In-Hye Park
So-Young Jeon
Hee-Ju Lee
Sang-In Kim
Kyung-Sik Song

A β-Secretase (BACE1) Inhibitor Hispidin from the Mycelial Cultures of *Phellinus linteus*

Abstract

In the course of screening for anti-dementia agents from natural products, a β-secretase (BACE1) inhibitor was isolated from the culture broth of *Phellinus linteus* and identified as hispidin. It showed an IC_{50} value of 4.9×10^{-6} M and a *Ki* value of 8.4×10^{-6} M. The compound was a non-competitive inhibitor. Hispidin also inhibited a prolyl endopeptidase (IC_{50} = 1.6×10^{-5} M, *Ki* = 2.4×10^{-5} M), but it was less inhibitory to α-secretase (TACE) and other serine proteases such as chymotrypsin, trypsin, and elastase.

Key words
Phellinus linteus · Hymenochaetaceae · mycelial culture · phenylpropanoid · hispidin · β-secretase inhibitor · Alzheimer

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder clinically characterized by progressive dementia that inevitably leads to incapacitation and death. A major histopathological characteristic of AD is the deposition of amyloid protein (amyloid plaques) in the parenchyma of the amygdala, hippocampus, and neocortex [1]. The major component of the amyloid plaques is β-amyloid peptide (Aβ), a 39 – 43 amino acid peptide composed of a portion of the transmembrane domain and the extracellular domain of the amyloid precursor protein (APP) [2]. The Aβ peptide is generated by endoproteolysis of the large type I membrane protein APP [3]. A protease called β-secretase initially cleaves the APP to form the *N*-terminus of Aβ at the Asp+1 residue of the Aβ sequence. Following β-secretase cleavage, C99 is the substrate of a second protease, γ-secretase, which cleaves the APP to generate the *C*-terminus of Aβ, and the mature peptide is secreted from the cell. A third protease, α-secretase, non-pathologically cleaves the APP in the middle of the Aβ domain; thus, precluding the formation of Aβ [4].

Induction of α-secretase, which is regulated by phosphorylation, via activated protein kinase C (PKC), might be a target for reducing Aβ formation in the brain. However, the strategy of increasing α-secretase via PKC results in activation of unrelated receptors and systems downstream of PKC. Activation of receptors upstream of PKC might result in problems that could be worse than those of AD. In addition, since α-secretase has relaxed sequence specificity and a range of substrates, any therapeutic strategy aimed at increasing activity of this enzyme could be damaging [5].

An alternative target might be an inhibition of γ-secretase activity, but the identity of this enzyme is still subject to debate and the detailed structure remains unknown [5]. Additionally, a potential difficulty for therapeutic use of γ-secretase is that Notch,

Affiliation
Division of Applied Biology and Chemistry, College of Agriculture and Life Sciences, Kyungpook National University, Daegu, Korea

Correspondence
Prof. Kyung-Sik Song · Division of Applied Biology and Chemistry · College of Agriculture and Life Sciences · Kyungpook National University · 1370 Sankyuk-Dong · Daegu 702-701 · Korea · Fax: +82-53-956-5715 · E-mail: kssong@knu.ac.kr

Funding
This work was supported by a grant from the Ministry of Health and Welfare, Korea (grant No. 02-PJ2-PG3 – 21604 – 0002).

Received June 2, 2003 · **Accepted** November 8, 2003

Bibliography
Planta Med 2004; 70: 143–146 · © Georg Thieme Verlag Stuttgart · New York · ISSN 0032-0943 · DOI 10.1055/s-2004-815491

not APP, signaling receptors are the major physiological substrates of this enzyme [6]. In addition, the C99-terminal stubs contain the complete amyloid sequence and are known to be neurotoxic in cellular models; therefore, they are expected to be neurotoxic *in vivo*[6].

Among the secretases, a novel transmembrane aspartic protease BACE1 (for β-site APP cleaving enzyme 1) [7], [8], also known as Asp2 (for novel aspartic protease 2) and memapsin2 (for membrane aspartic protease/pepsin 2), is at present the most attractive target for the inhibition of amyloid production. There is strong evidence that it is the major β-secretase in neurons and that the absence of BACE1 inhibits production of amyloid and the C99 stubs without any major side-effect [5]. The lack of Aβ production in BACE1 deficient mice clearly indicates that BACE1 is the β-secretase and that BACE1 inhibitors should reduce Aβ levels. Accordingly, as the key enzyme that initiates Aβ formation *in vivo*, BACE1 is a prime drug target for inhibition of Aβ production.

