-DE^{TM}, Perceptive Biosystem. Resveratrol, rhapontin, acetylthiocholine iodide (ASCh), butyrylthiocholine iodide (BuSCh), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), neostigmine bromide, tetraisopropylpyrophosphoramide (TPPA), 1,5-bis(4-allyldimethylammonium phenyl)penta-3-one (AAPP), AChE (Type V-S) and BuChE (from human serum) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Organic solvents used in the isolation of the AChE inhibitory compounds were purchased from Duksan Chemical Co. (Seoul, Korea).

Plant Material Underground parts of *C. chamlague* were purchased in Kyoung-dong market for Oriental medicine in 1998 and identified by Dr. Jong Hee Park, professor, College of Pharmacy, Pusan National University. Voucher specimens (SNUPH-0511) have been deposited in the herbarium of the College of Pharmacy, Seoul National University.

Extraction and Isolation Dried plant material (6.0 kg) was extracted six times with MeOH in an ultrasonic apparatus which, upon removal of the solvent *in vacuo*, yielded a methanolic extract (64 g). This methanolic extract was then suspended in H_2O and partitioned successively with CH_2Cl_2. Silica gel column (100 g, 2×30 cm, Merck) chromatography of CH_2Cl_2 fraction (5g) was carried out using a mixture of $CHCl_3$–MeOH with increasing polarity and yielded thirteen subfractions (fr.1—fr.13). Among these subfractions, fr.12 showed the most significant AChE inhibitory activity (58% inhibition at a concentration of 5 μg/ml, $p<0.01$). Silica gel column chromatography of fr.12 with a solvent gradient of MeOH in $CHCl_3$ yielded ten subfractions (fr.12-1—fr.12-10). Among the ten subfractions of fr. 12, fr.12-7 yielded **1** and **2** by additional purification steps on Sephadex LH-20 gel.

Compound **1**: $C_{42}H_{30}O_9$, a pale yellow amorphous solid.

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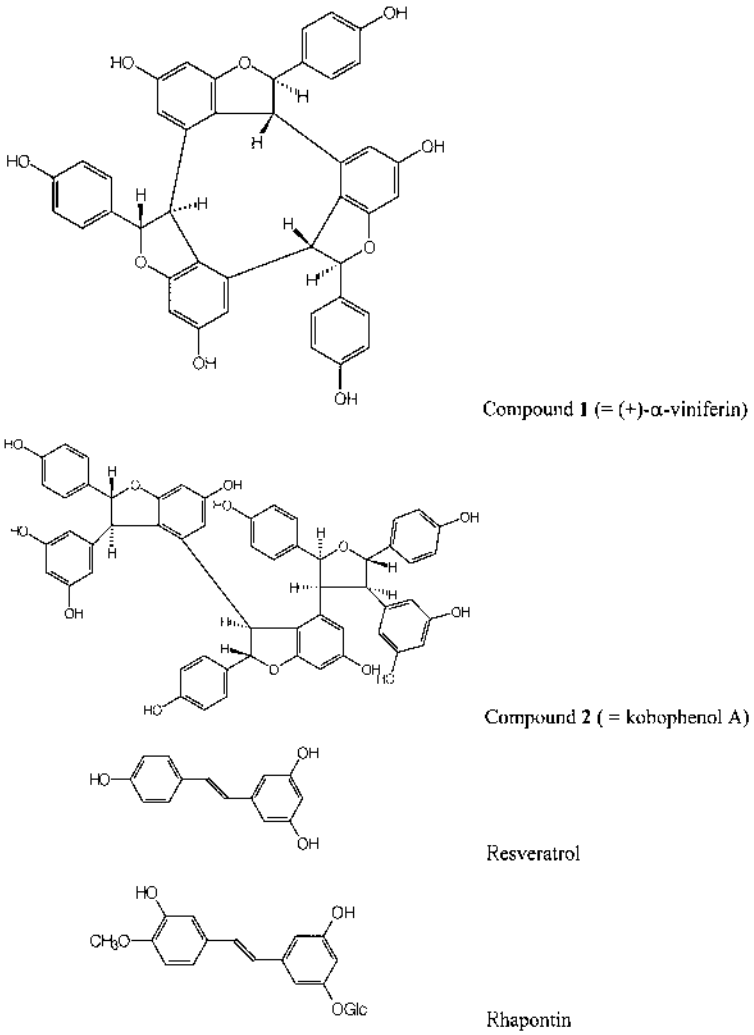

