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Amino acid-based enantiomerically pure 3-substituted 1,4-benzodiazepin-2-ones: A new class of anti-ischemic agents☆

Jitendra Kumar Mishra,[a] Puja Garg,[b] Preeti Dohare,[b] Ashutosh Kumar,[c] Mohammad Imran Siddiqi,[c] Madhur Ray[b] and Gautam Panda[a,*]

[a]*Medicinal and Process Chemistry Division, Central Drug Research Institute, Lucknow 226001, UP, India*
[b]*Pharmacology Division, Central Drug Research Institute, Lucknow 226001, UP, India*
[c]*Molecular and Structural Biology Division, Central Drug Research Institute, Lucknow 226001, UP, India*

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Abstract—A series of 3-substituted 1, 4-benzodiazepin-2-ones derived from *S* and *R* amino acids were evaluated for their anti-ischemic activity in vitro. Treatment with compounds **7h**, **16**, **9d**, and **17** decreased the apoptotic neuronal number, however increased the neuronal viability. The compounds decreasing apoptosis could protect neurons from the ischemic injury. The difference in the activities of 1,4-benzodiazepin-2-ones derived from *S*- and *R*-amino acids is discussed and explained on the basis of molecular modeling studies.

Brain injury by transient or permanent ischemia afflicts a large number of patients with death or permanent disability.[1] With the onset of ischemia, critical balance between the demand and supply of oxygen and substrates fails, leading to the damage of reversible or irreversible cellular interdependent pathways and it can be managed by timely protection offered by the drugs.

Benzodiazepine derivatives are of considerable interest because of their wide range of biological activities such as anticonvulsant,[2] cholecystokinin receptor A and receptor B antagonists,[3] platelet-activating factor antagonists,[4] GPIIb/IIIa inhibitors,[5] and Ras farnesyltransferase inhibitors.[6] Another well-known member of benzodiazepine family is pyrrolo[2,1-*c*][1,4]benzodiazepines (PBDs), known for their potential as antitumor agents, gene regulators, and DNA probes.[7] Although diverse biological activities of 1,4-benzodiazepines are known, to the best of our knowledge there is no report on anti-ischemic efficacy of 3-substituted 1,4-benzodiazepin-2-ones (Fig. 1).

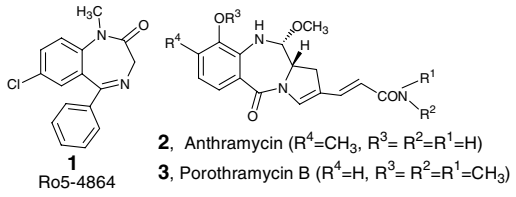

Figure 1. Biologically active 1, 4-benzodiazepine derivatives.

Toward the objective of finding new anti-ischemic agents, we have synthesized a series of *S* and *R*-amino acid-based enantiomerically pure 3-substituted 1,4-benzodiazepin-2-ones[8] (Schemes 1 and 2) and screened for anti-ischemic activity in vitro following the literature methods.[9–12] Compounds **7a**–**h**, **14**, **15**, and **16** derived from *S*-amino acids were evaluated for apoptotic/necrotic quantification of neuronal population in vitro (Fig. 2 and Table 1). Control neurons (LL) showed no FITC/PI-positive staining (0.05%/0.6%). The number of FITC-positive neurons (LR) was increased to 30.22% in ischemic neurons. Compounds **7h** and **16** derived from *S*-methionine and 4-hydroxy-*S*-proline decreased the number of FITC-positive neurons to 9.08% and 3.79%, respectively. The number of viable neurons was also increased by the treatment of compounds. In the ischemic group without treatment,

Keywords: Anti-ischemic agents; 1,4-Benzodiazepin-2-ones; Amino acids.
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* Corresponding author. Tel.: +915222364635; fax: +915222623938; e-mail: gautam.panda@gmail.com

