DR OM SILAKARI (Orcid ID : 0000-0002-1174-1125)

Article type : Research Article

Identification of 2-benzoxazolinone derivatives as lead against molecular targets of diabetic complications

Bhawna Vyas[a], Shalki Choudhary[b], Pankaj Kumar Singh[b], Baldev Singh[a], Renu Bahadur[c], Ashok Kumar Malik[a], Om Silakari[b*]

[a]Department of Chemistry, Punjabi University, Patiala, Punjab, India, 147002

[b]Molecular Modeling Lab, Department of Pharmaceutical Sciences and Drug Research, Punjabi University, Patiala, Punjab, India, 147002.

[c]Indian Council of Medical Research, New Delhi, 110029.

[*]E-mail: omsilakari@gmail.com

Telephone: +919501542696

Abstract

Diabetic complications follow multiple pathophysiological pathways involving aldose reductase (ALR2) mediated polyol pathway, advanced glycation end products (AGEs) and reactive oxygen species formation. Literature suggests ALR2 inhibitors such as Epalrestat to possess significant potential in retinopathy and neuropathy. Thus in this study multiple pathophysiology directed molecules targeting ALR2,AGEs and free radicals formation were designed using in-silico techniques. Initially, database was screened via in-silico tools to obtain hitswith affinity for the catalytic domain of ALR2. Additional focus was laid on the presence of structural attributes responsible for AGE's inhibitory and anti-oxidant potential. Out of obtained hits, 2-benzoxazolinone scaffold was selected and ten derivatives were synthesized accordingly. Finally, the synthesized molecules were evaluated for their ALR2 and AGEs inhibitory activities along with free radical scavenging potency.

Keywords: neuropathy; retinopathy; 2-benzoxazolinone; in-silico analysis

1. Introduction

Chronic diabetes is associated with persistent hyperglycaemia, which further leads to secondary complications. These diabetic complications (DC) are multi-factorial disorders postulated to cause various irreversible pathological conditions viz. macrovascular and microvascular complications (1). The microvascular complications such as retinopathy, neuropathy and nephropathy progress through different pathways such as polyol pathway, advanced glycation end product (AGEs) pathway, protein kinase c pathway (PKC) and hexosamine pathway, which are less active under normal condition (2, 3). Additionally in diabetic complications, oxidative stress increases the production of reactive oxygen species through mitochondrial electron transport chain and triggers all the above mentioned pathways through unifying mechanism(4).

In the last couple of decades, the management of diabetic complications has been tried via development of aldose reductase inhibitors (i.e. epalrestat, tolrestat), protein kinase Cβ inhibitors (i.e. ruboxistaurin) but none of them has received approval by US-FDA. The major road block in the management of diabetic complications is the identification of new chemical entities to target multiple pathological processes underpinning the pathogenesis of diabetic complications. In this article, we report molecular modelling based designing of some 2-benzoxazolinone derivatives as inhibitors of multiple molecular targets of diabetic complications. Based on the in-silico results, the designed molecules were synthesized and evaluated against molecular targetssuch as ALR2, AGEs and oxidative stress using in-vitro assays.

2. Experimental

2.1. Pharmacophore based virtual screening

Initially a previously published ligand based pharmacophore model was utilized to screen an in-house heterocycle database (5). Fit value of least “fit” active molecule was considered as a cut-off value to screen hits. Further obtained hits were filtered to obtain molecules having structural requirements for anti-AGE and anti-oxidant potential.The basic features required for AGEs inhibition include a hetero-aromatic nucleus with hydrogen bonding capability, one or more hydrophobic region and a protonable nitrogen atom which is connected to central core with alkyl linker (6).