Fig. 1. Structures of Stilbenes

$[\alpha]_D$ +50° (c=0.2, MeOH). IR (KBr) cm^{-1}: 3400 (–OH), 1613 (aromatic –C=C–), 1597, 1514, 829, 574. MALDI MS: 701 $[M+Na]^+$, 678 $[M]^+$. ^1H- and ^{13}C-NMR spectral data of compound **1** were in good agreement with those values reported for (+)-α-viniferin.[9)]

Compound **2**: $C_{56}H_{44}O_{13}$, a pale yellow amorphous solid $[\alpha]_D$: +218° (c=0.2, MeOH). IR (KBr) cm^{-1}: 3430 (–OH), 1617 (aromatic –C=C–), 1241 (–O–), 1514, 1453, 1364, 1167, 1121, 1000, 833, 574. MALDI MS: 947 $[M+Na]^+$, 924 $[M]^+$. ^1H- and ^{13}C-NMR spectral data of compound **2** were in good agreement with those values reported for kobophenol A.[13)]

Determinations of Cholinesterases Activities Activities of AChE and BuChE were determined by a slightly modified method of Ellman.[14)] AChE and BuChE were diluted in phosphate buffer (pH 8.0, 0.1 M) at 4.3 units/ml and 1.2 units/ml, respectively. The color reagent, DTNB, sample and authentic AChE or BuChE were added to a spectrophotometric cuvette and preincubated for 5 min, at 25°C or 37°C, respectively. ASCh or BuSCh was added as a substrate, and incubation continued for 3 min. The activity of AChE or BuChE was terminated by the addition of 100 μM neostigmine or 100 μM TPPA. Finally, the absorbance at 412 nm was measured.

RESULTS AND DISCUSSION

The total methanolic extract of the underground parts of *C. chamlague* was found to exhibit significant anti-AChE activity. To isolate the AChE inhibitory constituents of *C. chamlague*, the total methanolic extract was suspended in H_2O and partitioned with CH_2Cl_2. As a result, the activity was found in the CH_2Cl_2 fraction. Using several chromatographic techniques, compounds **1** and **2** were isolated as active constituents and identified as (+)-α-viniferin and kobophenol A, respectively, from spectral data in comparison with that of published values.[9)]

Compounds **1** and **2** inhibited AChE activity in a dose-dependent manner (Fig. 2). The concentrations of **1** and **2** required for half-maximal AChE inhibition (IC_{50}) were determined to be 2.0 and 115.8 μM, respectively (Table 1), while the IC_{50} value of a positive control, velnacrine, was 0.4 μM.

The characteristics of AChE inhibitory action of **1** were determined as follows. The anti-AChE activity of **1** was independent of incubation time (up to 60 min, data not shown). This result suggests that **1** inhibited AChE reversibly. The kinetic analysis of AChE inhibition of **1** is shown in Fig. 3. The K_m and V_{max} values were calculated from a Lineweaver–Burk plot. The V_{max} value of AChE as plotted against [ASCh] was

