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Article

Isolation of 6,6'-Bieckol from *Grateloupia elliptica* and its Antioxidative and Anti-Cholinesterase Activity

Bong Ho Lee*, Byoung Wook Choi, and Soo Young Lee

Department of Chemical and Biological Engineering, College of Engineering
Hanbat National University, Daejeon 34158, Korea

Abstract : During the search for anticholinesterase compounds from marine organisms, we were able to isolate 6,6'-bieckol from a red alga, *Grateloupia elliptica*. This compound showed moderate acetylcholinesterase (AChE) inhibitory activity in a micromole range (IC_{50} 44.5 μM). However, for butyrylcholinesterase (BuChE), a new target for the treatment of Alzheimer's disease (AD), it showed particularly potent inhibitory activity (IC_{50} 27.4 μM), which is more potent compared to AChE. It also inhibits BACE-1, a new target for reducing the generation of β-amyloid.

Key words : *Grateloupia elliptica*, bieckol, cholinesterase inhibition, BACE inhibition, Alzheimer's disease

1. Introduction

Alzheimer's disease (AD) is a chronic and progressive neurodegenerative disease that is characterized symptomatically by progressive deteriorations of activities of daily living, behavioral disturbances and cognitive loss. The neurodegenerative features of AD include classical pathological changes in the brain, such as the formation of β-amyloid plaques, neurofibrillary tangles, neuronal cell death and a dramatic synaptic loss (Selkoe 2005; Sambamurti et al. 2002). However, the initiating factors that underpin this pathology remain to be elucidated (Sambamurti et al. 2006). In particular, AD is associated with early substantial reductions in presynaptic markers of the cholinergic system. Specifically, the activity of choline acetyltransferase, critical in the synthesis of acetylcholine (ACh), is depleted, together with ACh levels, as cholinergic neurons are lost and cholinergic neurotransmission increasingly declines with disease progression (Greig et al. 2001, 2003; Lahiri et al. 2004; Sambamurti et al. 2002). Strategies to augment cholinergic neurotransmission, which is fundamental to memory and learning processes (Drachman and Leavitt 1974), have thus represented the primary approach to treat AD (Lahiri et al. 2004). Cholinesterase (ChE) inhibitors retard the inactivation of ACh after synaptic release and represent a mainstay treatment for AD, to which the glutamatergic antagonist, memantine, has recently been added. Whereas ChE inhibitors provide consistent improvements in cognitive performance in mild to moderate AD (Cummings 2000), these are generally modest and these are current debates as to the clinical relevance of current agents of this drug class (Courtney et al. 2004; Lopez et al. 2005).

Butyrylcholinesterase (BuChE) in the regulation of ACh level had been largely ignored. However, there is growing evidence that acetylcholinesterase (AChE) and BuChE both play important roles in the regulation of ACh level and may also have an important role in the development and progression of AD (Greig et al. 2001, 2003). Indeed, selective BuChE inhibition may provide all the cognitive benefits associated with classical AChE inhibition without the characteristic and dose-limiting adverse effect profile (Greig et al. 2005a).

Generation of the 40 or 42 amino acid-long β-amyloid peptides that aggregate in the brain of Alzheimer's disease patients requires two sequential cleavages of the amyloid precursor protein (APP). Extracellular cleavage of APP by beta amyloid cleaving enzyme (BACE) creates a

*Corresponding author. E-mail : lbh011@hanbat.ac.kr

soluble extracellular fragment and a cell membrane-bound fragment referred to as C99 (Vassar et al. 1999). Cleavage of C99 within its transmembrane domain by γ-secretase releases the intracellular domain of APP and produces β-amyloid. Since the γ-secretase cleaves APP closer to the cell membrane than BACE1 does, it removes a fragment of the β-amyloid peptide. Initial cleavage of APP by γ-secretase rather than BACE1 prevents eventual generation of amyloid-β. Drugs to block this enzyme (BACE inhibitors) in theory would prevent the buildup of β-amyloid and may help to slow or stop Alzheimer's disease (Walker 2006).

