J. Korean Soc. Appl. Biol. Chem. **53**(3), 371-374 (2010)

Short Communication

Secoiridoid Glycoside from the Flowers of *Osmanthus fragrans* var. *aurantiacus* Makino Inhibited the Activity of β-Secretase

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Received July 28, 2009; Accepted March 15, 2010

One secoiridoid glycoside was isolated from the EtOAc-soluble fraction of flowers of *Osmanthus fragrans* var. *aurantiacus* by repeated silica gel, octadecyl silica gel (ODS) and Sephadex LH-20 column chromatographies. It was identified as (8*E*)-ligstroside based on spectroscopic methods including NMR and MS. This compound has been first isolated from *Osmanthus fragrans* var. *aurantiacus,* and showed BACE1 inhibition activity.

Key words: (8*E*)-ligstroside, β-secretase, *Osmanthus fragrans*, secoiridoid

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by extracellular deposition of amyloid plaques, accumulation of intracellular neurofibrillary tangles and neuronal cell loss in the brain [Selkoe, 2001; Citron, 2004]. Although the pathogenesis of AD remains unclear, the theory that β-amyloids induce AD is widely accepted. β-amyloids are produced from amyloid precursor protein (APP) by an amyloidogenic pathway in AD. Two proteases: β- and γ-secretase are utilized in this APP processing. In contrast, APP can also be processed by α-secretase to block the formation of β-amyloids by the non-amyloidogenic pathway. Therefore, these proteases are considered promising options for the therapeutic treatment of AD and many attempts have been made to identify the modulators of APP processing, directly or indirectly. All of the drugs used for the treatment of AD must cross the blood-brain barrier and plasma membrane. The low molecular weight and high lipophilicity of secondary metabolites [Kwak *et al*., 2005] make them favorable for use in AD drugs. Therefore, we initiated the screening of hundreds of compounds isolated from several plants for use as *beta*-site APP-cleaving enzyme (BACE)1 inhibitors. As a result of this screening, one iridoid glycoside from the flowers of *Osmanthus fragrans* (*O. fragrans*) showed inhibitory activity.

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doi:10.3839/jksabc.2010.057

O. fragrans (Oleacea), an evergreen tree, grows in China, Japan and the southern portion of Korea. The flowers bloom from June to August [Choi *et al*., 1999] and they are used by the Chinese to give a pleasant aroma to tea or wine [Deng *et al*., 2004; Lee *et al.,* 2007] and in cosmetics for hair and skin. There are few reports about the biological and phytochemical research on this plant. Our ongoing work has led to the identification and isolation of one secoiridoid glycoside from the EtOAc fraction of the *O. fragrans* (OSE) flower. This paper describes the procedures for the isolation of the compound using silica gel (SiO_2) and octadecyl silica gel (ODS) column chromatography (c.c.), the structure determination using spectroscopic methods such as NMR and FAB/MS, and also the screening for BACE1 inhibition activity.

The flowers of *O. fragrans* were collected in September 2007, in Masan, Korea, and were identified by Prof. Dae-Keun Kim, College of Pharmacy, Woosuk University, Jeonju, Korea. The dried and powdered flowers (800 g) were extracted with 80% aqueous MeOH (3 L×3) and the concentrated extracts were partitioned with EtOAc (3 L×3) and H_2O (3 L). The EtOAc extract (30 g) was applied to a SiO_2 c.c. (8×15 cm) and eluted with *n*-hexane-EtOAc (5:1→1:1, 5.0 L of each) which was monitored by thin-layer chromatography (TLC) to produce 30 fractions (OSE-1 to OSE-30). Fraction OSE-22 (elution volume/total volume (V_e/V_t) 0.62-0.64, 1 g) was applied to the SiO_2 c.c. (4.5×10 cm) and eluted with $CHCl_3$-MeOH-H_2O (30:3:1, 1.2 L) to produce 5 fractions (OSE-22-1 to

OSE-22-5). Fraction OSE-22-4 (V_e/V_t 0.65-0.85, 500 mg) was subjected to ODS c.c. (4×6 cm) and eluted with MeOH-H_2O (1:2, 0.9 L) to give 3 fractions (OSE-22-4-1 to OSE-22-4-3) including a purified compound **1** (OSE-22-4-1, 367 mg, V_e/V_t 0.18-0.77; ODS TLC R_f 0.25, MeOH-H_2O=1:2).

