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RESEARCH ARTICLE

Synthesis of piperidine-4-one Derivative Containing Dipeptide: An Acetyl cholinesterase and β-secretase Inhibitor

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Abstract: ***Background*:** With the goal of developing Alzheimer's disease therapeutics, we have designed and synthesized novel piperidone fused dipeptide (DPPS) derivatives possessing dual action such as acetylcholinesterase (AChE) and beta-amyloid peptide (Aβ) aggregation inhibition. Designed peptide was synthesized by solid phase peptide synthesis using FMOC chemistry protocol and characterized by mass spectroscopy.

***Methods*:** The amino acid sequence in peptide was analyzed by LC-MS-MS. *In silico* docking analysis was carried out using GLIDE software. The docking score using GLIDE was found to be -7.88 against AChE and -9.74 against BACE1 enzyme. *In vitro* enzyme inhibition assay was carried out for AChE enzyme and BACE1 enzyme.

***Results*:** The IC_{50} values of AChE inhibition and BACE1 of DPPS were found to be 0.4796 μM/ml and 0.0154 μM/ml, respectively. The correlation of *in silico* and *in vitro* results showed that DPPS possessed a greater ability to inhibit BACE1 enzyme.

Keywords: Alzheimers disease, peptides, DPPS, enzyme inhibition, AChE, BACE1.

1. INTRODUCTION

Alzheimer's Disease (AD) is the most common form of dementia which is a complex neurodegenerative disorder [1, 2]. Until now, most AD approved drugs are inhibitors of AChE which increase the ACh level by decreasing hydrolysis of ACh in the brain. Literature review indicates that in AD pathogenesis several pathways and targets are involved, but available drugs hit only a single target out of many available. Hence, these agents are therefore intrinsically inadequate to treat complex diseases such as AD, which have several pathogenic factors. However, the possibilities of side effects are increased proportionally with two or more drugs combination. It includes side effects due to drug-drug interactions (unpredicted) and side effects of an individual drug (known) [3]. Recently, a single drug possessing various pharmacological properties has been designed rationally based on the paradigm of the alternative therapeutic option. Particularly when compared to combination therapy, single multimodal drug therapy exhibits more benefits [4]. Administration of many single drug moieties differs in pharmacokinetics, metabolism, and bioavailability, which is prevented by this single multimodal drug therapy. The other advantage of this therapy is reduced drug-drug interactions (possible), greatly simplified therapeutic regimen and improved patient compliance. In AD, there are many stages of progression; hence evaluation of the relationship between specific molecular target and progression timeline is important when designing a new drug molecule [5].

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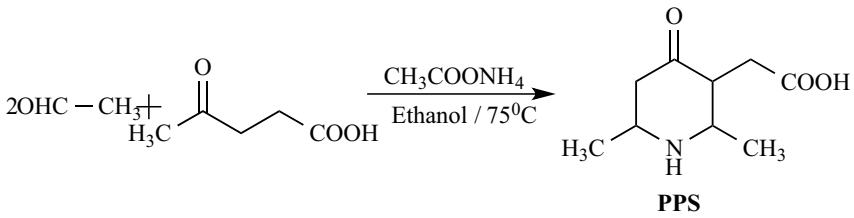


PPS

Scheme 1. Synthesis of PPS.

Peptides are reasonable alternatives to chemicals which are defined as molecules (linear) possessing of 2 or many (less than 100) residues of amino acids. Peptides are key biological function controllers with a high degree of specificity, pharmacological activity and low toxicity. The major disadvantage behind the development of peptide moieties is their short *in vivo* half-life as proteases degrade them rapidly, and their nature poses challenges for delivery and administration particularly in the brain. By using D-enantiomeric amino acid residues or many available methods for modifying peptides, the above problems can be partially solved [6-8]. A β-aggregation and its toxic effects are reduced by various small peptides and a fraction of them in AD rodent animal models has been found effective. β-binding peptides are also developed for *in vivo* imaging. AD is diagnosed early by using β-binding peptides [9].

The piperidone nuclei selected has anticholinesterase activity and acts as a pharmacophore like Donepezil. Since lysine is a cationic amino acid and can easily be connected to the hydrophilic portion, alanine is a hydrophobic amino acid that can be used to bind to the hydrophobic region. Ionic / charge interactions play an important role in age - related degenerative diseases such as hydrophobic and hydrophilic / amphiphile balancing of AD. Therefore, our work aimed at designing and synthesizing multi-targeted ligand molecule to inhibit the pathogenic enzymes involved in AD by means of solid phase peptide synthesis.

2. MATERIALS AND METHODS

2.1. Materials

All the chemicals were procured from Sigma Aldrich, Chennai. Using a Jasco FT-IR 410 spectrometer the IR spectra were recorded using KBr films or disks. Bruker Avance-500 NMR spectrometer was used to record NMR spectra in $CDCl_3$ using tetramethylsilane (TMS) as an internal standard. Using FAB positive (fast atom bombardment) mass spectra were recorded on a JEOL-SX-102 instrument.