The marine red algae *Grateloupia* is the largest genus in the family Halymeniaceae and widely distributed from tropical to warm temperature regions of the world. In the genus *Grateloupia*, especially *G. elliptica* and *G. lanceolata* have common features of bladelike thalli with leather in texture and crucially divided tetrasporangia (Liu 2008). *G. elliptica* extracts were reported to have anti-inflammatory effects by the decrease of production of pro-inflammatory mediators. Bromophenols of *G. elliptica* showed high α-glucosidase inhibitory activity and seem to have potential to prevent diabetes mellitus (Kim et al. 2008).

In the course of our elucidation of ChE inhibitory activity of natural products from Korean marine algae, we found potent ChEs-inhibitory activity in the methanol extract of *Grateloupia elliptica* and purified a marine polyphenol. Thus, herein, we report the isolation, structural characteristics, and ChEs and BACE inhibitory activity of the polyphenol from *Grateloupia elliptica*.

2. Materials and Methods

Material

The red alga, *Grateloupia elliptica* was collected offshore of Jeju island, Korea, in 2002. A voucher specimen of the alga has been deposited at the Laboratory of Natural Products Chemistry, Hanbat National University.

Isolation of 6,6'-bieckol

The methanol extract (10 g) of the dried algal sample (600 g) was suspended in 80% aqueous MeOH and washed with *n*-hexane three times to remove nonpolar constituents. The 80% aqueous MeOH was fractionated to 30% aqueous MeOH and chloroform. The 30% aqueous MeOH fraction was further fractionated to water and *n*-BuOH fractions. The *n*-BuOH fraction (2.5 g) was subjected to a reversed phase ODS open column chromatography [MeOH/H_2O = 0.2 to 1.0] to give 6 fractions. The 90% aqueous MeOH fraction (300 mg) was subjected to a series of size exclusion chromatography to give two fractions. One of the fractions was subjected to size exclusion chromatography [30% aqueous MeOH eluent] to give 5.47 mg of a pure compound, 6,6'-bieckol: white solid; UV (EtOH) λmax 232 nm;

^{1}H-NMR (DMSO-d_6, 400 MHz) δ 6.04 (s, 2H) 6.09 (s, 2H), 8.63 (s, 2OH), 9.07 (s, 2OH), 9.13 (s, 2OH), 9.14 (s, 4OH), 9.26 (s, 2OH); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ 94.3, 96.7, 98.3, 98.4, 100.2, 122.5, 123.2, 124.1, 137.8, 141.9, 142.4, 145.0, 145.9, 151.8, 159.3, 161.0. FABMS m/z 743.1 $(M+H)^+$.

UV spectra were recorded with a HP8453 UV/VIS spectrophotometer. IR spectra were performed on a Perkin-Elmer model 1750 FT-IR spectrophotometer. MS spectra were measured on a JEOL JMX-SX 102 mass spectrometer. High resolution mass measurement was carried out with a JEOL AX-505H mass spectrometer at high resolution. ^{1}H-NMR and ^{13}C-NMR spectra were recorded in DMSO-d_6 at 25°C on a Bruker ARX-400 NMR spectrometer.

Chemicals

Enzymes, substrates, and buffer agents used in the bioassay were purchased from SIGMA Co.: AChE (EC 3.1.1.7), BuChE (EC 3.1.1.8), C-1057; BuTCh (butyryl-thiocholine chloride), B-3128; DTNB [5,5'-dithio-bis(2-nitrobenzoic acid)], and D-8130. Other Chemicals were also purchased from SIGMA Co.

Free radical scavenging activity

The DPPH (1,1-dipheny-l,2-picrylhydarzyl) radical scavenging effect was carried out according to the method first employed by M.S. Blois (Blois 1955). The 100 μL of sample solution was added to 900 μL of DPPH solution in ethanol (1.01×10^{-4} M). After incubating at room temperature for 30 min, the absorbance of this solution was determined at 518 nm using a spectrophotometer and remaining DPPH was calculated. All experiments were carried out in triplicates. Results were expressed as percentage decreased with respect to control values. Each fraction was evaluated at the final oncentration at 100 μg/mL in the assay mixture.

