*Bulgarian Chemical Communications, Volume 41, Number 2 (pp. 143–148) 2009*

# New peptide mimetics with potential β-secretase inhibition activity

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Received July 10, 2008; Revised September 25, 2008

Alzheimer's disease (AD) is affecting about 20 millions people worldwide. The last forecast shows that during the next 20–30 years their number will double because of the human life-span increasing. The transformation of amyloid β (Aβ) soluble from the insoluble β-fibers is a critical stage in the AD progression. Aβ resulted from the precursor protein cleavage which is catalyzed by different proteinases named α, β and γ-secretases. That is why the inhibition of some of these enzymes is a promising alternative for AD treatment. The shortest peptide structure found with β-secretase inhibition activity is Glu-Val-Asp-Leu-Ala. A lot of investigations show the role of various amino acids in different positions for the inhibition activity and reveal important structure-activity relationships. Based on these investigations, we synthesized ten peptide mimetics with potential β-secretase inhibition activity by means of conventional peptide synthesis in solution. All newly synthesized compounds were characterized by TLC, m.p. and $[\alpha]_{546}^{22}$. The biological trials are in progress.

**Key words:** Alzheimer's disease; β-secretase inhibitors, peptide mimetics.

## INTRODUCTION

Alzheimer's disease (AD) is affecting about 20 millions people worldwide. The last forecast shows that during the next 20–30 years their number will double because of the human life-span-increasing. The transformation of amyloid β (Aβ) soluble from the insoluble β-fibers is a critical stage in the AD progression. Aβ resulted from the precursor protein containing 695 to 770 amino acid residues. This process starts with four cleavages of this protein catalyzed by different proteinases, named α, β and γ-secretases. The tearing in $A\beta_1$ and $A\beta_{11}$ is catalyzed by the enzyme β-secretase. There is no data in literature whether this enzyme is a part of the other life process in the organism.

Last ten years provide a number of different investigations on the β-secretase inhibition process. Tung et al. revealed that the shortest peptide structure with β-secretase inhibition activity is Glu-Val-Asp-Leu-Ala [1]. Their experiments show that Leu or Phe at $P_1$ position, Val or Leu at $P_3$ position and Asp or Asn at $P_2$ position is compulsory for inhibition activity availability. Additional investigations reveal that the minimal substrate with passable β-secretase inhibition activity is Boc-Val-Asp-Leu-Ala-OH. Based on that structure, we investigate the influence of different amino acid residues at $P_4$, $P_3$ and $P_2$ positions on the inhibition activity. At some structures the residues at $P_4$ and $P_3$ position were removed. It is well-known that the enzyme β-secretase includes high hydrophobic $S_1$ and $S_3$-pockets [2]. That is why the availability of hydrophobic amino acids at these positions is obligatory for inhibition activity. In our new structure, we choose Leu residue for these two positions. $S_2$ and $S_4$-pockets are hydrophilic [3]. Tung *et al*. investigated the residues Asn and Met for $P_2$ position. Their X-ray analysis revealed that $P_2$ Asn residue in the substrate made a hydrogen bond with $Arg^{235}$ in the enzyme binding site [1]. In our structure, Asp in this position was used because we suppose that its hydrogen bond formation potential is better than Asn. For $P_4$ position Tung *et al*. choose Glu or Boc residue and they proved that –C=O function participates in the strong hydrogen bond obtaining. There is no data whether the bonds made by Boc group are stronger than those obtained by the δ-COOH function of Glu [1]. We decided to investigate the length of the chain of the amino acid in this position and choose Asp.