2.2. Methods

2.2.1. Molecular Modeling Studies

The *in silico* modeling [10] of designed compounds were studied by GLIDE program (version 11, Schrodinger, LLC, New York, 2016) by extra precision (XP) mode. The Research Collaboratory for Structural Bioinformatics (RCSB) protein data bank was used to retrieve the X-ray crystal structures of acetyl cholinesterase (**PDB: 3LII**) and β-secretase (**PDB: 3U6A**).

2.2.2. Synthesis

2.2.2.1. Synthesis of piperidine-4-one derivative (PPS)

To a 0.2 mol of acetaldehyde in RBF, 0.1 mol of levulinic acid (13.27 ml) and 0.1 mol of ammonium acetate (7.7 gm) and ethanol (100 ml) was added. The mixture was refluxed for 50 min at 75 °C using a hot plate and kept at room temperature overnight. Recrystallization was done to obtain product (PPS) using ethanol. The synthetic route for the prepared piperidine-4-one (PPS) has been represented in Scheme **1**.

2.2.2.2. Synthesis of piperidine-4-one derivative containing dipeptide (DPPS)

Step I: Resin activation

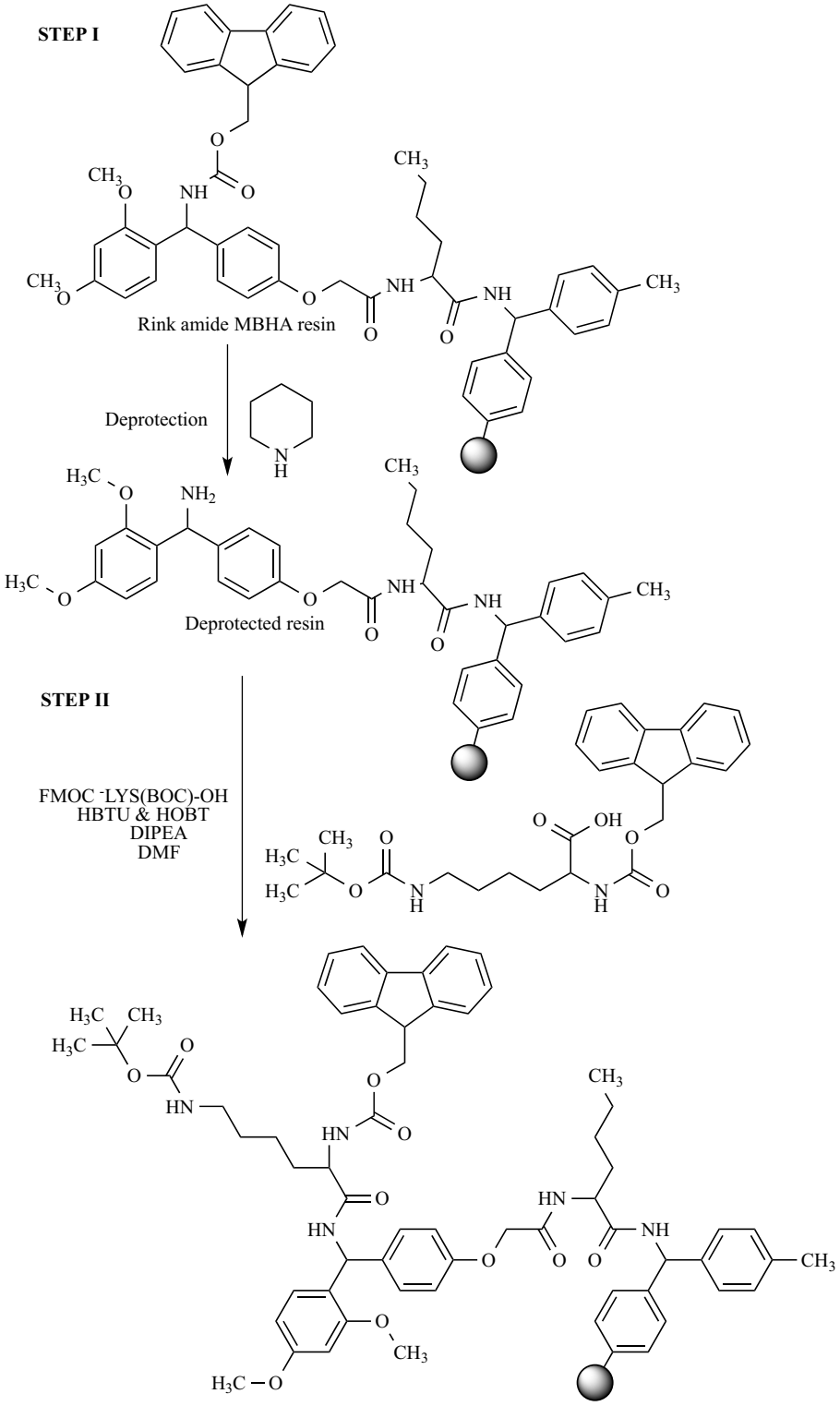
In dry dimethyl formamide (DMF), 250 mg of rink amide methyl benzhydryl amine (MBHA) resin was allowed to swell for about an hour. 10 ml 20% piperidine was added to the resin and swirled in DMF for 8 minutes. It was repeated once more to ensure that the FMOC protective group is completely removed. With an interval of 2 minutes six times, the resin was washed with 6 ml dry DMF before the first amino acid was attached to the resin.