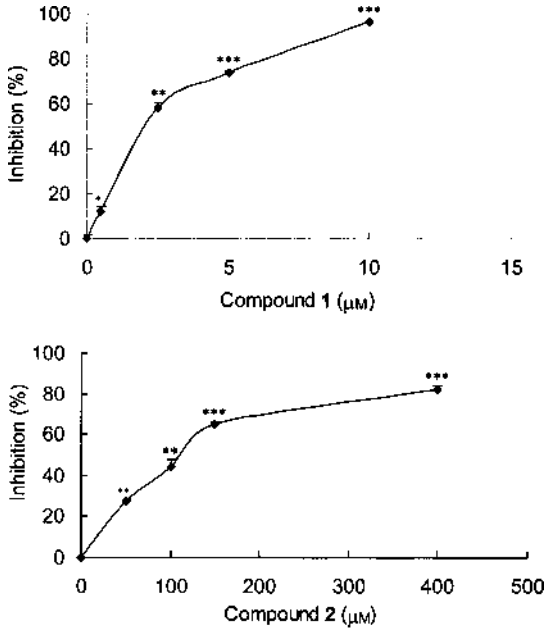

Fig. 2. The Inhibitory Activities of Compounds **1** and **2** on AChE

Differs significantly from the control, effective $*p<0.05$, $**p<0.001$, $***p<0.0005$.

Table 1. The Inhibitory Activities of Stilbenes on AChE

Compound	IC_{50} (μM)
1	2.0
2	115.8
Resveratrol	>500
Rhapontin	>500
Velnacrine	0.4

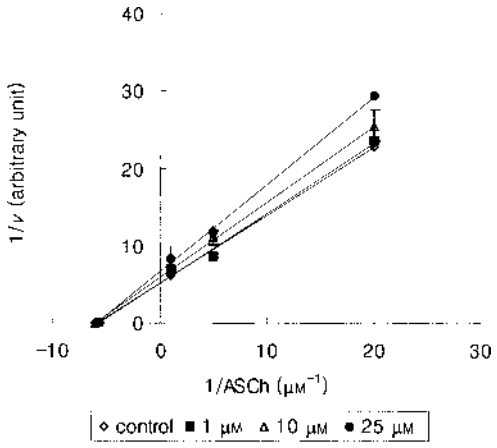

Fig. 3. Lineweaver–Burk plot of 1/*v* *vs.* 1/ASCh in the Presence or Absence of Compound **1**

decreased significantly by the addition of **1**; however, the K_m value was not changed. These results indicate that **1** inhibited AChE in a noncompetitive manner. The selectivity of **1** for AChE as opposed to BuChE was also tested and compared with that of a positive control, velnacrine, *in vitro* (Table 2). Compound **1** did not appreciably inhibit BuChE. BuChE, which is present in glial cells of the nervous system and in non-nervous tissues, was unaffected by **1** at the concentration of 10 μM, while BuChE was more efficiently inhibited than AChE by velnacrine at the concentration of 1 μM. Based on these results, we would expect **1** to have no significant side effects attributable to cross-reactivity to BuChE, in contrast to velnacrine.

Table 2. The Inhibitory Activities of Compound **1**, TPPA and AAPP on AChE and BuChE

Compound (10 μM)	Inhibition	
	AChE (%)	BuChE (%)
1	96.4**	16.9*
TPPA	0.0	70.0**
AAPP	100.0**	0.0
Velnacrine (1 μM)	59.8**	89.1**

TPPA and AAPP were used as reference inhibitors to mediate the specific inhibition of BuChE and of AChE, respectively. Differs significantly from the control, effective $*p<0.05$, $**p<0.001$.

The above results led us to investigate the effect of stilbene-olgomerization on AchE inhibitory activity. The inhibition of AChE was determined to be caused by a stilbene monomer, resveratrol, which is the structural unit of compounds **1** and **2**, and by a different type of stilbene monomer, rhapontin. However, AChE inhibition by resveratrol or rhapontin was not significant (Table 1). Based on the results, **1** might be a valuable AChE inhibitor because it has an appropriately bulky structure that masks AChE and prevents ASCh from binding to AChE in a noncompetitive manner. In contrast, in the case of a tetramer, **2**, while it has a bulky structure, its activity may come to be lowered due to the simple difficulty of accessibility to AChE. Our hypotheses may be verified with further study. The anti-amnestic activity of **1** *in vivo* is being studied in our laboratory.

Acknowledgements This work was supported in part by a research grant (HMP-98-D-6-0053) from the Ministry of Health and Welfare of Korea and by the 2000BK21 Project for Medicine, Dentistry and Pharmacy.

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