Arch Pharm Res Vol 28, No 7, 799-803, 2005

Archives of Pharmacal Research
http://apr.psk.or.kr

β-Secretase (BACE1) Inhibitors from Sanguisorbae Radix

Hee-Ju Lee, Yeon-Hee Seong[1], Ki-Hwan Bae[2], Soon-Ho Kwon, Hye-Min Kwak, Si-Kab Nho[3], Kyung-A Kim[3], Jong-Moon Hur, Kyung Bok Lee[4], Young-Hwa Kang, and Kyung-Sik Song

School of Applied Biosciences, College of Agriculture and Life Sciences, Kyungpook National University, 1370 Sankyuk-Dong, Daegu 702-701, Korea, [1]College of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk 361-763, Korea, [2]College of Pharmacy, Chungnam National University, Yusung, Daejon 305-764, Korea, and [4]Department of Biochemistry, School of Medicine, Konyang University, Nonsan, and [3]Department of Natural Fiber Science, College of Agriculture and Life Sciences, Kyungpook National University, 1370 Sankyuk-Dong, Daegu 702-701, Korea

(Received March 10, 2005)

In the course of screening anti-dementia agents from natural products, two β-secretase (BACE1) inhibitors were isolated from the ethyl acetate soluble fraction of Sanguisorbae Radix by the activity-guided purification using silica gel, Sephadex LH-20, and RP-HPLC. They were identified as 1,2,3-trigalloyl-4,6-hexahydroxydiphenoyl-β-D-glucopyranoside (Tellimagrandin II, **1**) and 1,2,3,4,6-pentagalloyl-β-D-glucopyranoside (**2**) and were shown to non-competitively inhibit β-secretase (BACE1) with the IC_{50} values of 3.10×10^{-6} M and 3.76×10^{-6} M, respectively. The *Ki* values of **1** and **2** were 6.84×10^{-6} M and 5.13×10^{-6} M. They were less inhibitory to α-secretase (TACE) and other serine proteases such as chymotrypsin, trypsin, and elastase, suggesting that they were relatively specific inhibitors of BACE1.

Key words: β-Secretase (BACE1), Inhibitor, Sanguisorbae Radix, Tellimagrandin II, 1,2,3,4,6-Pentagalloyl-β-D-glucopyranoside, Alzheimer's disease

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder clinically characterized by progressive dementia that inevitably leads to incapacitation and death (Terry *et al.*, 1999). Two characteristic brain lesions define AD at the microscopic level: (1) amyloid plaques, extracellular deposits primarily composed of 4 kDa, 40-42 amino acid Aβ peptide (Glenner and Wong, 1984), a product of APP (amyloid precursor protein) proteolysis, and (2) neurofibrillary tangles, and intracellular aggregates of the microtubule associated protein tau (Lee *et al.*, 1991). The relationships between amyloid plaques, neurofibrillary tangles, and the pathogenic mechanisms of AD are controversial. Evidence, however, suggests that Aβ is critically involved at an early stage in AD pathology (Ghosh *et al.*, 2001).

Two proteolytic cleavage events are required to generate Aβ from its precursor, one at the *N*-terminus by an enzyme termed β-secretase and one at the *C*-terminus by an enzyme termed γ-secretase. At least three distinct protease activities are involved in processing the membrane protein APP along two major pathways, the α-secretase and the amyloid-forming β-secretase pathway. A relatively small minority of APP molecules enters the β-secretase pathway in which β-secretase cleaves APP and releases a soluble fragment, β-APPs. The *C*-terminal membrane-bound C99 peptide is then cleaved by γ-secretase within the transmembrane domain and two major isoforms of 40 and 42 amino acid length with different *C*-termini, $A\beta_{40}$ and $A\beta_{42}$, are generated. In the α-secretase pathway, α-secretase cleaves in the middle of the Aβ region (thus precluding Aβ formation) and releases a soluble fragment, α-APPs. The remaining membrane-bound *C*-terminal fragment C83 is then cleaved by γ-secretase to give rise to $p3_{40}$ and $p3_{42}$, shortened versions of Aβ that do not seem to be major plaque components (Citron, 2002).