Several transition-state analogue peptide inhibitors of BACE1 modeled on the β-secretase cleavage site of the Swedish mutation have been reported with relatively low *Ki* values [9], [10]. However, natural product inhibitors have rarely been investigated. All the drugs considered for treatment of Alzheimer's disease must cross the blood-brain barrier and the plasma membrane. For BACE1 inhibitors, this requirement might be difficult to meet because currently available BACE1 inhibitors are peptidomimetics of the β-cleavage site of APP. In this sense, secondary metabolites of plants and microbes with relatively low molecular weight and high lipophilicity might be BACE1 inhibitors for the drug candidate. In the course of screening natural products for small molecule BACE1 inhibitors, we tested 256 plant and fungal extracts. A broth of a mycelial culture of *Phellinus linteus* showed the highest inhibitory activity. We here report on the isolation and structure elucidation of an active compound, hispidin, and on its inhibitory activity on additional proteolytic enzymes.

Materials and Methods

General

Fluorescence was measured with a Bio-TEK ELISA microplate fluorescence reader ELx 800 (VT, USA). Optical density was measured by a Bio-TEK ELx 808 (VT, USA). ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker Avance Digital 400 spectrometer (Karlsruhe, Germany) at 400 and 100 MHz, respectively. Chemical shifts are given in δ (ppm) from TMS. EI-MS was recorded on a Micromass VK QUATTRO II (Hertsfordshire, UK). TLC was performed on a precoated silica gel plate (Kiesel gel 60F254, Merck, NJ, USA). Silica gel column chromatography was carried out using a Kiesel gel 60 (Merck, NJ, USA). Authentic hispidin, (–)-epigallocatechin gallate (EGCG), Sephadex LH-20, chymotrypsin, trypsin, elastase, and their substrates were purchased from Sigma (MO, USA).

Enzyme assays

A BACE1 (recombinant human BACE1) assay kit was purchased from PanVera, WI, USA. The assay was carried out according to the manufacturer's instructions with modifications. Briefly, a mixture of 10 μL of assay buffer (50 mM sodium acetate, pH 4.5), 10 μL of BACE1 (1.0 U/mL), 10 μL of the substrate (75 μM Rh-EVNLDAEFK-Quencher in 50 mM ammonium bicarbonate), and 10 μL of sample dissolved in assay buffer was incubated for sixty min at 25 °C in the dark. 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 was 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 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. Hispidin had only a negligible quenching effect. α-Secretase activity was measured by an α-secretase assay kit with TACE according to the manual from R & D Systems, MN, USA. Prolyl endopeptidase (PEP, from *Flavobacterium meningosepticum*) and its substrate (*Z*-Gly-Pro-*p*NA) were purchased from Seikagaku (Tokyo, Japan). *Z*-Pro-prolinal was synthesized according to the method of Bakker et al. [11] and used as a positive control. The PEP activity and the inhibition percent of all samples were determined according to the previously reported method [12]. Chymotrypsin, trypsin, and elastase were assayed according to the protocol described in the references [13], [14], [15] 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 presented are the mean values of triplicate experiments.