Compound **1** [(8*E*)-ligstroside]: An amorphous powder (MeOH); $[\alpha]_D$ −184.8° (*c*=3.7, MeOH); positive FAB/MS *m/z* 547 $[M+Na]^+$, negative FAB/MS *m/z* 523 $[M-H]^-$, ^{1}H-NMR (400 MHz, CD_3OD) δ_H 7.50 (1H, br. s, H-3), 7.03 (2H, d, *J*=8.4 Hz, H-2'', 6''), 6.71 (2H, d, *J*=8.4 Hz, H-3'', 5''), 6.06 (1H, br. q, *J*=7.2 Hz, H-8), 5.90 (1H, br. s, H-1), 4.80 (1H, d, *J*=8.0 Hz, H-1'), 4.20 (1H, m, H-α_a), 4.09 (1H, m, H-α_b), 3.95 (1H, dd, *J*=9.2, 4.4 Hz, H-5), 3.88 (1H, dd, *J*=11.2, 1.2 Hz, H-6'$_b$), 3.69 (3H, s, 11-$COOCH_3$), 3.63 (1H, br. d, *J*=11.2 Hz, H-6'$_a$), 3.41-3.29 (4H, m, 2',3',4',5'), 2.80 (2H, dd, *J*=6.8 Hz, H-β), 2.69 (1H, dd, *J*=14.0, 4.4 Hz, H-6$_a$), 2.41(1H, dd, *J*=14.0, 9.2 Hz, H-6$_b$), 1.62 (3H, d, *J*=7.2 Hz, H-10); ^{13}C-NMR (100 MHz, CD_3OD) δ_C 172.9 (C-7), 168.4 (C-11), 156.8 (C-4''), 154.9 (C-3), 130.8 (C-2'',6''), 130.2 (C-1''), 129.8 (C-9), 124.7 (C-8), 116.1 (C-3'', 5''), 109.2 (C-4), 100.6 (C-1'), 95.0 (C-1), 78.3 (C-5'), 77.8 (C-3'), 74.6 (C-2'), 71.3 (C-4'), 66.8 (C-α), 62.6 (C-6'), 51.9 (C-OCH_3), 41.2 (C-6), 35.1 (C-β), 31.7 (C-5), 13.6 (C-10).

HEK 293 (BA-3), which stably express human wild type APP695 and BACE1 (BA-3) [Yeon *et al*., 2007], were used for the analyses of APP metabolites, sAPPα (a soluble and extra-cellular N-terminal fragment of APP cleaved by α-secretase) and sAPPβ (a soluble and extra-cellular N-terminal fragment of APP cleaved by β-secretase). The cells were maintained in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum. The cells were grown on 35-mm tissue culture dishes in a 5% CO_2 incubator at 37.8°C, until they became 80-100% confluent. The cells then were washed once with serum-free DMEM, and serum-free DMEM with or without (8*E*)-ligstroside then was added, followed by culture for 24 h. To analyze sAPPáand sAPPâin the conditioned medium, the medium was collected, and subjected to western blot analysis. sAPPα and sAPPβ were detected by using primary antibodies, MAB1560 (Chemicon International Inc, Temecula, CA) and Rb53 (generous gift from Cephalon Inc., West Chester, PA) recognizing C-terminus of sAPP, respectively. All blots were probed with either anti-rabbit or anti-mouse HRP-conjugated (Amersham Biosciences, Little Chalfont, UK) secondary antibodies and detected by chemiluminescence (GE Healthcare, Piscataway, NJ). For the analysis of β-secretase inhibition activity *in vitro*, the extracts of BA-3 were used as a β-secretase source. The cells were grown on 100-mm tissue culture dishes in a 5% CO_2 incubator at 37.8, harvested in cold PBS, re-suspended in lysis buffer (10 mM Mes, pH 6.0, 0.5% TritonX-100) and kept on ice for 15 min. After brief sonication, extracts were clarified by centrifugation at 14,000 rpm at 4.8°C. Supernatant was recovered and used as β-secretase sources. Briefly, β-secretase assays were carried out as follows. (8*E*)-Ligstroside was pre-incubated with 20 μg cell extracts containing buffer in 20 mM sodium acetate, pH 5.0 for 10 min. β-Secretase substrate (DABCYL-VNLDAE-EDANS, Bachem, Torrance, CA) was then added with 20 μM and incubated for 15 min at 37.8°C. At the end of the incubation, fluorescence was recorded at 360/40 and 485/20 nm as excitation and emission wavelengths, respectively (Synergy HT, BioTek, VT).