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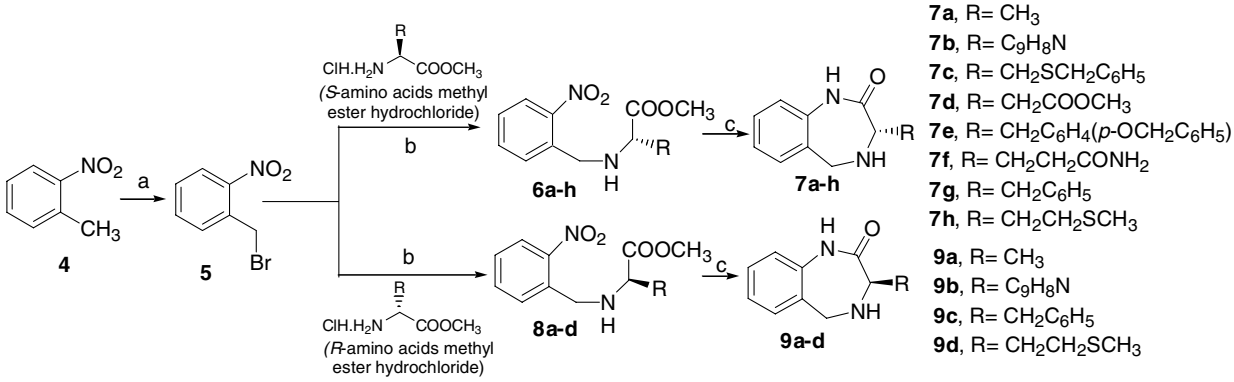
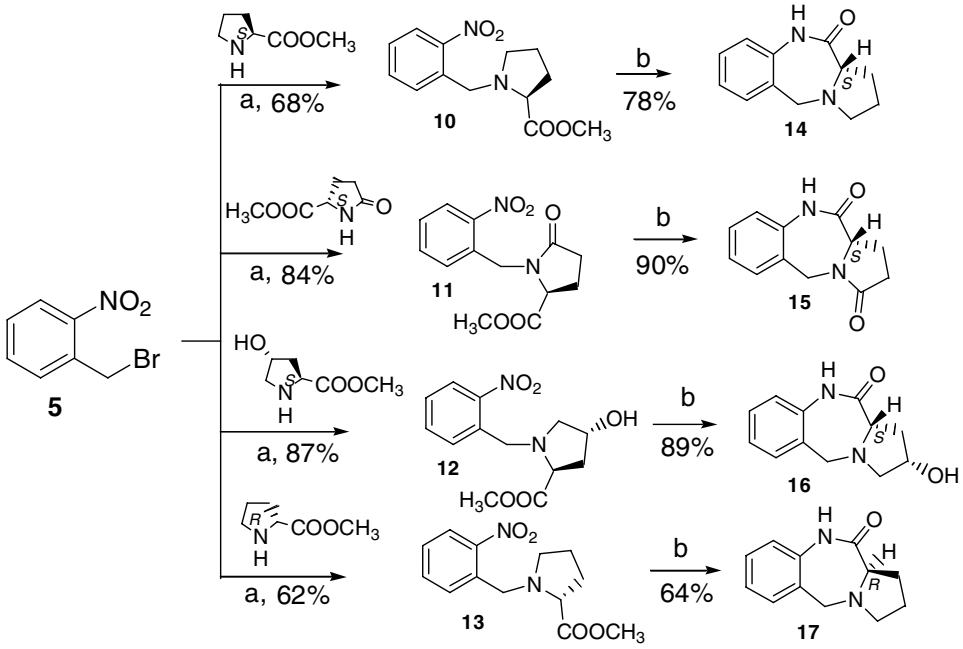


Scheme 1. Syntheses of 3-substituted 1,4-benzodiazepin-2-ones derived from *S*- and *R*-amino acids. Reagents and conditions: (a) NBS, $(C_6H_5CO)_2O_2$, CCl_4, 80 °C, 69%; (b) DIPEA, DMF, rt, 2 h, 62–78%; (c) Fe, AcOH, 110 °C, 30 min, 52–85%.



Scheme 2. Reagents and conditions: (a) DIPEA, DMF, rt, 2 h; (b) Fe, AcOH, reflux, 30 min.