Step II: First amino acid attachment

FMOC-Lysine in 1 mL of DMF was mixed with tetramethyl uranium hexa-fluorophosphate (HBTU) and hydroxybenzotriazole (HOBT). To obtain a clear solution in addition to the milky colloidal solution, di-isopropyl ethyl amine (DIPEA) was sonicated. The solution became of transparent yellow colour after one minute. Later, to the activated resin, the above contents were added and shaken continuously for 90 min. To remove the excess active ester with an interval of 2 minutes six times, the resin was washed with 6 ml dry DMF. In the ninhydrin test, the negative response indicates that the resin was 100% attached.

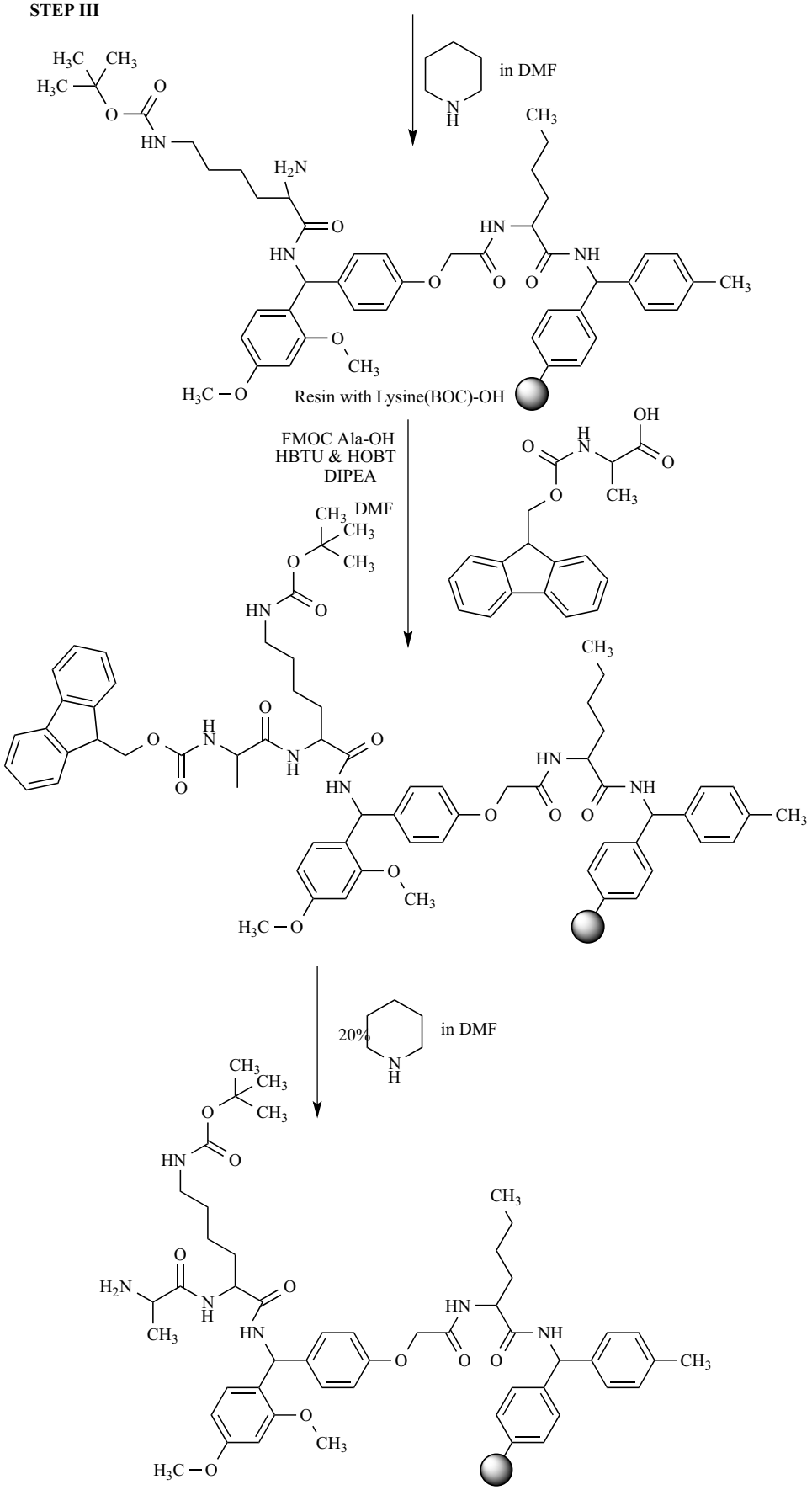
Step III: FMOC deprotection, coupling of remaining amino acid and piperidine-4-one derivative