The additional investigations on the high-potent β-secretase inhibitors conformation show that at $P_2$' position the chain of the compounds is turned off and the side chains of the residues at $P_3$' and $P_4$' positions do not participate in the enzyme-substrate interaction [4]. Based on the latter conclusion, Ghosh *et al*. removed and replaced the residues at $P_3$' and $P_4$' positions by different C-terminal residues [2]. They investigated the activity of derivatives based on the sequence Boc-Val-Asp-Leu-

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Ala-Val-Glu($P_3$')-Phe($P_4$')-OH. They succeeded to increase β-secretase inhibition activity replacing C-terminal amino acids at $P_3$' and $P_4$' positions by benzylamine. The latter results to fourfold inhibition activity increasing ($IC_{50}$ = 28 nM, $K_i$ = 2.5 nM). The other potential residue they determined was piridinylmethylamine ($IC_{50}$ = 70 nM) [1]. Because of the better activity of the compounds with benzylamine C-terminus in our new structure the same fragment as C-terminal moiety was used. On the other hand, it is well-known that high hydrophobic moieties play a key role in the molecule for blood brain barrier passing. Based on this fact, a series of analogues with C-terminal 3,4-dimethoxybenzylamine residue was synthesized.

The X-ray investigations on the crystal structure of the enzyme-substrate complex revealed that COOH group of the residue at $P_2$' position made a strong hydrogen bond with OH group of $Tyr^{198}$ which plays key role in the enzyme-substrate interaction. That is why even in the shortest peptide structure with β-secretase inhibition activity Glu-Val-Asp-Leu-Ala($P_1$') the availability of $P_2$' residue is obligatory. The question is: which is the best choice for amino acid residue in this position? In 2005 Hanessian *et al*. synthesized three peptides differing only by their residue at $P_2$' position – NH-Butyl ($IC_{50}$ = 1.82 μM), Ala ($IC_{50}$ = 0.6 μM) and Val ($IC_{50}$ = 0.19 μM) [5]. The obtained results show that the most powerful is the analogue with Val at $P_2$' position. That is why our design with Val in this position was done.

## EXPERIMENTAL

All newly synthesized compounds were characterized by TLC, m.p. and $[\alpha]_{546}^{22}$. Their structures were proved by ES/MS. The purity of the products was checked by TLC on precoated plates of Silica gel 60 $F_{254}$ (MERCK) with the following solvent systems: $CHCl_3$:AcOH 9:1 (v/v); *n*-BuOH:AcOH:$H_2O$ 3:1:1 (v/v/v) and *n*-BuOH:AcOH:pyridine:$H_2O$ 60:6:24:20. Spots on TLC chromatograms were detected by chlorine/*o*-tolidine reaction. The melting points were deter-mined on a Kofler apparatus and were uncorrected. The optical rotation was measured on a Quick Russel-Jouan Type SL1D polarimeter.

### *General procedure for preparation by the N,N'-dicyclohexylcarbodiimid (DCC)/1-hydroxybenzotriazol (HOBt) method*

1.00 mmol of the peptide (obtained from Boc-peptide ester by treatment with 10-fold excess of TFA) was dissolved in minimal amount of DMF and after cooling to –5ºC neutralized to pH 7–7.5 with $Et_3N$. 1.20 mmoles of Z- or Boc-amino acid, 1.20 mmoles of DCC and 1.40 mmoles of 1-HOBt were added. The reaction mixture was stirred for 24 h at 0ºC and for another 24 h at room temperature. The obtained DC-urea was removed by filtration and then 30 ml of water were added. The product was extracted into EtOAc (3×10 ml) and the organic layer was washed with 5% $NaHCO_3$ (3×10ml), $H_2O$ (3×10ml), 10% citric acid (3×10ml) and $H_2O$ to pH = 7. The solvent was dried with $Na_2SO_4$ and removed in *vacuo* followed by recristallization.

### *General procedure for preparation by the TBTU or TCTU method*

1.00 mmol of the peptide (obtained from Boc-peptide ester by treatment with 10-fold excess of TFA) was dissolved in minimal amount of DMF and after cooling to –5ºC neutralized to pH 7–7.5 with diisopropylethylamine (DIEA). 1.20 mmoles of Z- or Boc-amino acid, 1.20 mmoles of 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) or O-(6-chloro-1-hydroxybenzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TCTU) and 1.20 mmoles of DIEA were added. The reaction mixture was stirred for 24 h at room temperature. Finally, 30 ml of water were added. The product was extracted into EtOAc (3×10 ml) and the organic layer was washed with 5% $NaHCO_3$ (3×10ml), $H_2O$ (3×10ml), 10% citric acid (3×10ml) and $H_2O$ to pH = 7. The solvent was dried with $Na_2SO_4$ and removed in *vacuo* followed by recristallization.