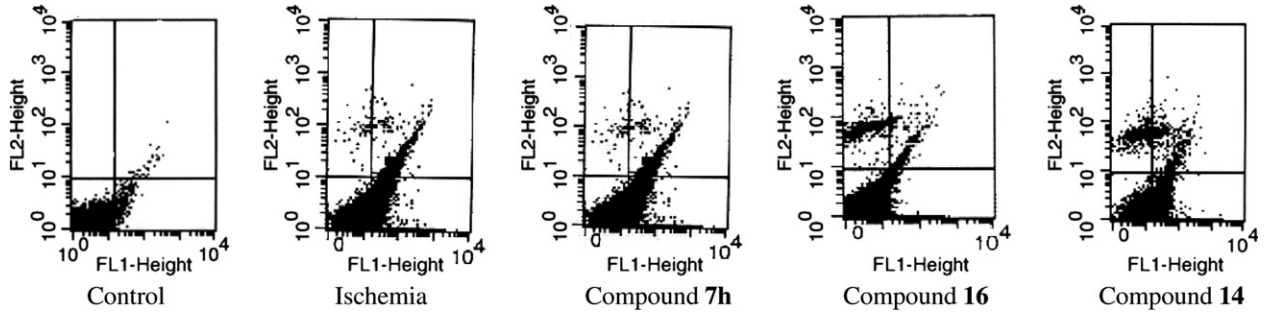
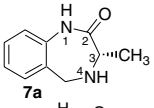
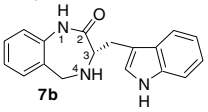
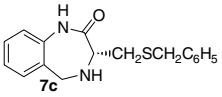
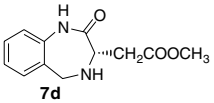
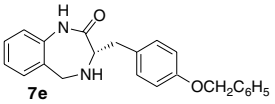
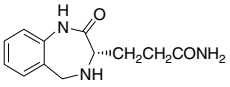
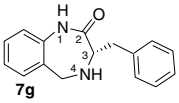
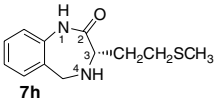
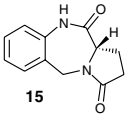
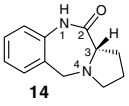
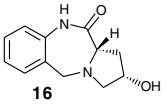


Figure 2. Representative dot plots of Flow cytometric profile observed in **7h**, **16** and **14**. The staining of cells simultaneously with FITC-Annexin V (green fluorescence) and the nonvital dye propidium iodide (red fluorescence), that is, bivariate analysis allows the discrimination of intact cells. The lower left quadrant (LL) represents viable cells (FITC−PI−) whereas lower right quadrant (LR) is for early apoptotic (FITC+PI−). On the other hand, the upper right quadrant (UR) is for late apoptotic (FITC+PI+) and the upper left quadrant (UL) is for necrotic cells (FITC−PI+). The dot plots represent two measurement parameters on the *x*- and *y*-axes. Cell count height and the particle counts are shown by density gradient and dot density, respectively. Dot plot displays FL1-FITC on the *x*-axis and FL2-PI on the *y*-axis. Logarithmic amplification is used to measure fluorescence in cells as this expands the scale for weak signals and compresses the scale for strong or specific fluorescence signals.

Table 1. In vitro assay of 1,4-benzodiazepin-2-ones derived from *S*-amino acids for apoptotic/necrotic quantification of neuronal population

	UL (necrosis)	UR (necrosis + delayed apoptosis)	LL (normal viable)	LR (apoptosis)
Control	0.88 ± 0.4	2.34 ± 0.8	91.31 ± 3.8	6.47 ± 0.9
Ischemia	1.57 ± 0.2	7.82 ± 1.3	60.39 ± 2.9	30.22 ± 1.5
 **7a**	5.35 ± 0.6	4.8 ± 0.9	60.38 ± 3.1	29.47 ± 2.1
 **7b**	4.33 ± 1.2	3.55 ± 1.1	61.26 ± 4.3	30.86 ± 0.9
 **7c**	5.05 ± 0.8	4.58 ± 1.3	64.36 ± 5.2	26.01 ± 1.7
 **7d**	4.73 ± 0.5	4.28 ± 0.8	65.24 ± 5.6	25.75 ± 1.8
 **7e**	4.99 ± 0.5	4.83 ± 1.2	66.55 ± 5.7	23.63 ± 1.4
 **7f**	4.75 ± 0.3	3.12 ± 0.7	67.2 ± 3.7	24.93 ± 2.1
 **7g**	2.07 ± 0.3	1.21 ± 1.3	70.14 ± 4.6	25.58 ± 1.1
 **7h**	4.73 ± 0.7	3.5 ± 0.8	82.69 ± 6.3	9.08 ± 0.4
 **15**	5.56 ± 0.6	3.75 ± 0.8	66.07 ± 4.8	24.62 ± 1.1
 **14**	5.51 ± 0.4	5.04 ± 1.4	66.18 ± 5.1	23.27 ± 1.2
 **16**	5.68 ± 0.8	3.08 ± 1.0	87.45 ± 6.7	3.79 ± 0.3

