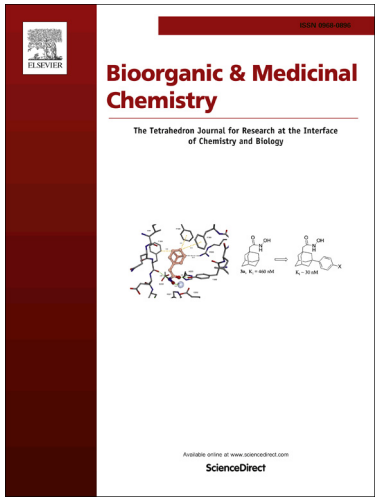
Accepted Manuscript



Novel agonists of benzodiazepine receptors: design, synthesis, binding assay and pharmacological evaluation of 1,2,4-triazolo[1,5-a]pyrimidinone and 3-amino-1,2,4-triazole derivatives

Mehrdad Faizi, Sara Dabirian, Hamed Tajali, Fatemeh Ahmadi, Elham Rezaee Zavareh, Soraya Shahhosseini, Sayyed Abbas Tabatabai

PII: S0968-0896(14)00864-5
DOI: http://dx.doi.org/10.1016/j.bmc.2014.12.016
Reference: BMC 11948

To appear in: *Bioorganic & Medicinal Chemistry*

Received Date: 11 October 2014
Revised Date: 10 December 2014
Accepted Date: 11 December 2014

Please cite this article as: Faizi, M., Dabirian, S., Tajali, H., Ahmadi, F., Zavareh, E.R., Shahhosseini, S., Tabatabai, S.A., Novel agonists of benzodiazepine receptors: design, synthesis, binding assay and pharmacological evaluation of 1,2,4-triazolo[1,5-a]pyrimidinone and 3-amino-1,2,4-triazole derivatives, *Bioorganic & Medicinal Chemistry* (2014), doi: http://dx.doi.org/10.1016/j.bmc.2014.12.016

Novel agonists of benzodiazepine receptors: design, synthesis, binding assay and pharmacological evaluation of 1,2,4-triazolo[1,5-a]pyrimidinone and 3-amino-1,2,4-triazole derivatives

Mehrdad Faizi[a], Sara Dabirian[b], Hamed Tajali[a], Fatemeh Ahmadi[b], Elham Rezaee Zavareh[b] Soraya Shahhosseini[b], Sayyed Abbas Tabatabai[b,*]

[a)] Department of Pharmacology and Toxicology, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran

[b)] Department of Pharmaceutical Chemistry, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Corresponding author: Sayyed Abbas Tabatabai*

Tel: 00982188200093

Fax: 00982188665341

Email: sa_tabatabai@sbmu.ac.ir

Keywords: Synthesis, Benzodiazepine agonists, Binding assay, Hypnotic activity.

Abstract

Agonists of benzodiazepine (BZD) binding site in GABA receptors are widely used in clinical practice. In spite of their benefits they have several side effects, so synthesis of new agonists of these receptors to get more specific effect and better profile of adverse drug reactions is still continued. Novel BZD agonists were designed based on the pharmacophore/receptor model of BZD binding site of $GABA_A$ receptor. Energy minima conformers of the designed compounds and estazolam, a known BZD receptor agonist, were well superimposed in conformational analysis. Docking studies revealed that the carbonyl group of the compound **4c** ,3-(2-chlorobenzyl)-5-methyl-2-phenyl-[1,2,4]triazolo[1,5-a]pyrimidin-7(3H)-one, was near the nitrogen moiety of triazole ring of estazolam providing the hydrogen bond acceptor in proper direction in the BDZ-binding site of $GABA_A$ receptor model ($\alpha1\beta2\Upsilon2$). The designed compounds were synthesized and their *in vitro* affinity for the central BZD receptor was determined. Most of the novel compounds had better affinity for the BZD site of action

on $GABA_A$ receptor complex than diazepam. Finally, the novel compound **4c** with the best affinity in radioligand receptor binding assay (K_i=0.42 nM and IC_{50}= 0.68 nM) was selected as candidate for *in vivo* evaluation. This compound showed significant hypnotic activity and weak anticonvulsant effect with no impairment on learning and memory performance in mouse. The pharmacological effects of the compound **4c** were antagonized by flumazenil, a BZD antagonist, which confirms the involvement of BZD receptors in the biological effects of the novel ligand.