2.2. Molecular docking and Dynamics Simulations

To perform molecular docking analysis, X-ray crystallographic structures of ALR2 (PDB ID: 1US0) was downloaded and prepared using automated Preparation Wizard in Schrodinger. The ligands were sketched using drawing tools and were optimized using Ligprep tool (pH 6.5-7.5). Docking analysis was carried out to identify the putative binding pose of the molecules within the active site of the proteins. Glide was used to perform the docking simulations, while Desmond software working on OPLS_2005 forcefield was used for molecular dynamics simulations. The protein complex was neutralized by adding Na+ ions and the system was then solvated in orthorhombic box of TIP3P water molecules having a salt concentration of 0.15M. A temperature of 310K was maintained using Nose-Hoover Chain thermostat and a pressure of 1.01 bar using Martyn-Tobias-Klein barostat(7, 8).

2.3. MMGBSA and ADME calculation

The ligand binding energies (kcal/mol) of final hits complexed with respective proteins were calculated using Prime MM/GBSA module employing an equation:

MMGBSA ΔGbind = ER: EL – EL – ER

Where ER: EL, EL and ER are the prime energies of the optimized complex, free ligand and free receptor, respectively (9).

Additionally, the ADME properties of all synthesized compounds were also calculated using QikProp module of Schrödinger software.

2.4. Chemical synthesis

All the organic/inorganic chemicals, solvents and reagents used for the synthesis were of analytical grade and purchased from Merck, Himedia, SD fine and Sigma Aldrich with >99% purity as certified by the manufacturer. Progress of the reaction was monitored at each step with thin layer chromatography using pet ether:ethyl acetate (8:2) as a solvent system. Meltingpoint of all compounds was recorded on Labtronics digital automatic melting point apparatus and was uncorrected. Finally purified compounds were characterized using IR, NMR and mass techniques. IR spectra were recorded using Bruker (Alpha E) FTIR spectrometer. Proton and carbon NMR spectra were recorded at 400MHz and 100MHz respectively on Bruker advance II NMR spectrometer using

$CDCl_3$ as a solvent and tetramethylsilane (TMS) as an internal standard. Mass spectra were recorded on waters Q-TOF micro MS spectrometer at ESI^+mode as m/z ratio.

2.4.1. General procedure for the synthesis of BV (1-10)

Protocol followed for the synthesis of all compounds is displayed in scheme 1. In first step, 4mmol of different benzyl chlorides (2) and 10 mmol of K_2CO_3 were added to a solution of 2 mmol of 2-benzoxazolinone (1) in acetone at room temperature. After completion of reaction, as monitored by TLC, the reaction mixture was filtered and concentrated to obtain crude products. Resulting products were then purified by column chromatography using pet ether:ethyl acetate (8:2) as solvent system.

2.5 *In vitro* antioxidant activity (DPPH assay)

1,1-diphenyl-2-picryl hydrazyl (DPPH) radical was used for the determination of free radical-scavenging activity of the synthesized compounds. DPPH free radical is reduced to the corresponding hydrazine by abstracting a hydrogen atom from antioxidant (10). Like other free radicals, it do not dimerize and give rise to deep violet color with an absorption in ethanol solution at around 520 nm. When mixed with antioxidants, DPPH gives rise to the reduced form (yellow color) by accepting hydrogen atom from the antioxidant (11).

The 0.1 mM solution of DPPH in methanol (39.4 mg in 1000 ml) was freshly prepared and covered with black paper. Different concentrations of test compounds were prepared in methanol and added with an equal volume to the methanolic solution of DPPH. After 30 min the absorbance was recorded at 517 nm. Ascorbic acid was used as a standard or positive control in the assay. EC_{50} values were calculated for standard and all test compounds by plotting concentration versus % inhibition graph. EC_{50} value is the concentration of antioxidants required to scavenge 50% of DPPH free radicals (12).