Among the secretases, a novel transmembrane aspartic protease BACE1 (for β-site APP, cleaving enzyme 1) (Anderson *et al.*, 1999; Vassar *et al.*, 1999), also known as Asp2 (for novel aspartic protease 2) and memapsin 2 (for membrane aspartic protease/pepsin 2), is at present

Correspondence to: Kyung-Sik Song, School of Applied Biosciences, College of Agriculture and Life Sciences, Kyungpook National University, 1370 Sankyuk-Dong, Daegu 702-701, Korea
Tel: 82-53-950-5715, Fax: 82-53-956-5715
E-mail: kssong@knu.ac.kr

the most attractive target for the inhibition of amyloid production. This initial work has been followed by several publications describing substrate-based peptidic inhibitors of β-secretase (Ghosh *et al.*, 2000, 2001; Tung *et al.*, 1999). Such peptidic compounds are unlikely to have the required metabolic properties of a drug, however, and in particular, will not penetrate the blood-brain barrier to a sufficient extent. Instead, it is necessary to generate small organic molecules that specifically inhibit β-secretase. In this sense, secondary metabolites of plants and microbes with relatively low molecular weight and high lipophilicity, might be a good candidate for BACE1 inhibitors. In the course of screening natural products for small molecule BACE1 inhibitors, Saguisorbae Radix showed the highest inhibitory activity out of the 260 tested plant extracts. We will report here on the isolation and structure elucidation of the active compounds and their inhibitory activity on BACE1 with additional proteolytic enzymes.

MATERIALS AND METHODS

General

Optical density was measured with a Bio-TEK fluorescence ELISA autoreader FLx 800 (U.S.A.). ^{1}H- and ^{13}C-NMR spectra were recorded on a UnityInova 500 spectrometer (Varian, U.S.A.) at 500 and 125 MHz, respectively and on a Bruker Avance Digital 400 spectrometer (Germany) at 400 MHz. Chemical shifts were given in δ (ppm) from TMS. TLC was performed on a precoated silica gel plate (Merck, Art. 5715). Silica gel column chromatography was carried out using Kieselgel 60 (Art. 7734, Merck, Germany) and Sephadex LH-20 was supplied by Sigma (U.S.A.).

Enzyme assays

A BACE1 (recombinant human BACE1) assay kit was purchased from PanVera (U.S.A.). The assay was carried out according to the manufacturer's instructions with modifications. Briefly, a mixture of 10 μL of an assay buffer (50 mM of sodium acetate, pH 4.5), 10 μL of the substrate (75 μM Rh-EVNLDAEFK-Quencher in 50 mM ammonium bicarbonate), and 10 μL of sample dissolved in an assay buffer was incubated for sixty min at 25 under the dark condition. The mixture was excited at 530 nm and the light emitted at 620 nm was collected. The inhibition ratio was obtained by the following equation: inhibition (%) = $[1 - \{S - S_0\}/\{C - C_0\}] \times 100$, where C is the fluorescence of a control (enzyme, assay buffer, and substrate) after 60 min of incubation, C_0 was the fluorescence of a control at zero time, S was the fluorescence of the tested samples (enzyme, sample solution, and substrate) after 60 min of incubation, and S_0 was the fluorescence of the tested samples at zero time. To check the quenching effect of samples, the sample solution was added to reaction mixture C, and any reduction in fluorescence by the sample was investigated. α-Secretase activity was measured by an α-secretase assay kit with TACE according to the manual from R&D Systems (U.S.A.). Chymotrypsin, trypsin, and elastase were assayed according to the protocol described in the references using *N*-benzoyl-L-Arg-*p*NA, *N*-benzoyl-L-Tyr-*p*NA, and *N*-succinyl-Ala-Ala-Ala-*p*NA as substrates, respectively. All data are represented as the mean value of duplicated experiments.