20% Piperidine (approximately 10 ml) was added to the resin after washing with dry DMF, and shaken for 8 minutes. The procedure was repeated two times. Finally, with an interval of 2 minutes, the resin was washed six times with 6 ml dry DMF. In the ninhydrin test positive results indicated that FMOC was 100% separated from the resin. DPPS has been synthesized by manual peptide synthesizer using a solid phase peptide synthesis (SPPS).



Scheme 2. Contd…

STEP III



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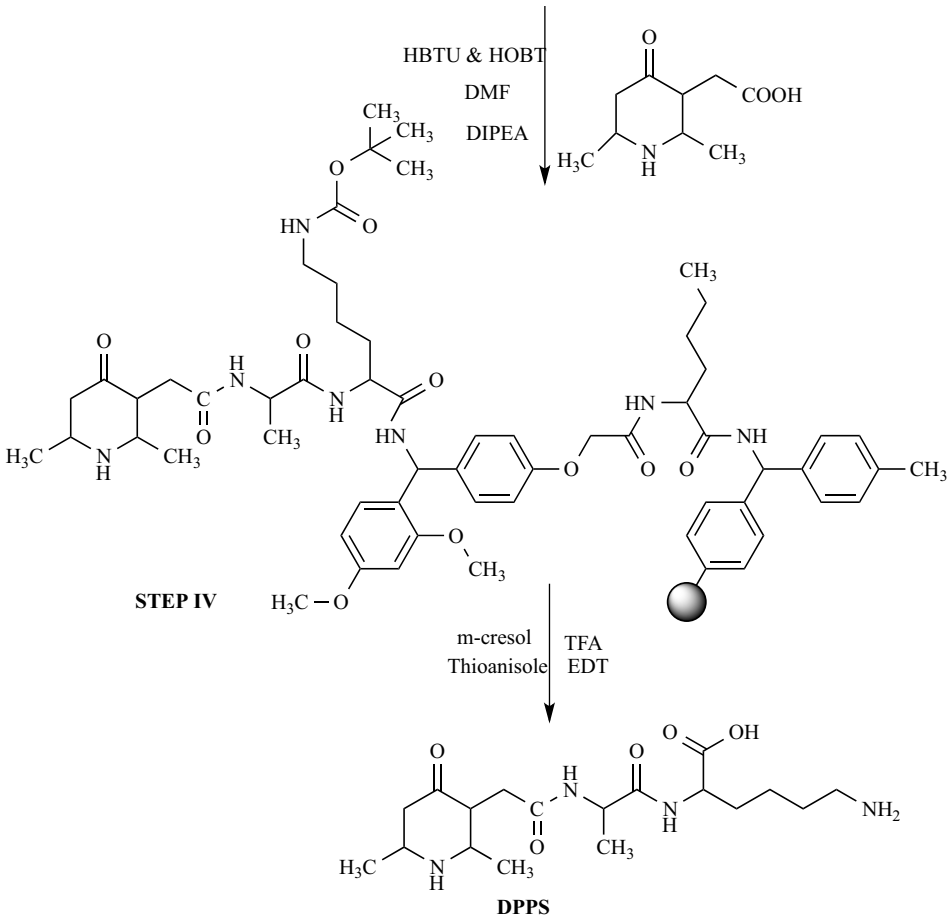

Scheme 2. Synthesis of DPPS.

Step IV: Cleavage of the Peptide DPPS from Resin

Peptide resin was washed successively with dichloromethane (10 ml) two times, followed by acetic acid twice (10 ml) and solvent ether twice (10 ml). Later, peptide bound resin was treated with a mixture of *m*-cresol (0.2 ml), ethane di-thiol (0.2 ml) and trifluoroacetic acid (7 ml). For 3 h the contents were shaken occasionally and filtered using sintered crystal. The obtained resin was then washed using trifluoroacetic acid. To remove the residual trifluoroacetic acid, the filtrate was concentrated under high vacuum. The slurry mass obtained was triturated and centrifuged with ice - cold ether, under the stream of nitrogen and the white precipitate of peptide obtained was dried and stored in the refrigerator.