ChE inhibition assays

Acetylcholinesterase and butyrylcholinesterase assays. Enzyme activities were determined at room temperature. Ultraviolet absorbance change was measured spectrophotometrically by a modification of the previously described

method (Ellmans et al. 1961). To each cuvette was added DTNB (900 μL of 5.55 mM DTNB in 50 mM potassium phosphate buffer, pH 7.4) followed by the addition of ATCh (25 μL of a buffer of ATCh of varying concentration). The enzymatic reaction was initiated at 25°C by the addition of enzyme (75 μL of AChE, appropriately diluted in 50 mM, pH 7.4, potassium phosphate buffer to give 0.005 unit), and the absorbance change was monitored at 412 nm for 60 s. The slope of the absorbance change for this time is the initial rate of an enzyme reaction. Enzyme inhibition assay for BuChE was performed by the same method as that of AChE using butyrylthiocholine as a substrate. Effect of inhibition for each sample was calculated as inhibition (%) = 100 − (ST/CT) × 100. CT stands for the initial rate for a control and ST for the initial rate of a sample.

BACE-1 inhibition assay

The assay was carried out according to the manufacturer's instruction with modifications. A mixture of 10 μL of substrate (75 μM Rh-EVNLDAEFK-Quencher, in 50 mM ammonium bicarbonate), 10 μL of BACE-1 (1 U/mL), 10 μL of assay buffer (50 mM sodium acetate, pH 4.5), and 10 μL of sample dissolved in assay buffer was incubated for 60 min at 25°C in the dark. The mixture was excited at 528 nm, and the light emitted at 620 nm was collected. Fluorescence was measured with a Bio-Tek Microplate fluorescence reader FLx 800 (VT, USA). The inhibition ratio is calculated by following the equation, inhibition (%) = [1 − {(S − S0)/(C − C0)}] × 100, where C was the fluorescence of control (substrate, assay buffer, and enzyme) after 60 min of incubation, C0 was the fluorescence of control at zero time, S was the fluorescence of tested samples (substrate, sample solution, and enzyme) after 60 min of incubation, and S0 is the fluorescence of tested samples at zero time. The BACE-1 inhibitor, Z-Val-Leu-Leu-CHO, is used as a positive control. A buffer containing 10% aqueous DMSO was a negative control.

3. Results and Discussion

Fig. 1 shows the chemical structure of the isolated compound from *Grateloupia elliptica*. The isolated compound is 6,6'-bieckol, two eckols are connected by 6-6' covalent carbon-carbon bond. 6,6'-Bieckol is an eckol-type phlorotannin found in the brown algae *Ecklonia cava* (Artan et al. 2008) and *Ecklonia stolonifera* (Lee et al. 2012). It was isolated by solvent fractionations and a series of column chromatographies. Briefly, the initial