Plant material, extraction, and isolation

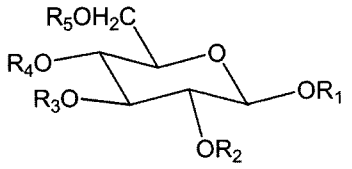
Sanguisorbae Radix was purchased from a market in Daegu, Korea in July, 2003. The voucher specimen (knunpc-so) is stored at the Natural Products Chemistry Lab., Division of Applied Biology and Chemistry, Kyungpook National University, Daegu, Korea. The dried material (1 kg) was refluxed in 2 L MeOH twice at room temperature. The MeOH extract (150.23 g) was suspended in water and the suspension was consecutively partitioned with the same volume of dichloromethane, ethyl acetate (EtOAc), and *n*-butanol for the activity-guided purification. The most active (more than 95% of inhibition at 1 ppm) EtOAc soluble fraction (9.75 g) was chromatographed on a Sephadex LH-20 column (3.6×68.4 cm, 10%-100% MeOH) to yield Fr. 1~12.

The active Fr. 11 (440 mg) was re-chromatographed on a Sephadex LH-20 column (1.2×28 cm, 30%-50% MeOH), followed by a silica gel column [1×30 cm, CH_2Cl_2:MeOH= 30:1→1:1 (1% HOAc)]. The HPLC [μBondapak C18, Waters, U.S.A., 7.8300 mm, 1% HOAc in 25% MeOH, UV254 nm, 1.5 mL min^{-1}] of the active fraction afforded 9.0 mg of compound **1**. Compound **2** (13.6 mg) was obtained by the HPLC [SUPELCOSIL™ LC-18, SUPELCO, U.S.A., 21.2250 mm, 1% HOAc in 10%-60% MeOH, UV254 nm, 5 mL min^{-1}] of Fr. 12 (400 mg).

RESULTS AND DISCUSSION

Structure determination

Compound **1** was obtained as an amorphous brown powder that was positive to the $FeCl_3$ reagent, suggesting that it had phenolic OH group(s) in its structure. In the ^{1}H-NMR spectrum, three aromatic singlets (δ 7.07, 6.96, and 6.93, each 2H) were predicted to be in the aromatic protons in a symmetrical structure of galloyl moiety. Two singlet protons (δ 6.60 and 6.46, each 1H), characteristic of the HHDP (hexahydroxydiphenic acid) group, were also detected. Signals corresponding to glucose moiety were found at δ 3.87 to δ 5.81. In the ^{13}C-NMR spectrum, five carbonyl carbons (δ 168.6, 168.2, 166.8, 166.1, and 165.5) were evident. Four couples of two aromatic carbons appeared at δ 144.8/145.6, 136.9/137.1, 108.1/108.4, and 125.9/126.5, which could be assigned to a



1 $R_1 = R_2 = R_3$ = Gal, R_4 and R_5 are connected to HHDP
2 $R_1 = R_2 = R_3 = R_4 = R_5$ = Gal

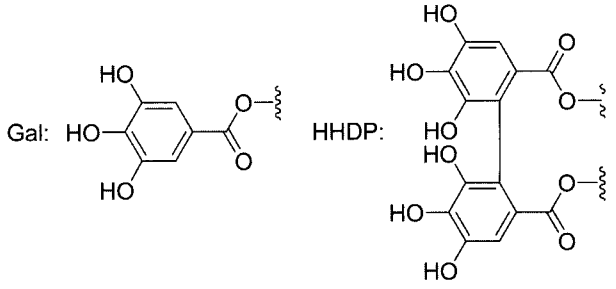


Fig. 1. Structure of compounds **1** and **2** HHDP: Hexahydroxydiphenoyl

symmetric structure of HHDP moiety. In addition, four sets of closely related three aromatic carbon resonances were found at δ 119.9/120.5/120.6, 110.1/110.4/110.6, 146.2/146.3/146.5, and 139.6/139.8/140.3, suggesting the existence of three galloyl groups. Considering these data, the active compound was assumed to have a HHDP and three galloyl moieties attached to glucose. The structure was identified as 1,2,3-trigalloyl-4,6-hexahydroxydiphenoyl-β-D-glucopyranoside (tellimagrandin II), by comparing its NMR data with those in literature (Feldman and Sahasrabudhe, 1999). The structure is presented in Fig. 1 and the NMR data are listed in Table I.