Step V: Purification of the Peptide

The gummy layer was collected and added to ether, shaken well and kept in ice-cold condition. After 10 minutes precipitate containing ether was centrifuged for 15-20 min. Ether was evaporated, glacial acetic acid (5 ml) was added with shaking and kept in the ice-cold condition. After 15 min, ether was added to get the white precipitate. Ether was centrifuged containing the precipitate for 20 min and was evaporated to get the peptide. It was then stored in the refrigerator. The peptides were synthesized by SPPS using FMOC chemistry protocols [11-14] and the sequence PPS-A-K-$CONH_2$. The prepared DPPS structural representation has been given in Scheme **2**.

2.2.3. Biological Activity

2.2.3.1. In vitro Acetylcholine esterase (AChE) assay

AChE activity was evaluated based on Ellman's method. The principle involved in this method is that the enzyme hydrolyzes the substrate acetylthiocholine iodide (ATCI) into acetic acid and thiocholine, which was then allowed to react with 5,5'-dithiobis-(2-nitrobenzoic acid (DTNB) to form yellow colour. The activity of the enzyme is directly proportional to the intensity of colour. 0.1M sodium phosphate buffer (pH 8.0; 150 µl), test compound (10 µl), and enzyme solution (0.1 units/ml; 20 µl) were added and incubated for 15 minutes at 25 °C. After that, it was mixed with 10 µl of DTNB (10 mM) and 10 µl of ATCI (14 mM) to initiate the reaction, and incubated further for 10 min. The intensity of the colour complex formed was measured at 410 nm, using solvent as control. Finally, by using the following equation, the percentage inhibition for each test solution was calculated [15].

Inhibition (%) = (1- absorbance of sample / absorbance of control) X 100

2.2.3.2. Screening β-secretase Inhibitors using Purified Enzyme

Using FRET (fluorescence resonance energy transfer) the compounds were assayed for BACE1 inhibition at 0.01-100 µg/ml concentration. Based on APP (amyloid precursor protein) mutation in Sweden, this assay uses peptide substrates and BACE1 *baculovirus*. The mutation was improved dramatically

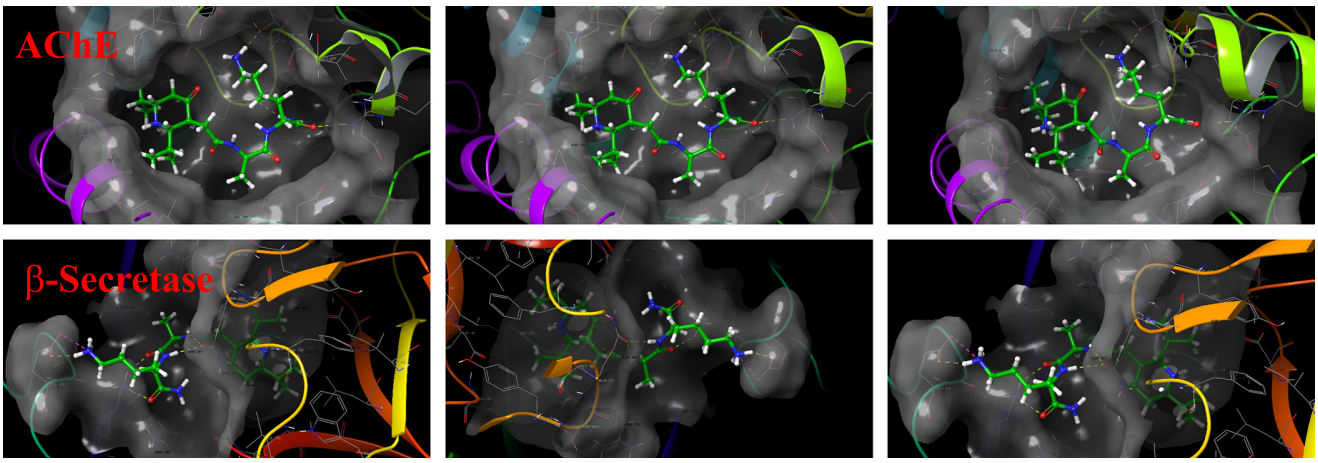

Fig. (1). (**a**) Ligand interaction of DPPS with AChE enzyme. (**b**) Ligand interaction of DPPS with β-Secretase enzyme.

due to cleavage of APP by BACE1 enzyme. Enzyme cleavage made the peptide substratum as highly fluorescent. In a dark condition at room temperature, the mixture of the test compound in buffer (10 µl), BACE1 substrate (Rh-EVNLDAEFK-quencher, 50 nM ammonium bicarbonate; 10 µl) and BACE1 enzyme (1.0 U/ml; 10 µl) were incubated for 1 h. The mixture was then added with 10 µl BACE1 stop buffer (2.5 M sodium acetate). Using 545 nm spectrofluorometer (TECAN) the fluorescence was measured at 585 nm emission. The percentage of BACE1 inhibition was measured using the below-mentioned equation.