### *Deblocking of Z- and OBzl groups by catalytic hydrogenation in the presence of Pd/C*

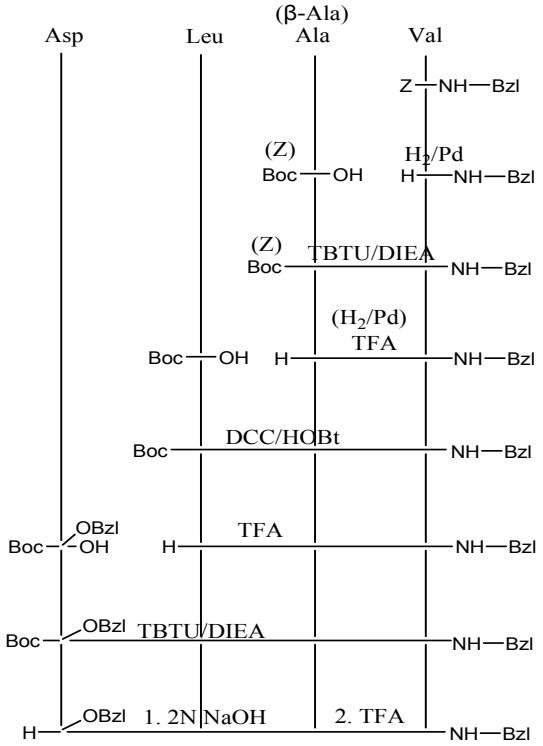
1.00 mmol of protected peptide was dissolved in MeOH and then Pd/C and 1.00 mmol (or catalytic amount) of HCl was added. Hydrogen was passed through the reaction mixture at room temperature. The deblocking of the protecting groups was controlled by TLC and after finishing the reaction, Pd/C was filtered out and MeOH was evaporated in *vacuo*. The formed oil was subjected to the next deblocking.

## RESULTS AND DISCUSSION

Based on the information above, a design of peptide mimetics including minimal substrate subunits with C-terminal benzylamine and 3,4-dimethoxybenzylamine functions was done. Ten peptide mimetics with potential β-secretase inhibition activity were synthesized by peptide synthesis in solution according to Scheme 1–5: H-Asp-Leu-Ala-Val-NH-Bzl; H-Asp-Leu-β-Ala-Val-NH-Bzl; Boc-Leu-Asp-Leu-Ala-Val-NH-Bzl; Boc-Leu-Asp-Leu-