Compound **2** was obtained as an amorphous brown powder that was positive to $FeCl_3$. In the ^{1}H-NMR spectrum, five aromatic singlets (δ 7.03, 6.96, 6.94, 6.90, and 6.87, each 2H) were characteristic to the aromatic protons in a symmetrical structure of galloyl moiety. Signals corresponding to glucose moiety were found at δ 4.20 to δ 6.17. In the ^{13}C-NMR spectrum, five carbonyl carbons at δ 169.4, 168.9, 168.7, 168.5, and 167.9 and overlapped complicated aromatic carbon signals were detected at δ 112.2 to δ 148.1. Considering the number of carbonyl carbon, **2** should have five galloyl groups. The active compound was finally identified as 1,2,3,4,6-pentagalloloyl-β-D-glucopyranoside (Fig. 1), by comparing its spectral data with those in literature (Kim and Song, 2000). The structure and the NMR data are presented in Fig. 1 and Table I, respectively.

Inhibitory activity against BACE1

Tellimagradin II (**1**) and 1,2,3,4,6-pentagalloloyl-β-D-glucopyranoside (**2**) inhibited the activity of β-secretase (BACE1) in a dose-dependant manner (Fig. 1). The inhibitory activity of **1** (IC_{50}=3.10×10^{-6} M) and **2** (IC_{50}=3.76×10^{-6} M) was less than that of the positive control, (-)-epigallocatechin gallate (IC_{50}=1.6×10^{-6} M) (Jeon *et al.*, 2003). Compounds **1** and **2** were non-competitive with a substrate in the Dixon plots (Fig. 3) and the inhibition constants

Table I. NMR data of compounds **1** and **2**

NO		1[a]		2[b]	
		^{1}H (δ)[c]	^{13}C (δ)	^{1}H (δ)	^{13}C (δ)
Glucose	1	6.17 (*d*, 8.0)	93.9	6.16 (*d*, 8.2)	96.9
	2	5.59 (*t*, 9.0)	71.9	5.54 (*dd*, 9.7, 8.2)	71.9
	3	5.81 (*t*, 9.8)	73.5	5.90 (*t*, 9.7)	73.5
	4	5.21 (*t*, 10.0)	71.2	5.60 (*t*, 9.7)	69.5
	5	5.35 (*dd*, 13.0, 6.5)	73.6	4.54 (*m*)	74.1
	6	4.51 (*dd*, 10.0, 6.5), 3.87 (*d*, 13.0)	63.3	4.22 (*dd*, 12.5, 4.1), 4.50 (*m*)	62.9
Galloyl	1		119.9-120.6		120.8-122.5
	2, 6	7.07, 6.96, 6.93 (2H, *s*)	110.1-110.6	7.03, 6.96, 6.94, 6.90, 6.87 (2H, *s*)	112.4-112.8
	3, 5		146.2-146.5		147.9-148.1
	4		139.6-140.3		141.7-142.2
HHDP[d]	1, 1'		125.9-126.5		
	2, 2'		116.3		
	3, 3', 5,5'		144.8-145.6		
	4, 4'		136.9-137.1		
	6, 6'	6.46, 6.60 (1H, *s*)	108.1-108.4		
C=O			165.5-168.6		167.9-169.4

[a] ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) data of compound **1** were measured in an acetone-d_6:methanol-d_4=4:1 mixture. [b] ^{1}H- (400 MHz) and ^{13}C-NMR (125 MHz) data of compound **2** were measured in an acetone-d_6:D_2O=3:1 mixture. [c] Integral and multiplicity. [d] Hexahydroxydiphenoyl.