The flowers of *O. fragrans* were extracted in 80% MeOH and the obtained extracts were partitioned between EtOAc and water. The repeated SiO_2 and ODS c.c. on the EtOAc fraction afforded one purified iridoid glycoside. Compound **1,** an amorphous powder, showed a dark brown color on the ODS TLC by spraying with 10% *aq*. H_2SO_4 and then heating. Pseudomolecular ion peaks, $[M-H]^-$ and $[M+Na]^+$ detected at *m/z* 523, 547 in the negative and positive FAB/MS, respectively, confirmed the molecular weight to be 524. In the ^{1}H-NMR (400 MHz, CD_3OD) spectrum, two doublet olefine methine protons [δ_H 7.03 (H-2'', 6''), 6.71(H-3'', 5'')] due to *para*-disubstituted benzene ring, one oxygenated methylene proton [δ_H 4.20 (H-α_a), 4.09 (H-α_b)] and one sp^3 methylene proton (δ_H 2.80 H-β) were observed suggesting the presence of *para*-hydroxyphenethyl alcohol (*p*-tyrosol) moiety. Other proton signals were very similar as those of oleoside, a secoiridoid glucoside, with the exception of an additional methyl ester. The proton signals of aglycon moiety included one oxygenated olefine methine (δ_H 7.50 H-3), one olefine methine (δ_H 6.06 H-8), one acetal (δ_H 5.90 H-1), one oxygenated methyl (v_H 3.69 H-$COOCH_3$), one methine (v_H 3.95 H-5), one methylene [δ_H 2.69 (H-6$_a$), 2.41 (H-6$_b$)] showing vicinal coupling, and one allyl doublet methyl (δ_H 1.62, H-10) proton signals. And one hemiacetal (δ_H 4.80, H-1') and one oxygenated methylene [δ_H 3.88 (H-6'$_b$), 3.63 (H-6'$_a$)] and lots of oxygenated methine (δ_H 3.41-3.29, H-2', 3', 4', 5') proton signals suggested the monosaccharide to be an aldohexose. And the anomer carbon of the sugar was determined to be β-configuration from the coupling constant (*J*=8.0 Hz) of the anomer proton signal. The ^{13}C-NMR (100 MHz, CD_3OD) spectrum showed twenty-five carbon signals. The multiplicity of each carbon was determined using a DEPT experiment. One oxygenated quaternary carbon (δ_C 153.5, C-4''), one carbonated quaternary carbon (δ_C 130.2, C-1''), four olefine methine carbon [δ_C 130.8 (C-2'',

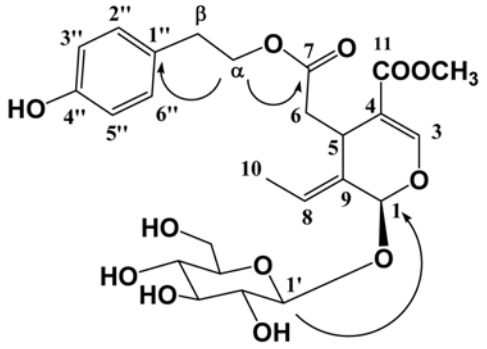

Fig. 1. ^{1}H and ^{13}C long-range correlations observed in the HMBC spectrum of compound 1. The arrows indicate the long-range correlations between proton and carbon signals in the HMBC spectrum.

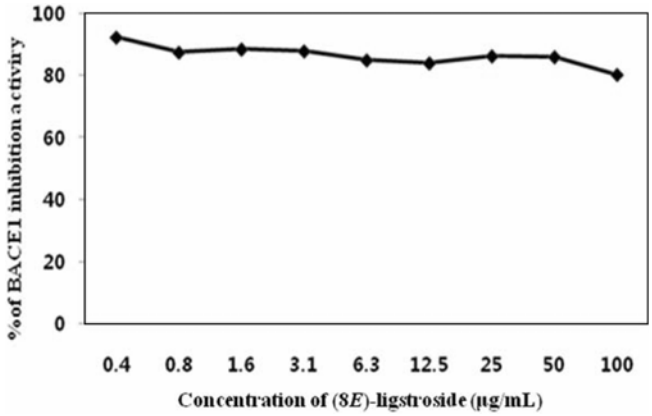

Fig. 3. Effects of (8*E*)-ligstroside on β-secretase inhibition activity *in vitro*.