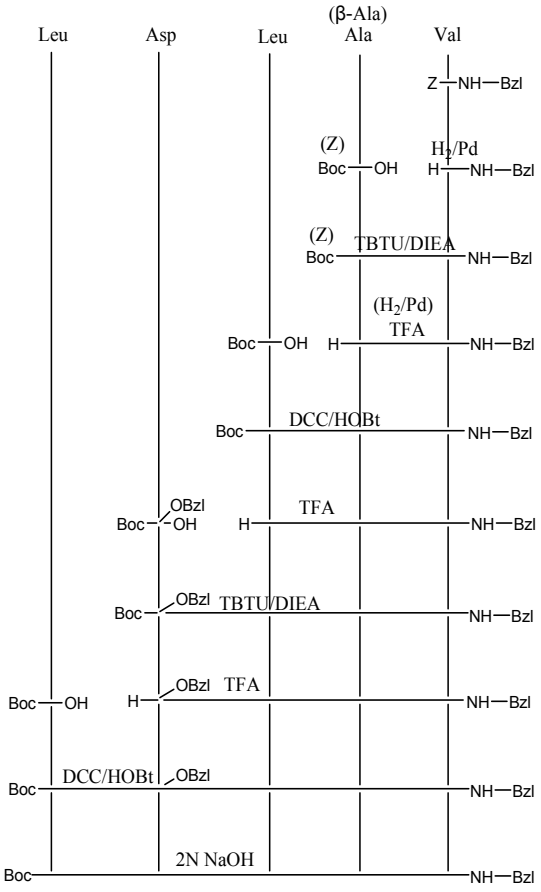
β-Ala-Val-NH-Bzl; H-Asp-Leu-Asp-Leu-Ala-Val-NH-Bzl; H-Asp-Leu-Asp-Leu-β-Ala-Val-NH-Bzl; Boc-Leu-Asp-Leu-Ala-Val-NH-3,4-dimethoxybenzyl; Boc-Leu-Asp-Leu-β-Ala-Val-NH-3,4-dimethoxybenzyl; H-Asp-Leu-Asp-Leu-Ala-Val-NH-3,4-dimethoxybenzyl; H-Asp-Leu-Asp-Leu-β-Ala-Val-NH-3,4-dimethoxybenzyl.

The first series of compounds include benzylamine moiety as C-terminal residue. Initially, a fragment condensation for all peptides was tried. Because of a lot of secondary products obtained during these reactions all newly compounds were synthesized by stepwise addition of amino acids starting from C- to N-terminus. The condensation reactions in the presence of variety of coupling reagent like TBTU, TCTU and DCC/1-HOBt were done. The best methods according to the purity and yields of the products were chosen. They are presented in the reaction schemes.




Scheme 1. Synthesis of tetrapeptides H-Asp-Leu-Ala(β-Ala)-Val-NH-Bzl.

The shortest needed fragments H-Asp-Leu-Ala-Val-NH-Bzl and H-Asp-Leu-β-Ala-Val-NH-Bzl were synthesized by stepwise attachment of Boc-Ala-OH or Z- β-Ala-OH, Boc-Leu-OH, Boc-Asp(OBzl)-OH and Boc-Leu-OH to the C-terminal H-Val-NH-Bzl residue according to Scheme 1. Most of the reactions ran with high yields and good purity. Some difficulties with Leu residue attachment were met. DCC/1-HOBt method finally was chosen as the most effective according to the yield of the target product (78%). Asp and Ala residues were subjected condensation in the presence of TBTU with quantitative yields.

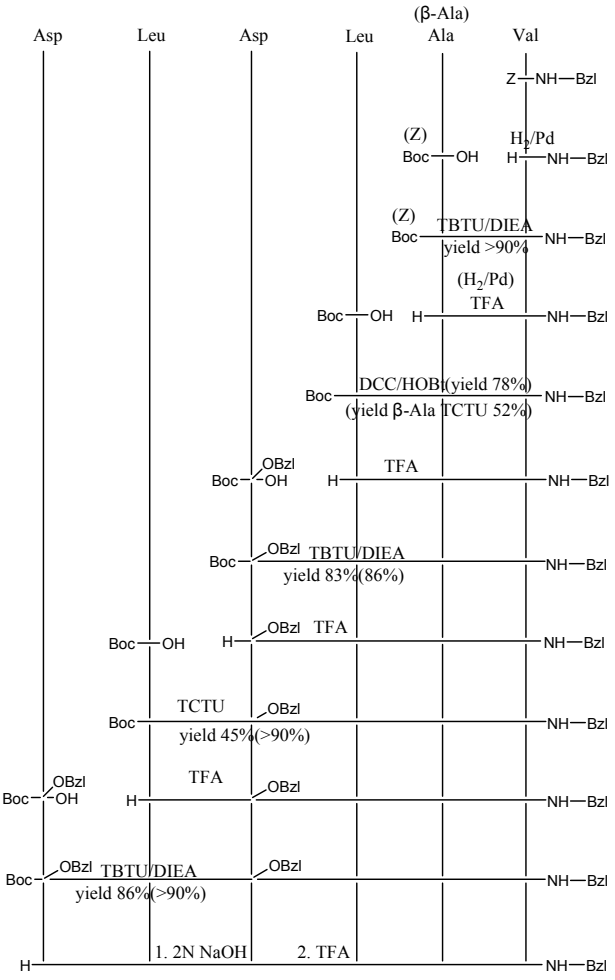



Scheme 2. Synthesis of pentapeptides Boc-Leu-Asp-Leu-Ala (β-Ala)-Val-NH-Bzl.