UL, upper left quadrant; UR, upper right quadrant; LL, lower left quadrant; LR, lower right quadrant.

neuronal viability was 61.11% (cells falling in lower left quadrant). Among all the compounds tested **7h** and **16** showed increase in neuronal viability to 82.69 and 87.45%, respectively. The 1,4-benzodiazepin-2-ones derived from *S*-alanine, *S*-aspartic acid, *S*-glutamine, and *S*-methionine showed 60.38%, 65.24%, 67.20% and 82.69% neuronal viability, respectively. On the other hand, 1,4-benzodiazepin-2-ones derived from *S*-amino acids containing aromatic side chains such as tryptophan, cysteine (S-Bn), tyrosine (–OBn), and phenylalanine showed 61.26%, 64.36%, 66.55%, and 70.14% neuronal viability, respectively.

To test the difference in the activities of enantiomers, we synthesized 1,4-benzodiazepin-2-ones derived from *R*-amino acids following the method reported by our group (Schemes 1 and 2).[8] In this context **9a**, **9b**, **9c**, **9d** and **17** were evaluated for apoptotic/necrotic quantification of neuronal population in vitro (Fig. 3 and Table 2). Careful analysis of Tables 1 and 2 suggests that

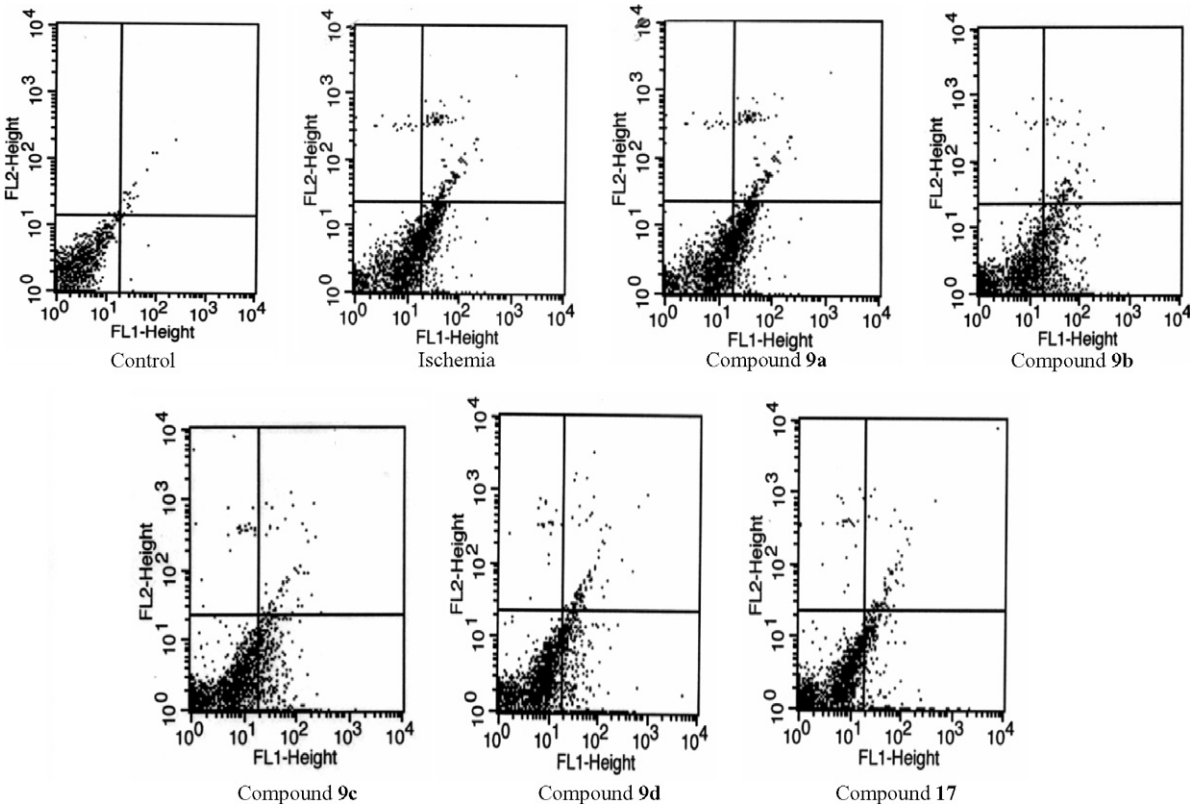
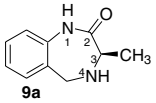
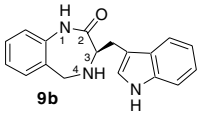
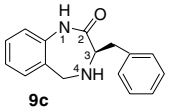
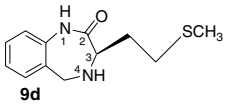
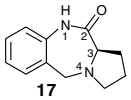