2.6. *In vitro* ALR2 inhibitory activity

The goat eyes were obtained from a local slaughter house just after slaughtering and stored in PBS pH 7 for transportation. Followed by extraction of enzyme aldose reductase from goat eye lens. The eye lenses from the freshly slaughtered goats were removedand washed with saline. 2.5 g of eye lenses were pooled and homogenized with 7.5 mL of 2-mercaptoethanol in cold water (1:3 W/V). Any insoluble material was removed from the homogenate by centrifugation at 10,000 rpm for 30 min (4°C) and obtained supernatant liquid was further saturated with 40% solution of ammonium sulfate to remove inert proteins from the mixture. The saturatedsolution was filtered and centrifuged again for 30 min at 4°C toobtain pure ALR2 enzyme, which was further used in the assay (13, 14).

2.6.1. Determination of enzymatic activity- Absorbance of a cuvette containing assay reaction mixture i.e. 1 mL of freshly extracted ALR2 enzyme, 1 mL of different concentrations of inhibitors in 1% DMSO and 1 mL of 0.067 M phosphate buffer (0.104mM NADPH, 10 mM glyceraldehydes and 0.4 M LiSO4) with final pH 6.2 was recorded againstnegative controlgroup containing all the components but no inhibitors using UV spectrophotometer at 340 nm for 3 min. The enzyme reaction is started upon the addition of a substrate (glyceraldehydes). The enzyme inhibitory activity was calculated using following formula:

$$\text{Activity U/ml} = \frac{\Delta A \text{ test per min} \times \text{total volume of assay mixture}}{(6.2\ \mu M, \text{extinction coefficient of NADPH at 340nm}) \times \text{volume of enzyme taken}}$$

Where, ΔA test/min is the rate of change of absorbance per minute of test compounds.

The percentage inhibition of all test compounds was calculated by considering 100% activity in negative control groupagainst ALR2. The 50% inhibitory concentration (IC_{50}) of each test compound was determined by plotting log concentration of test compounds versus percentage inhibition (15).

2.7. *In vitro*ALR1 inhibitory activity

Extraction and purification of ALR1 enzyme was performed by following standard procedure as reported in the literature (16). Freshly obtained rat kidney was kept in PBS solution, which was further homogenized by taking 3:1 ratio of 10mM sodium phosphate buffer (pH 7.2) having 2.5mM 2-mercaptoethanol 2.0mM EDTA, 0.25 M sucrose and rat kidney (1). The resulting homogenate was further centrifuged at 10,000 rpm for 20 min and supernatant solution was saturated with ammonium sulfate solution. Obtained precipitates were then filtered and filtrate was again centrifuged at 10,000 rpm for 20 minutes. Finally obtained supernatant was used as a source of ALR1 enzyme for the assay.

2.7.1. Determination of enzymatic activity

Absorbance of assay mixture (1ml of 50mM sodium phosphate buffer pH 7.2 containing 10 mM DL-glyceraldehyde, 0.1 mM NADPH, 0.2 M ammonium sulfate and 5mM β-mercaptoethanol + 1ml of enzyme supernatant + 1ml of different concentrations of inhibitors) was recorded spectrophotometrically at λ_{max} 340nm and 37°C temperatureat an interval of 1 minute for a period of 3 minutes. Reaction starts after the addition of substrate DL-glyceraldehyde and the ALR1 activity was monitored as a function of consumption of NADPH due to its oxidation via ALR1. Enzyme inhibitory activity and IC_{50} values werecalculated using the same formula as used in case of ALR2. Selectivity index of all compounds was calculated by taking the ratio of IC_{50} values of ALR1 and ALR2.

2.8. *In vitro* AGEs inhibitory activity

Glycation reaction between free amino group of biomolecules and carbonyl group of glucose leads to the production of advanced glycation end products (AGEs), which are implicated in diabetic complications(17). Amadori products obtained as a result of glycated protein were measured using bovine serum albumin (BSA)-glucose assay(18). In this assay, different concentrations of test compounds were prepared by dissolving in 1% DMSO. 100 µl of each test compound was incubated with 500 µl of Bovine serum albumin (BSA, 1mg/ml) and 400 µl of 500 mM glucose at 60°C for 24h. After the incubation period, 10 µl of 100% (w/v) trichloroacetic acid (TCA) was added to the reaction mixture in order to stop the reaction.Themixture was then keptat 4°C for 10 min and then centrifuged at 15,000 rpm at 4°C. Precipitates of glycated protein were redissolved in 1ml PBS (pH10) and immediately quantified in triplicate for glycated BSA on the basis of fluorescence