According to Scheme 2, N-terminal Boc-Leu-OH was linked to H-Asp-Leu-Ala-Val-NH-Bzl and H-Asp-Leu-β-Ala-Val-NH-Bzl obtained by Scheme 1. The same difficulties as with condensation of first Leu residue were met. The same methods were carried out and finally TCTU was chosen leading to quantitative yield of 96% for the reaction with H-Asp(OBzl)-Leu-Ala-Val-NH-Bzl (67% for the same reaction with DCC/1-HOBt method) and 45% for the reaction with H-Asp(OBzle)-Leu-β-Ala-Val-NH-Bzl (37% for the same reaction with DCC/1-HOBt method) and better purity of the target compounds.

The protected hexapeptides Boc-Asp(OBzl)-Leu-Asp(OBzl)-Leu-Ala-Val-NH-Bzl and Boc-Asp(OBzl)-Leu-Asp(OBzl)-Leu-β-Ala-Val-NH-Bzl were obtained by consecutive attachment of Asp residue to the products synthesized by Scheme 2 in quantitative yields (Scheme 3). The final products were obtained by deblocking the protecting groups by treatment with 2 N NaOH and TFA, subsequently.

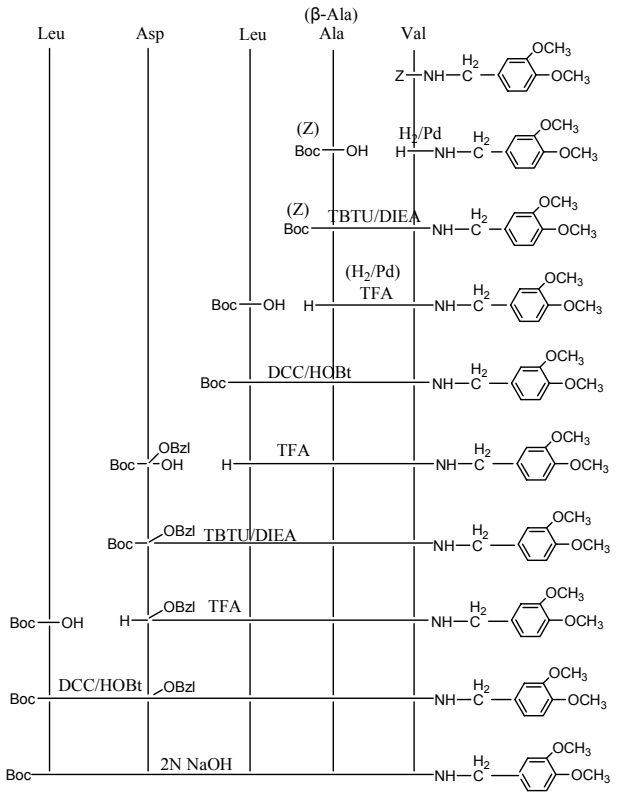
The second series of compounds include 3,4-dimethoxyphenylamine as C-terminal residue and were synthesized according to Schemes 4 and 5