Figure 3. Representative dot plots of flow cytometric profile observed in **9a**, **9b**, **9c**, **9d**, and **17**.

Table 2. In vitro assay of 1,4-benzodiazepin-2-ones **9a**, **9b**, **9c**, **9d**, and **17** derived from *R*-amino acids for apoptotic/necrotic quantification of neuronal population

	Yield, $[\alpha]_D^{20}$	UL (necrosis)	UR (necrosis + delayed apoptosis)	LL (normal viable)	LR (apoptosis)
Control		1.21 ± 0.7	2.8 ± 1.3	93.34 ± 2.3	2.6 ± 0.2
Ischemia		0.745 ± 0.3	6.6 ± 0.3	65.89 ± 2.79	26.76 ± 2.1
 **9a**	52%, −15° (*c* 1, CH_2Cl_2)	0.98 ± 0.14	3.2 ± 0.4	68.96 ± 2.42	26.85 ± 1.88
 **9b**	72%, −42° (*c* 1, CH_2Cl_2)	0.5 ± 0.2	3.47 ± 0.33	74.97 ± 2.04	21.06 ± 2.1
 **9c**	85%, +23° (*c* 1, CH_2Cl_2)	0.68 ± 0.41	0.68 ± 0.41	79.01 ± 0.58	17.26 ± 0.13
 **9d**	63%, −12° (*c* 1, CH_2Cl_2)	0.52 ± 0.29	3.98 ± 0.39	77.85 ± 2.29	16.96 ± 0.94
 **17**	64%, −21° (*c* 1, CH_2Cl_2)	0.64 ± 0.36	3.10 ± 0.34	84.53 ± 0.80	11.72 ± 0.79

S-methionine-derived 1,4-benzodiazepine-2-one **7h** is more active than its *R*-isomer **9d**, whereas in the case of proline *R*-isomer **17** is more active as compared to its *S*-isomer **14**. To gain insight into the difference in activities of *R*- and *S*-isomers, docking studies were carried out with ligand binding core of AMPA receptor

with the help of modern docking engine LigandFit available with Cerius 2 4.10.[13]

A number of benzodiazepines act as negative modulators of AMPA receptor[14] and inhibit AMPA-induced current in a noncompetitive fashion.[15] The negative allosteric modulators regulate the frequency and amplitude of the excitatory neurotransmitters independent of the concentration of excitatory amino acids or polarization state of the synaptic membrane. The negative modulators of AMPA receptor bind at the same site as the positive modulators of AMPA, so we have taken the crystal structure of ligand binding core of AMPA receptor in complex with noncompetitive positive modulator, aniracetum (PDB entry 2AL5, resolution: 1.65 Å), as the crystal structure in complex with noncompetitive negative modulator was not available. Reference protein coordinates for docking studies were taken from Protein DataBank (PDB). Charges were assigned to the protein molecule and the docked ligands using the Cff1.02 force field.[16]

The binding site of modulators is located in a solvent-filled crevice, at the dimer interface and it is proximal to the transdomain-strands that undergo conformational changes during agonist binding and domain closure.[17]

Strikingly, a key element of the modulator-binding site involves a U-shaped crevice that is formed by main-chain residues Pro 494 through Ser 497. Pro 494 forms the apex of the crevice, whereas Ser 497 and 729 define its base.