intensity measured on spectrofluorometer at 370 nm (excitation) and 440nm (emission). Fluorometer cuvette containing BSA, Glucose and different concentration of test compounds was read against negative control (BSA and glucose alone without any inhibitor) and aminoguanidine was used as a positive control (19). The percentage inhibition was calculated by using formula

$$\% \text{ Inhibition} = \frac{\text{Intensity of control - Intensity of test}}{\text{Intensity of control}} \times 100$$

IC_{50} of all test compounds and standard were calculated by plotting % inhibition versus log concentration curve.

3. Results and discussion

3.1. In-silico analysis

Previously reported Ligand-based pharmacophore models for ALR2(20), generated using Phase module of Schrödinger was employed to screen an *in-house* small molecule database. This screening yielded 2-benzoxazolinone derivatives as hits, which were then subjected to docking analysis using co-crystallized 3-D structure of ALR2 followed by molecular dynamic simulations and calculation of MM-GBSA score (binding energies). Molecular docking analysis in ALR2 showed that the carbonyl group in the core nucleus acts as an acceptor group and interacts via hydrogen bond with His110 in many of the designed compounds while the fused aromatic ring of the scaffold formed π-π interactions with various hinge region residues such as Trp111. All the compounds showed good docking scores (Glide XP G-score) ranging from -1.74 to -1.49 within the ALR2. Docking was followed with molecular dynamic simulations of the designed molecules in complex with ALR2, for a period of 10 ns. Simulations disclosed that almost all the molecules were maintaining key H-bond interactions with catalytic domain amino acids. The RMSD values of the protein and ligands in the complex with top compound (**BV-4)** were calculated to observe the overall stability of the complexfor the given period of time and the graphs for the same are represented in **fig.1**. The 3D interaction diagram of the designed molecules **BV-4** in ALR2 is shown in **Fig. 2**. Finally, binding energy scores (MM-GBSA scores) of the compounds lied in the range of -72.83 to -37.54 in ALR2 (Supplementary **table S2**).

Finally, ADME properties of synthesized compounds were calculated. Pharmacokinetic prediction resulted in the selection of ligands which are more likely to exhibit optimum ADME properties. Values of ADME properties for BV1-BV10 fall in the acceptable limits according to lipinski's rule of five (21) **(Supplementary table S3)**.

3.2. Chemistry

For the synthesis of molecules pertaining to **scheme 1** given in supplementary, in the step one, benzylation of 2-benzoxazolinone was performed by treating it with variedly substituted benzyl chlorides in the presence K_2CO_3 using acetone as solvent to yield different benzylated2-benzoxazolinone. The obtained compounds were purified via recrystallization. All the compounds were characterized by IR, ^{1}H-NMR and ^{13}C-NMR. In IR spectrum, the 2-benzoxazolinone derivatives showed the presence of strong absorption bands of C=O ~1750 and C=N from ~1610 to ~1620 cm^{-1}. The synthesis of final compounds was confirmed in ^{1}H-NMR. Almost each spectrum showed presence of singlet of two $-CH_2$ proton at ~5.0 ppm. Rest of the aromatic protons were observed in similar pattern from ~6.8 to ~ 7.8 ppm. ^{13}C spectrum exhibited characteristic peak at ~154 ppm for carbonyl and aromatic carbons were observed from 110 to 134 ppm with a characteristic peak at 55 ppm for $-CH_2$ carbon. Similarly, mass spectrometry also showed quasi ion peaks at expected *m/z* values.