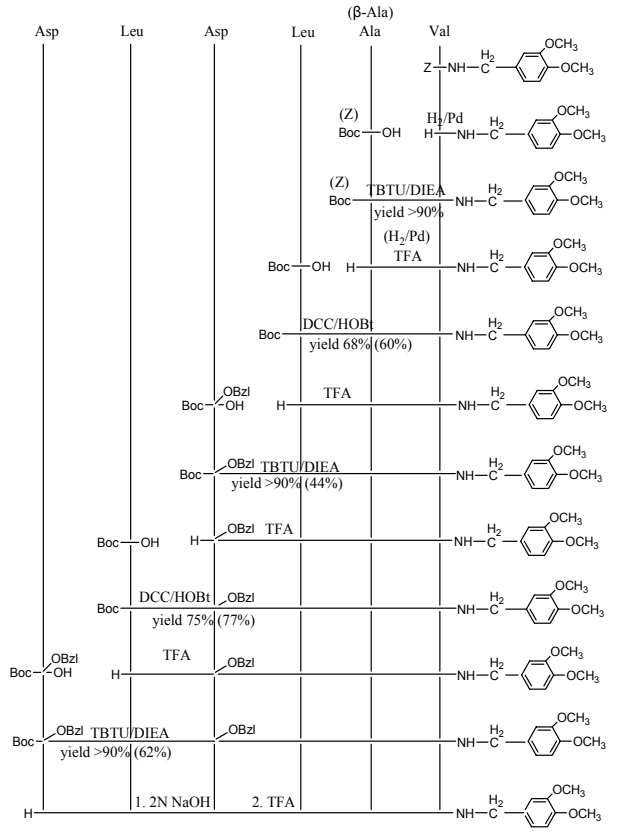

Scheme 3. Synthesis of hexapeptides H-Asp-Leu-Asp-Leu-Ala(β-Ala)-Val-NH-Bzl.

The same problems as with the first series of compounds, concerning the reactions of condensation of Leu residues were met. The reactions of condensation were carried out again at different conditions and in the presence of condensation agents with a view to get the best results with respect to yields and purity of products. Similar problems in this series of compounds were met with the reaction of condensation with participation of Boc-Asp(OBzl)-OH. The best results according to the condensation agent and yields are shown in the reaction schemes.

Boc-protecting group in all compounds was removed by treatment with 10-fold excess of TFA. Z- and –OBzl protecting groups were removed by catalytic hydrogenation in the presence of HCl (molar or catalytic amount) and Pd/C. The melting points and $[\alpha]_{546}^{22}$ are presented in Table 1:




Scheme 4. Synthesis of pentapeptides Boc-Leu-Asp-Leu-Ala(β-Ala)-Val-3,4-dimethoxybenzylamide.




Scheme 5. Synthesis of hexapeptides H-Asp-Leu-Asp-Leu-Ala(β-Ala)-Val-3,4-dimethoxybenzylamide.