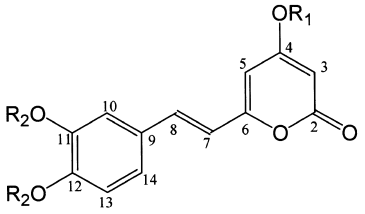
Material, extraction, and isolation

The mycelium of *P. linteus* Teng (Strain No. KCTC 6190, originally from ATCC 26719, USA) belonging to Hymenochaetaceae was obtained from KCTC (Korean Collection for Type Culture), Daejon, Korea. The stock culture was deposited and maintained at KCTC and the Innovative Research Laboratory of Natural Product Medicine, Kyungpook National University, Daegu, Korea. The strain was inoculated into 100 mL of a yeast-malt medium (pH 5.8, 25 °C, 100 rpm). Five mL of the above seed culture were transferred to 100 mL of the same medium in a 500 mL Erlenmeyer flask and cultured for 12 days. The culture broth of *P. linteus* (3 L) was filtered through filter paper and the filtrate was partitioned with 9 L of EtOAc. The EtOAc-soluble fraction (1.57 g) was chromatographed on a silica gel column [3.5 × 50 cm, benzene/EtOAc/HOAc (each 300 mL of 25 : 1 : 1, 20 : 1 : 1, 10 : 1 : 1, 5 : 1 : 1, and 2 : 1 : 1)]. Fractions of 15 mL were analyzed by TLC (SiO_2, benzene/EtOAc/HOAc, 10 : 1 : 1 and 5 : 1 : 1; $FeCl_3$). According to their TLC pattern, fractions were combined (fr 1 : 900 mL, fr 2 : 45 mL, fr 3 : 555 mL). HPLC [μBondapak C18, Waters, MA, USA, 7.8 × 300 mm, 1 % HOAc in 25 % MeOH, UV254 nm, 1.8 mL min^{-1}] of the active fraction (fr 2, 352.0 mg) afforded 12.0 mg of compound **1** (t_R: 24.3 min).

Synthesis of hispidin

Hispidin was synthesized according to the reported method [16] with some modifications. A suspension of $Mg(OMe)_2$ (120 mmol) in anhydrous MeOH (100 mL) was added to a solution of piperonal (40 mmol) and 4-methoxy-6-methyl-2-pyrone (28 mmol) in MeOH (80 mL) under N_2 gas. The mixture was refluxed for 7 h,

and the solvent was removed by evaporation. The residue was recrystallized from MeOH to afford 3.12 g (41% of yield) of **2** [4-methoxy-6-(3′,4′-methylenedioxystyryl)-2-pyrone] as yellow needles. A 1 M solution of BCl_3 in hexane (6.91 mmol) was added to a solution of **2** (2.23 mmol) in CH_2Cl_2 (100 mL) under N_2 gas. The reaction mixture was refluxed for 22 h. The solvent was removed and the residue was recrystallized from the MeOH to give 0.31 g (53% of yield) of **3** [4-methoxy-6-(3′,4′-dihydroxystyryl)-2-pyrone] as yellow needles. To a suspension of NaH (72.0 mg, 60% oil suspension) in 1 mL of DMF was added a solution of EtSH (1.73 mmol) in 2 mL of DMF under N_2 gas. The mixture was stirred for 10 min and a solution of **3** (0.384 mmol) in 3 mL of DMF was added. The mixture was refluxed for 1 h. The crude product was recrystallized from the MeOH to yield 42 mg (44% of yield) of hispidin (**1**) as light brown needles. The structures of **1**, **2**, and **3** were verified by comparing their ^{1}H-NMR data with those in reference [16]. The purities of **1 – 3** were checked by HPLC (μBondapak C18, 3.9 × 150 mm, linear gradient elution with 1% HOAc in water and 1% HOAc in MeOH for 50 min, 0.8 mL min^{-1}) equipped with PDA and ELSD (evaporative light scattering detector) (t_R′s: **1**; 17.28, **2**; 28.36, **3**; 20.18).



1, R_1=R_2=H
2, R_1=Me, R_2=linked to each other as dioxolane
3, R_1=Me, R_2=H

Fig. **1** Structures of hispidin and related compounds; **1**, hispidin; **2**, 4-methoxy-6-(3′,4′-methylenedioxystyryl)-2-pyrone; **3**, 4-methoxy-6-(3′,4′-dihycroxystyryl)-2-pyrone.

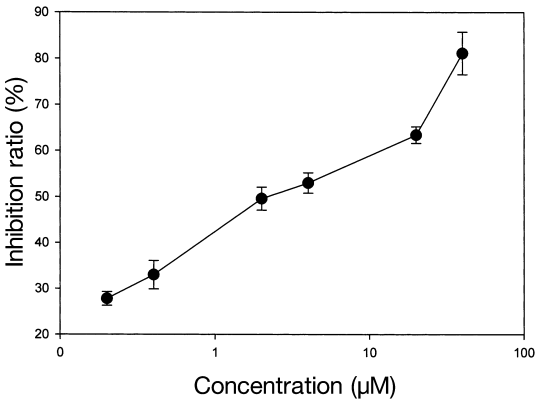


Fig. **2** Dose-dependant inhibition of BACE1 by hispidin. The values are the means of triplicate experiments.