BACE1 inhibition (%) = [1-test sample / positive control] x 100

3. RESULTS

3.1. Molecular Docking

Using the GLIDE docking software, docking analysis of ligands was performed with the target protein (β-secretase and acetyl cholinesterase) and the images of docking are presented in (Fig. **1**). (3D structures are presented in Fig. **1A** and **2D** structures are presented in Fig. **1B**). Docking score using GLIDE varied between -7.88 Kcal/mol against acetyl cholinesterase and -9.74 Kcal/mol against β-secretase. This proves that piperidine-4-one derivatives might be a potent drug for anti-Alzheimer drug development. The binding ability of ligands with specific protein receptor was semi-quantitatively conformed from the GLIDE score.

From the study, very good concurrence was found between the location of the inhibitors at the docking stage and the protein structures of acetyl cholinesterase and β-secretase. Conformational analysis of docked complex shows that the residues GLY 234, THR 238, HIS 405, ASN 233 and GLU 313 against acetyl cholinesterase and GLY 278, ASP 276, GLN 121, THR 120 and PRO 118 against β-secretase play a vital role in this receptor's activity.

3.2. Synthesis

3.2.1. Synthesis and Characterization of Novel Piperidine Analogue

By Mannich base condensation, 2,6-disubstituted piperidine-4-one derivative was synthesized. FT-IR, ^{1}H-NMR and Mass spectroscopy were used to characterize the synthesized compounds. The physico-chemical parameter of the synthesized compound is shown in Table **1**. A **s**atisfactory yield was obtained. The FT-IR, ^{1}H-NMR and Mass spectroscopy data of test compounds are shown in Tables **2** and **3**.

3.2.2. Synthesis, Characterization and Evaluation of Heterocyclic Dipeptides

The LC-MS-MS method confirmed the structural integrity and amino acid sequence of the peptides. The mass spectral data showed the chemical entity (DPPS) by the appearance of an M^{+1} peak at 384.1 (actual mass 383) with the molecular formula $C_{18}H_{35}N_5O_4$. The centroid and curve spectrum of DPPS is shown in the supplementary.

The amino acid sequence analysis of the DPPS compound was confirmed by the analytic peak called alanine and lysine in the 43300 and 418000 peak counts. The amino acid concentrations were found to be 0.111 µg/ml (A) and 0.452 µg/ml (K). The retention time of the compound DPPS was found to be 4.60 and 4.01 min. The blank and standard spectrum is shown in the supplementary table**.** The spectral data of blank, standard and dipeptides are shown in the supplementary table.

3.3. Biological Activity

3.3.1. In vitro AChE Inhibition of DPPS

The acetylcholinesterase assay was performed based on improved Ellman method using the Quanti Chrome test kit (USA) in a 96 well plate reader. One of the characteristic changes in AD is the increase in the activity of acetyl cholinesterase (AChE), the enzyme responsible for hydrolysis of acetylcholine from both cholinergic and non-cholinergic brain neurons. The results obtained from the DPPS against the inhibition activity of the AChE enzyme and the percentage inhibition were evaluated and tabulated in Table **4** and the graph is shown in Fig. (**2**). The plots were drawn between the percentage of AChE inhibition and various concentrations of DPPS (100, 10, 1 and 0.1 µM/ml).

3.3.2. In vitro BACE1 Inhibition of DPPS

A FRET (fluorescence resonance energy transfer) based BACE1 kit from Invitrogen (formerly Pan-Vera) was used to

Table 1. The physico-chemical parameters of the synthesized compound.

Compound Code	Molecular Formula	Molecular Weight	% of yield	Melting Point (°C)
PPS	$C_9H_{15}NO_3$	185	31.9	97-99

Table 2. The IR and MASS spectral data of 2, 6- disubstituted piperidine-4-one derivative.

Compound Code	Infrared Spectroscopy (cm^{-1})	Molecular Weight	M^++1 peak
PPS	3137.38 (-NH secondary amine), 2811.7 (CH_2 Methyl group), 1403..42 & 3017.50 (C=O &- OH carboxylic group), 1743.01(C=O cyclic ketone)	185	186.4 (M+1)

Table 3. The NMR spectra of 2, 6- disubstituted piperidine-4-one derivative.

Compound Code	^{1}H NMR
PPS	1.93 (s, NH), 1.03 (s, 6H,CH_3), 2.40 & 2.60 (d, carboxy CH_2), 2.50 & 2.62 (d, CH_2), 2.94 (s, CH), 3.03 (s, CH), 3.14 (s, CH), 10.28 (s, H, OH),

Table 4. *In vitro* AChE Inhibition of DPPS.