A qualitative analysis of the binding mode found in the docked benzodiazepin-2-ones-AMPA receptor complex revealed that both the enantiomers bind in different con-

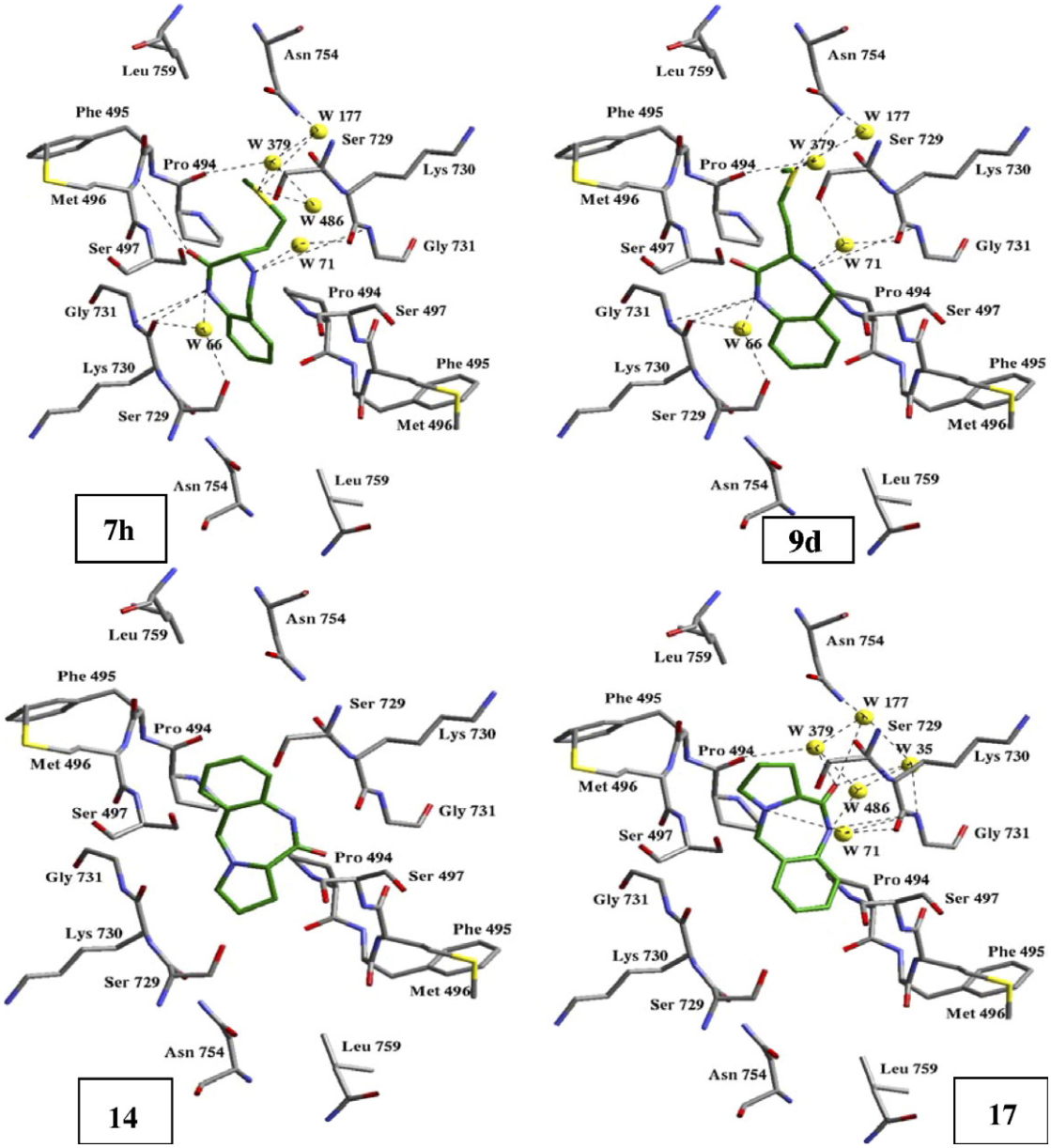


Figure 4. Docked conformations of 1,4-benzodiazepine-2-ones (green) **7h**, **9d**, **14**, and **17** with ligand binding core of AMPA receptor noncompetitive antagonist binding site.