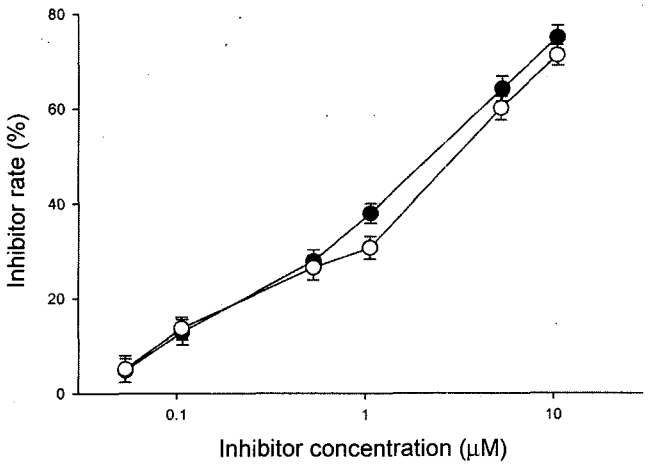


Fig. 2. Dose-dependant inhibition of BACE1 by compound **1** and **2** -■-: **1** (Tellimagradin II), -○-: **2** (1,2,3,4,6-pentagallolyl-β-D-glucopyranoside).

(*Ki*) were 6.84×10^{-6} M and 5.13×10^{-6} M, respectively. To check the enzyme specificity, the inhibitory activities on α-secretase (TACE) and other serine proteases such as chymotrypsin, trypsin, and elastase were compared with that of BACE1. Up to 0.1 mM, they did not show a significant inhibition of α-secretase, chymotrypsin, trypsin, and elastase (data not shown), suggesting that the inhibitory activity was not a false-positive reaction due to the protein-precipitating property of tannin. Thus, **1** and **2** appeared to be relatively specific inhibitors of BACE1, as is the case of other natural inhibitors (Jeon *et al.*, 2003).

Recently, two transition-state analog inhibitors of β-secretase, OM99-1 and OM99-2s with IC_{50} of ~1.6 nM, were reported (Ghosh *et al.*, 2001). They were synthesized on the basis of the model on the cleavage site on the β-secretase of the Swedish mutation. On the other hand, naturally occurring β-secretase inhibitors have rarely been achieved.

Table II. Inhibitory activity of compounds **1** and **2** against α-secretase and other serine proteases

Enzymes	IC_{50} (M)
α-secretase (TACE)	$>110^{-4}$
Trypsin	$>110^{-4}$
Chymotrypsin	$>110^{-4}$
Elastase	$>110^{-4}$

The isolated compounds might not be directly considered as a drug candidate since they are relatively hydrophilic and have large molecular weight structures for natural products, however, this is the first report on the BACE1-inhibiting activity of tellimagradin II and 1,2,3,4,6-pentagallolyl-β-D-glucopyranoside. The isolated compounds are expected to be useful in the study of the mechanisms of Alzheimer's disease.

ACKNOWLEDGEMENTS

This work was supported by a grant from the BioGreen 21 Program, Rural Development Administration, Republic of Korea. Thanks of the Post-doc. program, Kyungpook National University for the financial support of Dr. Kyung-Ah Kim.

REFERENCES

Anderson, J. P., Barbour, R., Basi, G. S., Caccavello, R., Davis, D., and Doan, M. *et al.*, Purification and cloning of amyloid precursor protein β-secretase from human brain. *Nature*, 402, 533-537 (1999).

Citron, M., β-Secretase as a target for the treatment of Alzheimer's disease. *J. Neurosci. Res.*, 70, 373-379 (2002).

Feldman, K. S. and Sahasrabudhe, K., Ellagitannin Chemistry.