1. Introduction

Agonists of benzodiazepine (BZD) receptor, are extensively used in the treatment of epilepsy, anxiety, muscle cramps and sleep disorders.[1] The pharmacological effects of BZDs result from their affinity for a specific binding site on the $GABA_A$ receptors, known as the BZD receptor.[2-4] BZD receptor agonists modulate the allosteric GABA binding site and increase the opening of a selective chloride ion channel that causes hyperpolarization of the post-synaptic membrane.[1] Since BZDs have some adverse effects, [5] development of the novel BZD receptor agonists is still continued and a great deal of attention. Among all pharmacophores proposed for binding to the BZD receptors, an aromatic ring and a coplanar proton-accepting group in suitable distance are essential for interaction with the receptor. In addition, binding to the receptor could be potentiated by second out-of-plane aromatic ring.[6, 7] According to this structure-activity relationship (SAR) and in continuance of our previous studies on some simple non-rigid derivatives with five member heterocycle rings such as triazoles, oxadiazoles, and thiadiazoles, [6, 8-16] We designed some structures with all suggested requirements (Fig. 1) and performed conformational analysis followed by superimposition of energy minima conformers of the designed compounds on estazolam, a known BZD agonist to confirm whether they could mimic the structure of a BZD agonist. The synthesized compounds were evaluated for their in vitro affinity to BZD receptor by radioligand receptor binding assay. The compound, which had the best affinity to BZD receptor, was studied *in vivo* for its pharmacological effects. The anticonvulsant action was determined using pentylenetetrazole (PTZ)-induced lethal convulsion and maximal electroshock (MES) tests. Mouse learning and memory impairment was evaluated by passive avoidance test and the hypnotic effect was measured through potentiating of pentobarbital sleeping time model. Finally, to confirm the mode of action of the synthesized compound, the effect of flumazenil,

a BZD receptor antagonist, on the pharmacological activity of the compounds was determined.

2. Results and discussion

2.1. Conformational analysis

Conformational analysis of the designed compounds and estazolam were performed through MMX force field method followed by AM1 calculation. Figure 2 represents the superimposition of the energy minima conformer of the compound **4c** on estazolam as a known BZD agonist. Obviously, the main BZD pharmacophores, aromatic rings and proton accepting groups are in the same orientation which means the designed compound could well mimic the agonistic shape of BZD ligands.

2.2. Chemistry

The designed compounds were synthesized according to scheme l. Methyl benzoate was reacted with hydrazine hydrate in methanol at room temperature to give corresponding hydrazide **1**[6] which was treated with cyanogen bromide to close the 1,3,4-oxadiazole ring ,compound **2**, with 62% yield.[6] Compounds **4a**-**4c** were prepared in acceptable yield by the reaction of **2** with ethyl acetoacetate to afford the intermediate **3** followed by treatment with proper substituted amine.[17] Finally, compounds **5a**-**5c** were synthesized through the reaction of the **4a**-**4c** with hydrazine hydrate in good yields.[18]

2.3. Affinity for the central BZD receptors

According to the Table 1, all of the synthesized compounds except **5b** showed more affinity for BZD binding site in rat brain than diazepam, a classic central BZD receptor (CBR) agonist. In triazolopyrimidinone series, the most potent analogue is the compound **4c** having chloro substituent on 2 position of the benzyl group with K_i and IC_{50} values of 0.42 nM and 0.68 nM respectively. This result is in agreement with SAR of BZD receptor ligands.[19] However in 1,2,4-triazole series, unsubstituted analogue **5a** showed the best affinity for the CBR with K_i value of 0.53 nM and IC_{50} value of 0.87. This difference between two series may be due to small dissimilarity of the benzyl group orientation in receptor binding site.