Table 3. Docking scores using different scoring functions

Scores	Compounds			
	7h	**9d**	**14**	**17**
Dock_Score	43.883	41.213	13.173	51.118
LigScore1	1.990	3.420	2.290	3.920
LigScore2	1.770	4.830	2.760	4.710
−PLP1	74.660	83.690	59.270	72.760
−PLP2	74.520	79.480	60.410	72.180
JAIN	1.690	1.610	3.200	1.280
PMF	−22.870	−30.960	−61.480	−33.250
LUDI	385	428	461	491
Cons_Score	11	12	6	12

formation and it may be responsible for the difference in the activities of 1,4-benzodiazepin-2-ones. The nitrogen atom N4 of **17** interacts with backbone nitrogen of Gly 731 forming a direct hydrogen bond and it also forms water-mediated hydrogen bonds with oxygen of Ser 729, oxygen of Lys 730, and nitrogen of Gly 731 while **14** binds in a different orientation (Fig. 4, **17** and **14**). The difference in binding affinity is also revealed by comparing the docking scores. The compound **17** is having LigandFit Dock_Score of 51.118 as compared to **14** with 13.173 (Table 3). *S*- and *R*-isomers of methionine-based 1,4-benzodiazepin-2-ones **7h** and **9d** exhibit similar LigandFit Dock score (Table 3, **7h**, 43.883; **9d**, 41.213) and bind with the receptor in almost similar fashion. Both isomers form direct hydrogen bonds with backbone oxygen of Lys 730 and backbone nitrogen of Gly 731 (Fig. 4, **7h** and **9d**).

In summary, a series of 3-substituted 1,4-benzodiazepin-2-ones derived from *S*- and *R*-amino acids were evaluated for anti-ischemic activity in vitro. Among the compounds tested **7h**, **16**, **9d**, and **17** showed promising activity. The molecular modeling studies demonstrate that the stereochemistry of 3-substituted benzodiazepin-2-ones may affect the anti-ischemic efficacy.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.12.001.

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12. Experimental procedure: The forebrain neurons were isolated. Three groups were assigned to the isolated neuron at 1×10^6 neuronal cells/100 μl: (1) no treatment, (2) hydrogen peroxide treated, and (3) Pretreated with test compound followed by H_2O_2 treatment. For H_2O_2 treatment neurons were treated with equal volume of H_2O_2 (concentration of 300 μM) for one hour at 37 °C. This treatment resulted in significant number of death of the neurons either by apoptosis or necrosis mode of death. For pretreated group neurons were treated with each compound (100 μM) separately for 20 min at 37 °C followed by H_2O_2 treatment. For no treatment group neurons were maintained for 1 h and 20 min at 37 °C. The concentration of no treatment, H_2O_2, and/or compound treated neurons is 1×10^6 cells/100 μl. Neurons were washed twice with cold PBS and treated with binding buffer, FITC-labeled Annexin V and Propidium iodide (PI) essentially following the manufacturer's protocol (Oncogene, Apoptosis Detection kit). After incubation at room temperature for 15 min in the dark, fluorescence of 10,000 neuronal cells was acquired, as it is possible to identify and quantitate apoptotic cells on a single-cell basis by flow cytometry. Neuronal fluorescence was determined[16] at an excitation wavelengths of 488 nm and emission wavelength of 530 and 670 nm with Flow Cytometer (Becton–Dickinson, UK) and analyzed in a Cell Quest program. Those compounds showing >80% viable population of neurons, falling in the left lower quadrant, are considered active. Acquisition and analysis were performed with the Cell Quest software package (Becton–Dickinson, UK, Ltd).
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