Compounds	% Inhibition				IC_{50}
Concentration of compound (μM/mL)	0.1	1	10	100	μM/mL
DPPS	15.32	57.66	69.37	91.89	0.4796
DONEPEZIL	90.65	93.07	95.17	96.19	0.0050

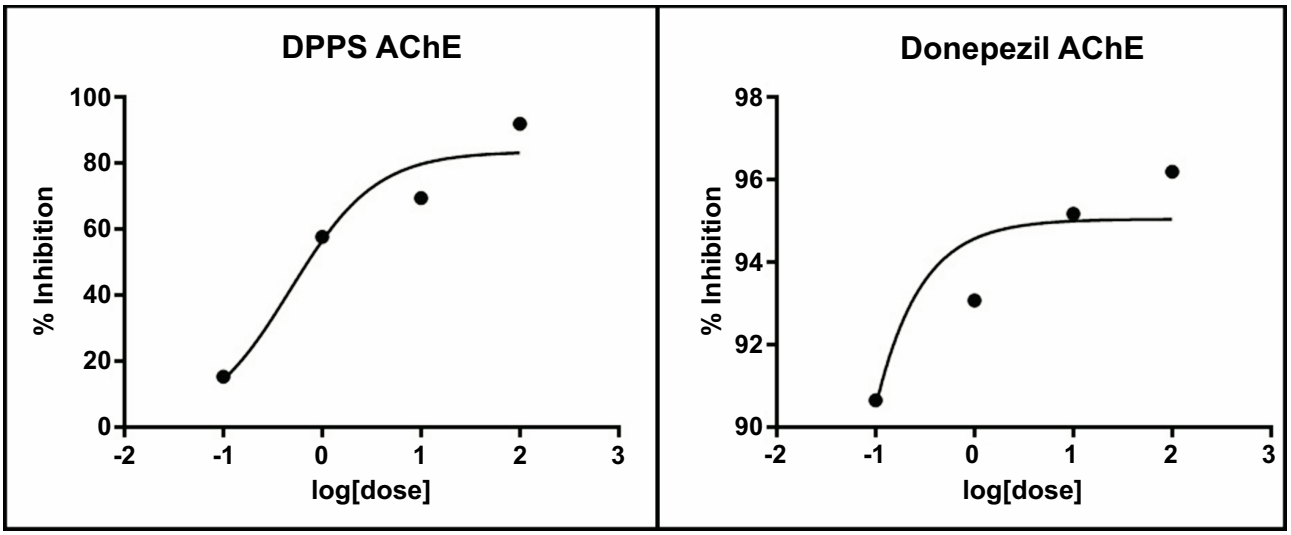

Fig. (2). *In vitro* AChE inhibition of DPPS.

evaluate the inhibitory activity of the synthesized DPPS derivatives. This kit uses the BACE1 enzyme expressed by purified *baculovirus* and a specific APP-based peptide substratum (Rh-EVNLDAEFK quencher). At different concentrations, the compounds were tested and IC_{50} values were calculated. Table **5** summarizes the results of *in vitro BACE1* inhibition of DPPS and the graphs are shown in Fig. (**3**). The plots were drawn between the percentage of BACE1 inhibition and various concentrations of DPPS (100, 10, 1 and 0.1 μM/ml).

4. DISCUSSION

Alzheimer's disease is a complex, multifactorial syndrome, unlikely to take place from a single causal factor; instead, a number of allied biological alterations are thought to put into its pathogenesis. Till today, developed drug paradigm was "one-molecule-one-target," that turned out to be palliative. Hence, drug combinations that can act at different levels of the neurotoxic cascade offer new avenues in the direction of curing Alzheimer's and other neurodegenerative

Table 5. ***In vitro*** **BACE inhibition of DPPS.**

Compound	% Inhibition				IC_{50}
Concentration of compound (μM/mL)	0.1	1	10	100	μM/mL
DPPS	89.93	91.45	98.54	99.20	0.0154

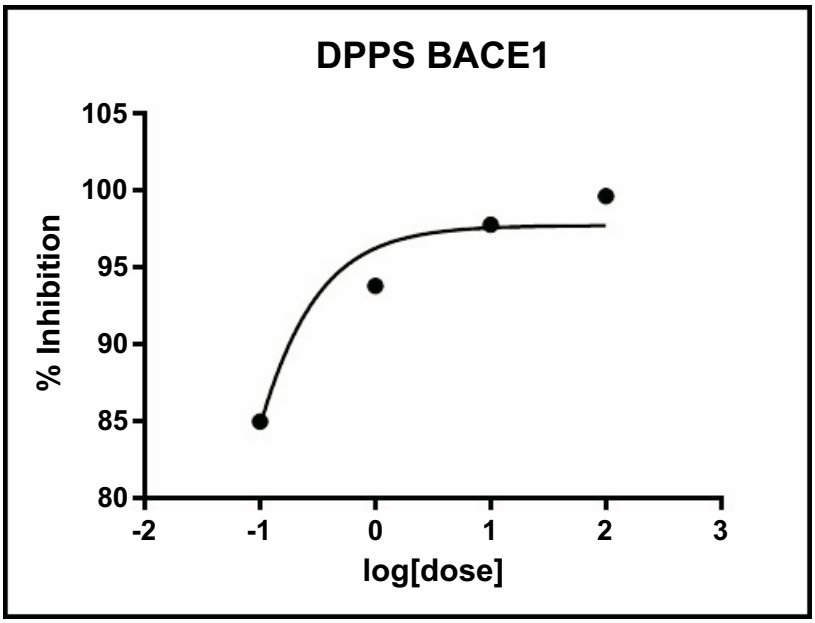

Fig. (3). *In vitro* BACE inhibition of DPPS.