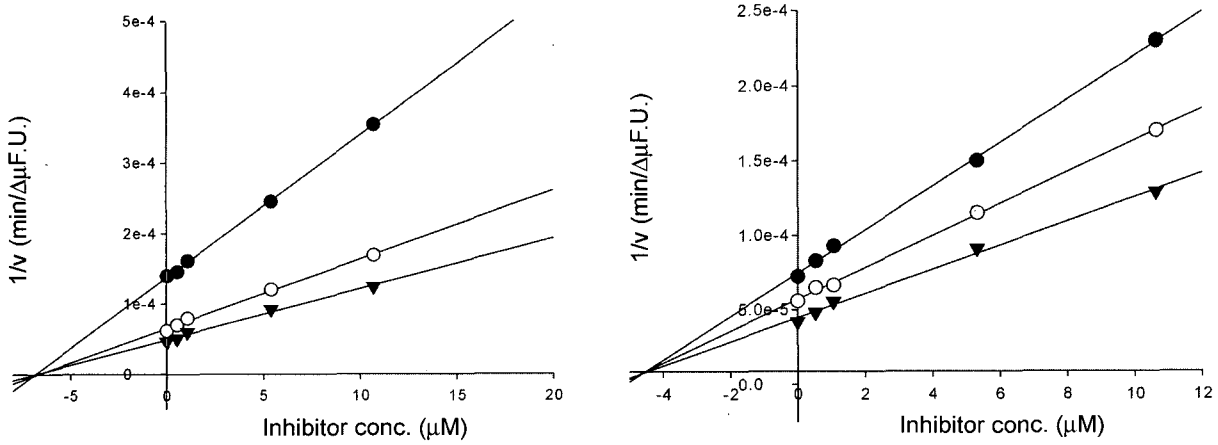


Fig. 3 Dixon plots of inhibition of compounds **1** and **2**. Inhibition of BACE1 by tellimagradin II (left) and 1,2,3,4,6-pentagallolyl-β-D-glucopyranoside (right). Concentration of substrate: 750 nM (-●-), 500 nM (-○-), and 250 nM (-▼-). The values are the mean of duplicated experiments.

Syntheses of tellimagrandin and a dehydrodigalloyl ether-containing dimeric gallotannin analogue of coriariin A. *J. Org. Chem.*, 64, 209-216 (1999).

Ghosh A. K., Bilcer G., Harwood C., Kawahama R., Shin, D., Hussain, K. A., Hong, L., Loy, J. A., Nguyen, C., Koelsch, G., Ermolieff, J., and Tang, J., Structure-based design: potent inhibition of human brain memapsin 2 (β-secretase). *J. Med. Chem.*, 44, 2865-2868 (2001).

Ghosh, A. K., Shin, D., Downs, D., Koelsch, G., Lin, X., Ermolieff, J., and Tang, J., Design of potent inhibitors for human brain memapsin 2 (β-secretase). *J. Am. Chem. Soc.*, 122, 3522-3523 (2000).

Glenner, G. G. and Wong, C. W., Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem. Biophys. Res. Commun.*, 120, 885-890 (1984).

Jeon, S.-Y., Bae, K.-H., Seong, Y.-H., and Song, K.-S., Green tea catechins as a BACE1 (β-secretase) inhibitor. *Bioorg. Med. Chem. Lett.*, 13, 3905-3908 (2003).

Kim, S.-I. and Song, K.-S., 1,2,3,4,6-Pentagalloyl-β-D-glucopyranose, a prolyl endopeptidase inhibitor from Moutan cortex. *J. Korean Soc. Agric. Chem. Biotechnol.*, 43, 158-161 (2000).

Lee, V. M.-Y., Balin, B. J., Otvos, L., and Trojanowski, J. Q., A major subunit of paired helical filaments and derivatized forms of normal tau. *Science*, 251, 675-678 (1991).

Terry, R. D., Masliah, E., and Hansen, L. A., The neuropathology of Alzheimer disease and the structural basis of its cognitive alterations, in: R. D. Terry *et al.* (Ed.), Alzheimer Disease, Lippincott, Williams and Wilkins, Philadelphia, pp. 187-206 (1999).

Tung J. S., Davis D. L., Anderson J. P., Walker D. E., Mamo S., Jewett N., Hom R. K., Sinha S., Thorsett E. D., and John V., Design of substrate-based inhibitors of human β-secretase. *J. Med. Chem.*, 45, 259-262, (2002).

Vassar, R., β-Secretase (BACE) as a drug target for alzheimer's disease. *Advanced Drug Delivery Reviews*, 54, 1589-1602 (2002).