**Table 1.** Melting points and $[\alpha]^{22}_{546}$ of the newly synthesized products.

| No | Product | M.p., °C | $[\alpha]^{22}_{546}$, ° |
|---|---|---|---|
| 1 | Z-Val-NH-Bzl | 167–169 | –40 |
| 2 | Boc-Ala-Val-NH-Bzl | 164–166 | –120 |
| 3 | Z-β-Ala-Val-NH-Bzl | 184–186 | - |
| 4 | Boc-Leu-Ala-Val-NH-Bzl | 139–141 | –130 |
| 5 | Boc-Leu-β-Ala-Val-NH-Bzl | 169–171 | –40 |
| 6 | Boc-Asp(OBzl)-Leu-Ala-Val-NH-Bzl | 121–123 | –0.2* |
| 7 | Boc-Asp(OBzl)-Leu-β-Ala-Val-NH-Bzl | 124–126 | +40 |
| 8 | Boc-Leu-Asp(OBzl)-Leu-Ala-Val-NH-Bzl | 197–199 | - |
| 9 | Boc-Leu-Asp(OBzl)-Leu-β-Ala-Val-NH-Bzl | 200–201 | - |
| 10 | Boc-Asp(OBzl)-Leu-Asp(OBzl)-Leu-Ala-Val-NH-Bzl | 193–195 | - |
| 11 | Boc-Asp(OBzl)-Leu-Asp(OBzl)-Leu-β-Ala-Val-NH-Bzl | 191–193 | –10 |
| 12 | Asp-Leu-Ala-Val-NH-Bzl | 119–121 | –50 |
| 13 | Asp-Leu-β-Ala-Val-NH-Bzl | 152–154 | +30 |
| 14 | Boc-Leu-Asp-Leu-Ala-Val-NH-Bzl | 125–127 | +30 |
| 15 | Boc-Leu-Asp-Leu-β-Ala-Val-NH-Bzl | 123–125 | +20 |
| 16 | Asp-Leu-Asp-Leu-Ala-Val-NH-Bzl | 117–119 | +40 |
| 17 | Asp-Leu-Asp-Leu-β-Ala-Val-NH-Bzl | 114–116 | +60 |
| 18 | Z-Val-3,4-dimethoxybenzylamide | 144–146 | - |
| 19 | Boc-Ala-Val-3,4-dimethoxybenzylamide | 151–153 | –90 |
| 20 | Z-β-Ala-Val-3,4-dimethoxybenzylamide | 187–189 | - |
| 21 | Boc-Leu-Ala-Val-3,4-dimethoxybenzylamide | 183–185 | –60 |
| 22 | Boc-Leu-β-Ala-Val-3,4-dimethoxybenzylamide | 169–171 | –20 |
| 23 | Boc-Asp(OBzl)-Leu-Ala-Val-3,4-dimethoxybenzylamide | 178–180 | –10 |
| 24 | Boc-Asp(OBzl)-Leu-β-Ala-Val-3,4-dimethoxybenzylamide | 191–193 | +36 |
| 25 | Boc-Leu-Asp-Leu-Ala-Val-3,4-dimethoxybenzylamide | 136–138 | - |
| 26 | Boc-Leu-Asp-Leu-β-Ala-Val-3,4-dimethoxybenzylamide | 210–212 | - |
| 27 | Asp-Leu-Asp-Leu-Ala-Val-3,4-dimethoxybenzylamide | amorphus | - |
| 28 | Asp-Leu-Asp-Leu-β-Ala-Val-3,4-dimethoxybenzylamide | 217–219 | - |

For all compounds $[\alpha]^{22}_{546}$ [°] is for C 1 MeOH except *C 0.5 MeOH.

Biological trials of all newly synthesized compounds are in progress.

***Acknowledgements**: This work is supported by the Ministry of Education and Science grant BY-16.*

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# НОВИ ПЕПТИДНИ МИМЕТИЗИ С ПОТЕНЦИАЛНА β-СЕКРЕТАЗНА ИНХИБИТОРНА АКТИВНОСТ

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Постъпила на 10 юли 2008 г.; Преработена на 25 септември 2008 г.

(Резюме)

Болестта на Алцхаймер (БА) е засегнала около 20 милиона души по целия свят. Последните проучвания показват, че през следващите 20–30 години броят им ще се удвои поради нарастващата продължителност на човешкия живот. Превръщането на разтворимия β амилоиден пептид (Aβ) в неговата неразтворима форма е ключов стадий в прогресията на БА. Aβ се получава като резултат от разкъсване на прекурсорния β амилоиден пептид, което е катализирано от различни протеази, известни като α-, β- и γ-секретази. Ето защо инхибирането на някой от тези ензими е обещаваща алтернатива при лечението на БА. Най-късата пептидна структура с установена инхибиторна активност срещу β-секретазата е Glu-Val-Asp-Leu-Ala. В литературата са публикувани множество изследвания, показващи ролята на различните аминокиселини в този пептид за инхибиторната му активност и са изведени някои зависимости структура–активност. Основавайки се на тези литературни данни ние направихме дизайн и синтезирахме 10 пептидни миметици с потенциална β-секретазна инхибираща активност, с помощта на стандартен пептиден синтез в разтвор. Всички новосинтезирани съединения бяха охарактеризирани чрез TLC, т.т. и $[\alpha]_{546}^{22}$. Биологичните изследвания са в ход.