6''), 116.1 (C-3", 5")], one oxygenated methylene carbon (δ_C 66.8, C-α). One methylene carbon (δ_C 35.1, C-β) due to the *para*-hydroxyphenethyl alcohol were observed. The aglycone was identified as ligstroside aglycone from the carbon resonances of two esters [δ_C 172.9 (C-7), δ_C 168.4 (C-11)], two carbonated olefine quaternary [δ_C 129.8 (C-9), 109.2 (C-4)], one oxygenated methine (δ_C 154.9, C-3), one olefine methine (δ_C 124.7, C-8), one acetal carbon (δ_C 95.0, C-1), one oxygenated methyl (δ_C 51.9, C-OCH_3), one methine (δ_C 31.7, C-5), one methylene (v_C 41.2, C-6), and one methyl (δ_C 13.6, C-10) signals. And the chemical shifts of the sugar moiety were identical with those of β-D-glucopyranose, that is a hemiacetal (δ_C 100.6, C-1'), four oxygenated methines [δ_C 78.3 (C-5'), 77.8 (C-3'), 74.6 (C-2') and 71.3 (C-4")], and one oxygenated methylene (δ_C 62.6, C-6') signals. The location of *para*-hydroxyphenethyl alcohol moiety in compound **1** was determined on the basis of ^{1}H-NMR data and HMBC experiment. The oxygenated methylene proton signal (δ_H 4.20, H-α) in compound **1** was shifted to low magnet field with δ_H 4.20 comparing to the chemical shift of *para*-hydroxyphenethyl alcohol, δ_H 3.68 [Kim *et al*., 2008] indicating the hydroxy group of *para*-hydroxyphenethyl alcohol was linked to a carboxyl group of oleoside through ester bond. And the J_3 correlations between oxygenated methylene proton signals (δ_H 4.20, H-α_a) and the carbonyl carbon signal (δ_C 172.9, C-7) observed in the the HMBC spectrum confirmed it. And the glucopyranose was determined to be linked to the hydroxy of C-1 acetal carbon from the J_3 correlation between the anomer proton (δ_H 4.80, H-1') and acetal carbon (δ_C 95.0, C-1) in the HMBC spectrum (Fig. 1). The stereostructure of chiral carbons, C-1 and C-5, and a double bond between C-8 and C-9 were determined as *S*, *S*, and *E*, respectively, by comparison of several spectroscopic data including specific rotation [Christophoridou and Dais, 2009] with those described in the literatures [Asaka *et al*., 1972a; 1972b; Lalonde *et al.,* 1976; Koichi *et al*., 2002]. Finally compound **1** was identified as (1*S*,5*S*,8*E*)-11-methyl oleoside-2-(4-hydroxyphenyl) ethyl ester, (8*E*)-ligstroside, which was first isolated from *Ligstrum obtusifolium* and reported it is common in olive oil [Asaka *et al*., 1972a; 1972b; Lalonde *et al.,* 1976; Koichi *et al*., 2002]. In the search of the blocking modulators of β-secretase activities using APP and β-secretase over-expressing HEK293 cells (BA-3), we found that (8*E*)-ligstroside dose-dependently inhibited β-secretase mediated APP processing (Fig. 2). (8*E*)-Ligstroside blocked the production of sAPPwith an IC_{50} of approximately 1 g/mL with no effect on the production of sAPP. To test whether the blockade of β-secretase mediated APP processing is due to direct inhibition of enzyme activities, β-secretase activity *in vitro* using the extracts of BA-3 as an enzyme source and fluorescent substrates was investigated. (8*E*)-

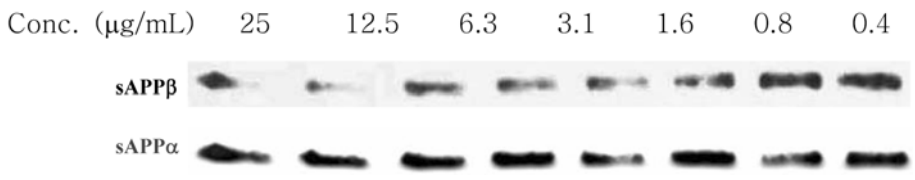

Fig. 2. Effects of (8*E*)-ligstroside on APP processing. Amounts of APP and APP from BA-3 cells treated with or without (8*E*)-ligstroside were measured by quantitative western blot analyses.

Ligstroside weakly inhibited β-secretase activity (about 20%) (Fig. 3) even at the highest concentration tested (100 M). From these results, it is proposed that (8*E*)-ligstroside affect β-secretase mediated APP processing by indirect manner rather than direct inhibition of β-secretase. And this study on *O. fragrans* could provide information to aid the treatment or prevention of Alzheimer's disease.

Acknowledgments. This work was supported by the BioGreen 21 program from Rural Development Administration, Republic of Korea (20080401-034-027-009-02-00) and Technology Development Program from Agricultural Research & Development Promotion Center (108056031HD110).

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