Results and Discussion

The mycelium of *P. multiplex* was cultured in a yeast-malt (YM) liquid medium. The time course of the fermentation showed that the BACE1 inhibition reached its maximum level 12 – 14 days after inoculation. The cultures were filtered through filter paper and the filtrate was partitioned with EtOAc. The activity-guided purification of the EtOAc soluble fraction with silica gel and Sephadex LH-20 columns afforded **1**. The compound was obtained as a light brown powder that was positive to the $FeCl_3$ reagent. The EI-MS, ^{1}H-, and ^{13}C-NMR data were identical with those of 6-(3,4-dihydroxystyryl)-4-hydroxy-2-pyrone (hispidin), which had been previously isolated from *P. pomaceus*[17] and studied as an anticancer agent due to its property as a selective PKC-βs inhibitor [18]. The structure of **1** (Fig. **1**) was confirmed by chemical synthesis and authentic hispidin. No recognizable impure peaks were found in the HPLC analysis (more than 99% of purity under the conditions described in Materials and Methods).

Compound **1** inhibited BACE1 activity in a dose-dependant manner with IC_{50} of 4.9×10^{-6} M in the current study (Fig. **2**). The inhibition of hispidin was non-competitive with a substrate in the Dixon plot (Fig. **3**). The *Ki* value was 8.4×10^{-6} M.

To check the enzyme specificity, the inhibitory activities on TACE (tumor necrosis factor alpha converting enzyme), which is a candidate for α-secretase [19], and other serine proteases such as PEP (prolyl endopeptidase), chymotrypsin, trypsin, and elastase were compared with those of BACE1 (Table **1**). Up to 40 μM, hispidin did not show significant inhibition against the above enzymes, but it inhibited more than 80% of both PEP and BACE1 at the same concentration, indicating that hispidin appears to be a relatively specific inhibitor of BACE1 and PEP. Hispidin inhibited the prolyl endopeptidase (PEP, IC_{50} = 1.6×10^{-5} M, *Ki* = 2.4×10^{-5} M) in a non-competitive manner with a substrate in the Dixon plot (Fig. **3**). Although it has much lower activity compared to that of a positive control, *Z*-Pro-prolinal (7×10^{-8} M), it is noteworthy that hispidin also inhibited PEP since this enzyme degrades proline-containing neuropeptides such as vasopressin, substance P, and thyrotropin-releasing hormone (TRH), which have been suggested to play an important role in learning and memory [20].

The hispidin analogues **2** and **3** (more than 99% of purity in HPLC analysis) obtained as intermediates in the course of hispidin synthesis were also active against BACE1 (IC_{50} = 4.0×10^{-5} M and 7.2×10^{-5} M, respectively), but not inhibitory toward PEP (IC_{50} > 1.0 mM, each). Compound **2**, which does not have a catechol moiety, showed inhibitory activity against BACE1, suggesting that a catechol might not be necessary for stronger activity.

Peptidomimetic inhibitors such as OM99 – 1 and OM99 – 2 [10] have been synthesized with *Ki* values of 6.84×10^{-8} to 2.86×10^{-10} M and IC_{50} values of 1.6×10^{-9} to 3.0×10^{-8} M. However, these large peptide-based inhibitors have not been developed as drug candidates because the obstacles for Alzheimer therapy are even higher in comparison to those for the inhibition of rennin and HIV protease, which also belong to the aspartic protease group [21]. Takeda reported that tetraline, which is not an obvious scaffold for protease inhibition, is likely to originate from high-throughput screening efforts. The inhibitory activity is poor and the mode of action is insecure [22]. Latifolin, isolated from the heartwood of *Dalbergia sisso*, has been found to inhibit Aβ synthesis with an IC_{50} value of 180 μM, again a rather weak and in-