3.3. *In vitro* antioxidant activity (DPPH assay)

Oxidative stress is the unifying mechanism which triggers all the pathways of diabetic complications. All the synthesized compounds **BV(1-10)** were screened for free radical scavenging activity using DPPH assay. Results in the form of EC_{50} values are summarized in **Table 1**. All compounds have shown activity in range of 180-620 nM in term of EC_{50}.The anti-radical power (ARP) of the standard ascorbate was found to be 0.126, the stoichiometry was found to be 0.398 and number of molecules of DPPH quenched was found to be 2.512.

3.4. *In vitro*ALR2 inhibitory activity:

The synthesized compounds were evaluated for their ALR2 inhibitory activity by considering negative control group (with no inhibitor) with 100% ALR2 activity. All experiments were performed in triplicate and results are summarized in **Table 1** in terms of IC_{50} values with standard error mean (SEM) at 0.05 level of significance.All compounds have shown promising ALR2 inhibitory activity which is in range of 1.53-9.77 µM. Out of all, compound BV-6 with fluoro substitution at 4^{th} position of benzyl ring and BV-4 with chloro substitution at 2^{nd} position of benzyl ring have shown best ALR2 inhibitory activity with IC_{50} value of 1.53±0.98 µM and 1.89±0.90 µM respectively.

3.5. *In vitro* ALR1 inhibitory activity:

ALR1 inhibitory activity was performed on freshly removed rat kidney.Valproic acid was used as a standard drug and was used as a positive control value (16, 22). The IC_{50} value and selectivity index of BV1-BV10 are displayed in **Table 1**. Our results clearly indicate that the compounds with nitro, chloro and methyl substitutions (BV2, BV4 and BV7) are less active on ALR1 when compared to their activity on ALR2. Furthermore, BV4 was found to be most selective ALR2 inhibitor with selectivity index 10.73.

3.6. In vitro advanced glycation end-product (AGEs) formation inhibitory activity:

In diabetic complications, AGEs formation leads to various pathological conditions. All the synthesized compounds **BV (1-10)** were evaluated for in vitro AGEs inhibitory activity and their corresponding results are shown in **Table 1**. Activity was found in range of 1.82-21.31 µM for all screened molecules. Compound BV-1 and BV-8 displayed best activity with IC_{50} values 2.157±1.44 and 1.82±1.14 respectively.

4. Conclusion

Present study is simply an in-silico guided effort to identify lead molecules having ALR2 inhibitory potential with additional ability to inhibit formation of AGEs and process of free radicals generation, which may be beneficial for diabetic complications. On the basis of this study it can be concluded that ten 2-benzoxazolinone derivatives, effective against ALR2, AGEs and oxidative stress were

identified. This scaffold can be further explored to develop molecules with better activity profile for the treatment of diabetic complications.

Acknowledgement

BV would like to thank ICMR for Research Associate Fellowship.

Conflict of interest

Declared none

Legends

Fig. 1 RMSD plot of BV-4 in complex with ALR-2 for the time period of 10 ns.

Fig. 2 Compound **BV-4** in the catalytic domain of ALR2 after the time period of 10 ns, i) 3D-view, ii) 2D-view.

Table 1 The Aldose reductase inhibitory, advanced glycation end products (AGEs) formation inhibitory and radical scavenging activities of synthesized compounds **(BV-1** to **BV-10)**.

Legends for supporting information

Scheme 1 General synthetic scheme for the synthesis of BV (1-10)

Table S1 All the synthesized molecules along with their physicochemical data

Table S2 MMGBSA score, predicted activity and fit value of BV1-BV10

Table S3 ADME properties of all synthesized compounds using Qikprop module of schrödinger

Fig. S1 ^{H}NMR spectra of BV-1

Fig. S2 C^{13} NMR spectra of BV-1

Fig. S3 Mass spectra of BV-1

Fig. S4 ^{H}NMR spectra of BV-2

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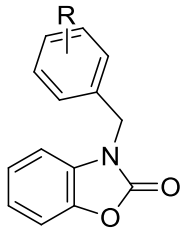
Table 1: The Aldose reductase inhibitory, advanced glycation end products (AGEs) formation inhibitory and radical scavenging activities ofsynthesizedcompounds **(BV-1** to **BV-10)**.