diseases. This has led to a new paradigm in medicinal chemistry, the "multi-target–directed ligand" design strategy, which has previously been successfully exploited at both academic and industrial levels. Based on this, the designed drug was synthesized and characterized. Molecular docking studies revealed the interaction and binding affinity of designed drug molecule towards protein targets AChE and BACE1. It properly fits the active sites of protein targets. The chemical structure of the compound was confirmed by spectral data. In IR spectra, at 1743.01 cm^{-1} appearance of strong peak confirms the cyclic ketone. A peak at 3137.38 cm^{-1} and 699.03 cm^{-1} was an evidence of secondary amine of the compound. A peak at 2811.70 cm^{-1} confirms the presence of -CH_2 methyl group. Peaks at 1403.42 cm^{-1} and 3017.50 cm^{-1} confirm the formation of carboxylic C=O & –OH group as further evidence for the confirmation of compound. The ^{1}H-NMR spectra of secondary amine group of synthesized analog showed broad singlet signals at 1.93 ppm, which were assigned to the N–H protons. Carboxylic -OH proton of compounds exhibited a singlet at 10.28 ppm. A sharp singlet at 1.03 ppm assigned for the –CH_3 of side chain. Signals at 2.40 ppm & 2.60 ppm were assigned for methyl proton, adjacent to carboxylic acid group. Signals at 2.50 ppm & 2.62 ppm were assigned to methyl protons adjacent to carboxy keto group. Signals at 3.03 ppm, 3.14 ppm were assigned to methyl proton adjacent to secondary amine. The mass spectral analysis of the synthesized compound PPS was performed, and the mass spectrum of the compound was 186.4 (M^{+1}), in agreement with its molecular weight 185**.** The observed value of spectral data is evident from the formation of piperidine analogue. The heterocyclic dipeptide was designed by SPPS using FMOC chemistry protocol. The same was confirmed by the LC-MS-MS and analytic peak of alanine and lysine by amino acid sequence analysis.

Domination of acetylcholine esterase inhibitors has been observed in the treatment of Alzheimer's disease. By inhibiting acetylcholine turnover, it compensates the cholinergic neuron death and restores the synaptic levels of this neurotransmitter. Additionally, numerous studies have suggested AChE inhibition; causes cholinergic modulation and other functional consequences will affect amyloid precursor protein processing and protect neurons against a variety of insults [16]. IC_{50} values of AChE inhibition of DPPS were 0.4796 μM/ml and 0.0050 μM/ml for standard donepezil. However, these compounds are shown to be nowhere near as effective as the well-known control AChE inhibitor donepezil. According to β-amyloid and the amyloid hypothesis, proteins called secretases, are involved in cutting APP into beta-amyloid. Those that have received the most attention are beta-secretase (also known as BACE1) and gamma-secretase. Changing the behavior of these proteins could prevent or reduce beta-amyloid production. Hence, we tested the synthesized compound against BACE1 enzyme. (https://www.alz.org/national/documents/topicsheet_betaamyloid.pdf). IC_{50} value for DPPS was 0.0154 μM/ml. As there are no standard drugs available for BACE1 enzyme, we were not able to compare the score. DPPS showed inhibition at low concentrations (IC_{50} values: 0.4796 μM/ml for AChE and 0.0154 μM/ml for BACE1). Hence, DPPS may be a dual inhibiting molecule. Greater ability of DPPS to inhibit BACE1 enzyme was proved from the results.

CONCLUSION

In silico docking analysis was done for the framed peptide. Using GLIDE software, the docking score was found to be -7.88 against AChE and -9.74 against BACE1 enzyme. The AChE inhibitory activity of synthesized DPPS derivative was evaluated using Ellmans method and the IC_{50} value of DPPS was 0.4796 μM/ml. However, these compounds were not as effective as the well-known control AChE inhibitor donepezil (0.0050 μM/ml). The inhibitory activity of the synthesized DPPS derivative BACE1 was evaluated with fluorescence resonance transfer energy. The IC_{50} value for DPPS against BACE1 was 0.0154 μM/ml. *In silico* results correlates with the *in vitro* results. The result showed that DPPS possessed a greater ability to inhibit BACE1 enzyme and further it can be evaluated using *in vivo* models.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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