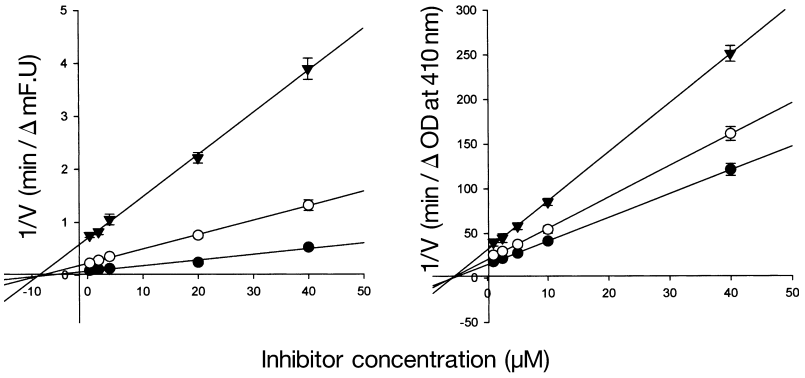


Fig. **3** Dixon plots of inhibition. *Left:* inhibition of BACE1 by hispidin. *Right:* inhibition of PEP by hispidin. Substrate concentration: *Left*; –▼– 250 nM, –○– 500 nM, –●– 750 nM. *Right*; –▼– 0.5 mM, –○– 0.75 mM, –●– 1.0 mM.

Table **1** Inhibitory activities[a] of hispidin against TACE, PEP, and other serine proteases

Conc. (μM)	*Chymotrypsin*	*Trypsin*	*Elastase*	*PEP*	*TACE*	*BACE1*
Hispidin						
4.0	0.6 ± 0.2	3.3 ± 0.3	5.2 ± 1.2	30.1 ± 1.2	0.1 ± 0.0	53.0 ± 0.8
20.0	1.4 ± 0.3	1.0 ± 0.2	3.1 ± 1.3	61.3 ± 7.0	1.1 ± 0.1	63.4 ± 3.1
40.0	0.6 ± 0.2	0.0 ± 0.0	8.2 ± 2.0	80.1 ± 5.8	3.1 ± 1.0	81.1 ± 4.9
Control[b]	4.4 ± 1.3	1.6 ± 0.2	8.1 ± 0.2	3.1 ± 3.2	2.1 ± 0.8	10.1 ± 0.8
EGCG[c]	–	–	–	–	–	81.2 ± 5.0
ZPPCHO[c]	–	–	–	50.0 ± 5.6	–	–

[a] The activities (%) are mean ± SE of three independent experiments. The inhibition ratio was calculated as described in Materials and Methods.

[b] Ten μL of 5% MeOH were added to the reaction mixture instead of a sample solution.

[c] Eleven μM of (–)-epigallocatechin gallate and 7 nM of *Z*-Pro-prolinal were used as positive controls for BACE1 and PEP, respectively.

secure activity [23]. The *Ki* and IC_{50} values of hispidin were much higher than those of synthetic peptidomimetics, but relatively similar to those of a natural inhibitor EGCG (IC_{50} = 1.6×10^{-6} M, *Ki* = 2.1×10^{-7} M) [24], which was introduced as a positive control for BACE1 inhibition in this study. Enzyme inhibitors with therapeutic potential are preferably smaller than 700 Da with an IC_{50} value in the nM level, so hispidin might not be directly considered as a drug candidate. However, it might be a starting point for rational non-peptidyl drug design and be a useful reagent for studying the enzyme properties of BACE1.

References

[1] Sisodia SS, Price DL. Role of the β-amyloid protein in Alzheimer's disease. FASEB J 1995; 9: 366 – 70

[2] Glenner GG, Wong CW. Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. Biochem Biophys Res Commun 1984; 120: 885 – 90

[3] Selkoe DJ. Alzheimer's disease: genes, proteins, and therapy. Physiol Rev 2001; 81: 741 – 66

[4] Vassar R. β-Secretase (BACE) as a drug target for Alzheimer's disease. Adv Drug Deliv Rev 2002; 54: 1589 – 602

[5] Dewachter I, Van Leuven F. Secretases as targets for the treatment of Alzheimer's disease: the prospects. Lancet Neurol 2002; 1: 409 – 16

[6] Wilson CA, Doms RW, Zheng H, Lee VM. Presenilins are not required for Aβ 42 production in the early secretory pathway. Nature Neurosci 2002; 5: 849 – 55