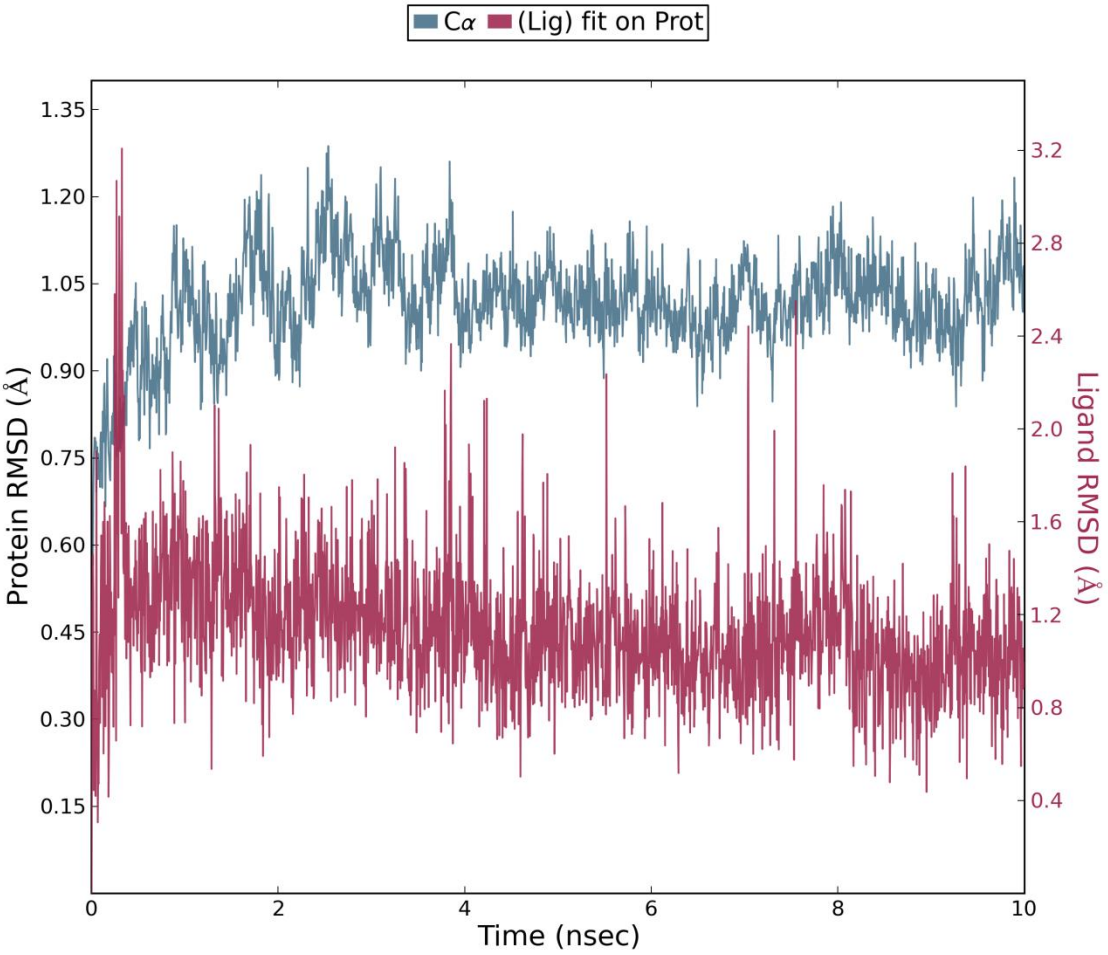
C. No.	Substituent (R)	ALR2 inhibitory activity (IC_{50}[a]±SD[a], µM)	ALR1 inhibitory activity (IC_{50}[a]±SD[a], µM)	Selectivity index[b]	AGEs inhibitory activity (IC_{50}[c]± SD[a], µM)	Radical scavenging activity (EC_{50}[d]± SD[a], nM)
BV-1	4-NO_2	2.46±0.67	9.58±1.27	3.89	2.157±1.44	254.49±0.60
BV-2	3-NO_2	3.16±0.71	15.86±0.77	5.01	11.76±3.28	180.93±1.17
BV-3	2-NO_2	5.84±0.64	14.19±1.12	2.42	5.06±1.52	246.08±1.13
BV-4	2-Cl	1.89±0.90	20.28±5.4	10.73	8.64±1.20	213.38±2.73
BV-5	3-Cl	6.73±1.49	14.69±1.59	2.18	21.31±1.89	387.63±1.30
BV-6	4-F	1.53±0.98	9.17±2.46	5.99	12.24±1.51	453.09±6.18
BV-7	4-CH_3	7.44±1.07	15.82±1.67	2.12	2.5±1.21	458.68±14.63
BV-8	4-$(CH3)_2CH$	7.45±0.24	9.04±0.95	1.21	1.82±1.14	267.61±6.67
BV-9	4-$(CH3)_3C$	9.77±0.53	10.67±1.92	1.09	8.544±1.75	409.95±5.09
BV-10	3-CF_3	7.15±1.15	11.64±2.95	1.62	3.299±0.97	620.62±10.60
Epalrestat	-	0.8±1.49			-	-
Valproic acid	-	-	56.1±2.7	-	-	-
Aminoguanidine	-	-	-	-	0.45±1.71	-
Ascorbic acid	-	-	-	-	-	199.4±0.466