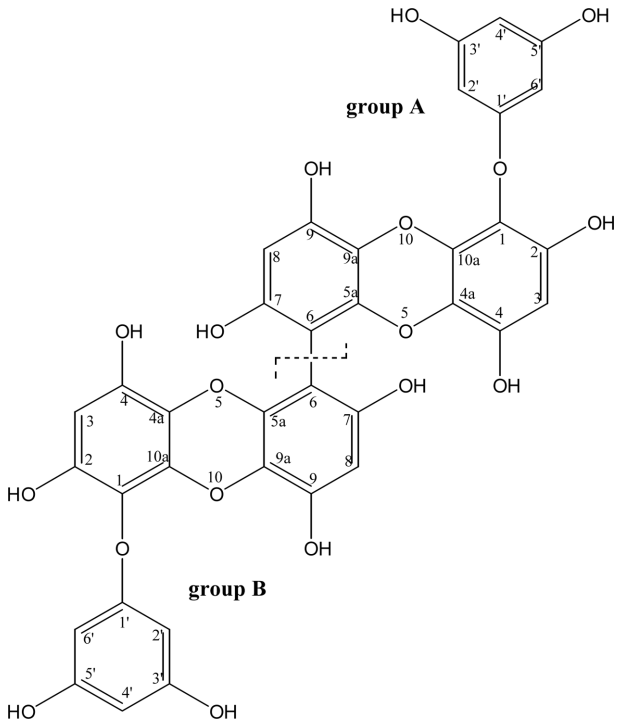

Fig. 1. The chemical structure of 6,6'-bieckol

methanol extract was partitioned to nonpolar and polar fractions. The ChEs inhibition activity went to the polar fraction. This fraction was partitioned to chloroform and 30% aqueous methanol. The enzyme inhibitory activity went to the chloroform and then the active compound was purified by a series of ODS flash and silica gel chromatographies. The detailed procedure to isolate the compound is described in Materials and Methods. Kim et al. isolated two bromophenols, 2,4,6-tribromophenol and 2,4-dibromophenol from *Grateloupia elliptica* and measured their IC_{50} values against *Saccharomyces cerevisiae* alpha-glucosidase (Kim et al. 2008). Besides these bromophenols, sugars of polysaccharide were also reported in this alga (Hirase et al. 1967).

Like the other polyphenols isolated from brown seaweeds, 6,6'-bieckol showed strong DPPH radical scavenging activity comparable to that of BHT and BHA. As shown in Table 1, the RC_{50} value of DPPH radical scavenging activity is 28 μM. The IC_{50} of anticholinesterase activities of 6,6'-bieckol is also in Table 1, as calculated concentrations required to inhibit 50% of the activity of either AChE or BuChE (IC_{50} values) to cleave their respective substrates. The compound showed moderate AChE and BuChE inhibitory activities with IC_{50} values of 44.5 μM and 27.4 μM, respectively. Although the compound have been isolated previously from *Ecklonia*

Table 1. Biological activities of 6,6'-bieckol

Sample	DPPH scavenging activity (RC_{50}, μM)	AChE inhibitory activity (IC_{50}, μM)[a]	BuChE inhibitory activity (IC_{50}, μM)[a]	BACE-1 inhibitory activity (%) (concentration 1 μM)[a]
6,6'-Bieckol	28 ± 3.6	44.5 ± 6.1	27.4 ± 5.3	18.6
		0.01 ± 0.0003[b]	3.8 ± 0.1[b]	75.0[c]

[a]Data from the previous author's report on Phytotherapy Research (Choi et al. 2015)
[b]AChE and BuChE inhibition by donepezil
[c]Reference for BACE inhibition by Z-Val-Leu-Leu-CHO

cava (Choi et al. 2015), this is the first report isolating 6,6'-bieckol from *Grateloupia elliptica*. 6,6'-Bieckol showed very strong BACE-1 inhibition comparable with a peptide inhibitor known to have strong BACE-1 inhibitory activity (Selkoe 2005; Sambamurti et al. 2002). Other polyphenols such as dieckol, eckol, and PFF-A, isolated from *Ecklonia cava* also showed strong BACE inhibitory activities a little bit higher than that of 6,6'-bieckol (Choi et al. 2015). It has been shown that terrestrial flavonols and flavonoids inhibit BACE-1. An important pathological feature of AD is the presence of extracellular senile plaques in the brain. Senile plaques are composed of aggregations of small peptides called Aβ. There are two secretases responsible for the production of Aβ, β-secretase (BACE-1) and γ-secretase (Selkoe 2005; Sambamurti et al. 2002). Many peptide analog secretase inhibitors are under clinical trials. The present results indicate that *Grateloupia elliptica* polyphenol have a strong potential of treating AD through the inhibition of BACE-1.

In general, the majority of potent ChE inhibitors that are the focus of clinical and scientific interest are alkaloids or quaternary ammonium salts (Brossi 1990; Giacobini 2000). Natural products, such as physostigmine and huperzine A, have proved to be useful as both clinical drugs (Giacobini 2000; Liu et al. 1986) and lead compounds for medicinal chemistry to provide effective drug candidates (Greig et al. 1995, 2003, 2005b; Yu et al. 1999), 6,6'-bieclol represent further intriguing candidates that may be added to this category.

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