[7] Anderson JP, Barbour R, Basi GS, Caccavello R, Davis D, Doan Met al. Purification and cloning of amyloid precursor protein β-secretase from human brain. Nature 1999; 402: 533 – 7

[8] Vassar R, Bennett BD, Babu-Khan S, Khan S, Mendiaz A, Denis Pet al. β-Secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE1. Science 1999; 286: 735 – 41

[9] Schmidt B. Aspartic protease involved in Alzheimer's disease. ChemBioChem 2003; 4: 366 – 78

[10] Ghosh AK, Shin D, Downs B, Koelsch G, Lin X, Ermolieff JJ et al. Design of potent inhibitors for human brain memapsin 2 (β-secretase). J Am Chem Soc 2000; 122: 3522 – 3

[11] Bakker AV, Jung S, Spencer RW, Vinick FJ, Faraci WS. Slow-tight binding of prolyl endopeptidase by benzyloxycarbonylprolinal. Biochem J 1990; 274: 559 – 62

[12] Toda S, Obi Y, Kimura K, Hamagishi Y, Tomita K, Komiyana Net al. Eurystatins A and B, new prolyl endopeptidase inhibitors. I. Taxonomy, production, isolation and biological activities. J. Antibiotics 1992; 45: 1573 – 9

[13] Chung CH, Ines HE, Almeda S, Goldberg AL. Purification of *Escherichia coli* of a periplasmic protein that is potent inhibitor of pancreatic protease. J Biol Chem 1983; 258: 11032 – 8

[14] Hubert F, Gaertner J, Antonie J, Puigserver AJ. Increased activity and stability of polyethyleneglycol-modified trypsin. Enzyme Microb Technol 1992; 14: 150 – 5

[15] Bieth J, Spiess G, Camille GW. The synthesis and analytical use of a highly sensitive and convenient substrate of elastase. Biochem Med 1974; 11: 350 – 7

[16] Adam W, Saha-Moeller CR, Veit M, Markus W, Welke B. A convenient synthesis of hispidin from piperonal. Synthesis, 1994: 1133 – 4

[17] Klaar M, Wolfgang S. Fungus pigments, XXVII. Isolation of hispidin and 3,14′-bihispidinyl from *Phellinus pomaceus* (Poriales). Chem Ber 1997; 110: 1058 – 62

[18] Gonindard C, Bergonzi C, Denier C, Sergheraert C, Klaebe A, Chavant Let al. Synthetic hispidin, a PKC inhibitor, is more cytotoxic toward cancer cells than normal cells *in vitro*. Cell Biol Toxicol 1997; 13: 141 – 53

[19] Buxbaum JD, Liu K-N, Luo Y, Slack JL, Stocking KL, Peschon JJet al. Evidence that tumor necrosis factor alpha converting enzyme is involved in regulated α-secretase cleavage of the Alzheimer amyloid protein precursor. J Biol Chem 1998; 273: 27765 – 7

[20] Katsube N, Sunaga K, Chuang D-M, Ishitani R. ONO-1603, a potential antidementia drug, shows neuroprotective effects and increases m3-muscarinic receptor mRNA levels in differentiating rat cerebellar granule neurons. Neurosci Lett 1996; 214: 151 – 4

[21] Ghosh AK, Bilcer G, Harwood C, Kawahama R, Shin D, Hussain KA et al. Structure-based design: potent inhibitors of human brain memapsin 2 (β-secretase). J Med Chem 2001; 44: 2865 – 8

[22] Miyamoto M, Matsui J, Fukumoto H, Tarui N. Preparation of 2-[2-amino- or 2-(N-heterocyclyl)ethyl]-6-(4-biphenylylmethoxy)tetralin derivatives as β-secretase inhibitors. PCT Int Appl, 2001 WO 087293

[23] Ramakrishna NVS, Kumar EKSV, Kulkarni AS, Jain AK, Bhat RG, Parikh Set al. Screening of natural products for new leads as inhibitors of β-amyloid production: Latifolin from *Dalbergia sissoo*. Ind J Chem 2001; 40B: 539 – 40

[24] Jeon S-Y, Bae K, Seong Y-H, Song K-S. Green tea catechins as BACE1 (β-secretase) inhibitor. Bioorg Med Chem Lett 2003; 13: 3905 – 8