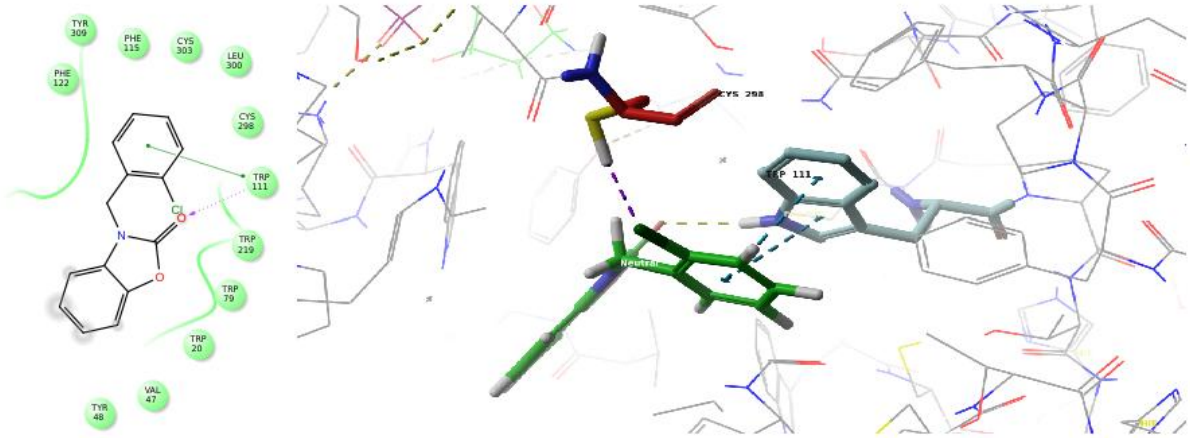
[a] SD: Data are expressed as mean ± SD (n=3). Data were statistically analyzed by one way ANOVA; *P<0.05 vs Std.

[b] Defined as IC_{50} [ALR1]:IC_{50} [ALR2].

[c] IC_{50} was defined as the concentration resulting in 50% inhibition of AGEs product formation.

[d] EC_{50} was defined as the effective concentration resulting in 50% scavenging activity.





Scheme 1 General synthetic scheme for the synthesis of BV (1-10)