2.4. Molecular modeling (docking) studies

Interaction of the most potent BZD ligand **4c**, 3-(2-chlorobenzyl)-5-methyl-2-phenyl-[1,2,4]triazolo[1,5-a]pyrimidin-7(3*H*)-one,with amino acids of diazepam-binding pocket of

$GABA_A$ receptor (α1β2ϒ2) and the orientation of this ligand along with estazolam in the BZD-binding site that examined by a flexible docking experiment using AutoDock 4.2.3 software were observed in Figure 3(a, b). It is obvious that the carbonyl group of the **4c** was in the same direction of the nitrogen moiety of estazolam for hydrogen binding with α1 Thr206 and α1 Tyr209 and also the phenyl ring of the **4c** may bind in a lipophilic pocket formed byϒ2 Phe77 and ϒ2 Val190 of the receptor. In addition, ligand **5a,** 3-amino-4-benzyl-5-phenyl-4*H*-1,2,4-triazole**,** was docked in the BZD-binding site of $GABA_A$ receptor and shown in Figure 4 (a, b). This figure indicated that the benzyl group of this ligand has different orientation relative to same group of **4c** in the active site.

2.5. Pharmacological evaluations

2.5.1. PTZ-induced lethal convulsion test and MES tests

Compound **4c** showed the highest affinity for the BZD receptors among the novel compounds and was selected for pharmacological tests to evaluate the efficacy of the compound. As shown in Table 2, compound **4c** showed weak anticonvulsant effect in both MES and PTZ models compared to diazepam. The anticonvulsant potency of the novel compound was significantly lower than diazepam in both tests (P<0.05). In both PTZ and MES tests, flumazenil (10 mg/kg) was able to prevent the anticonvulsant activity of the compound. The antagonistic effect of flumazenil indicates that BZD receptors are highly involved in the anticonvulsant effects of the novel compound.

2.5.2. Passive avoidance test

The latency to enter the dark compartment in passive avoidance test in testing day is shown in Figure 5. Midazolam as positive control showed significant decrease in latency compared to control (P<0.001) indicating anterograde amnesia. However, compound **4c** in doses of 0.325, 0.65, 1.25, and 2.5 mg/kg did not show any significant effect on passive avoidance test. These results indicate that compound **4c** has minimum effect on anterograde memory.

2.5.3. Potentiation of pentobarbital induced sleeping time

The duration of losing of righting reflex (considered as sleeping time) following injection of pentobarbital and the novel compound is shown in Figure 6. Diazepam (1 mg/kg) as positive control increased the sleeping time (P<0.001). The novel compound in dose of 0.625 mg/kg did not show any effect on sleeping time induced by pentobarbital. However, in doses of 1.25,

2.5, and 5 mg/kg, it was able to increase the sleeping time (P<0.001 for all three doses) and the effect was dose-dependent. Flumazenil (10 mg/kg) prevented the effect of the compound **4c** (P<0.001). This test revealed that compound **4c** has hypnotic effect by acting on BZD receptor.

2.5.4. Preliminary evaluation of toxicity

Injection of 50 and 150 mg/kg, which are 10 and 30 times more than the hypnotic dose of the compound **4c** respectively (ED_{50} = 5mg/kg), did not show any significant toxicity reactions except sedation and hypnosis. It seems that this compound is probably safe enough to precede future studies. Obviously, complete toxicity evaluation tests are necessary in future studies.

3. Conclusions

In summary, the derivatives of 1,2,4-triazolo[1,5-a]pyrimidinone and 1,2,4-triazole-3-amine as novel BZD agonists were investigated. Most of the novel synthesized compounds showed better affinity for CBR than diazepam in radioligand binding assay. Compound **4c** with the highest affinity for CBR was evaluated in biological assay. This compound revealed considerable hypnotic and weak anticonvulsant activity beside no impairment on learning and memory. Since most of the potent BZD agonists affect learning and memory, the developed scaffold could be a valuable lead compound in designing the new class of BZD receptor ligands with no memory defect. It seems that the synthesized compounds have binding affinity for the $GABA_A$ receptors with α_1 subunit due to the fact that the $GABA_A$ receptor with this subunit is responsible for the hypnotic effect and part of seizure protection of BZD agonists. [20] Since the effect on memory is mediated through the $GABA_A$ receptors with α_5 subunit, [20] the designed compounds may have no affinity for this subunit. In this study, we did not test the binding of the compound to the specific subunits of $GABA_A$ receptor and proving this hypothesis needs further studies.

4. Experimental

4.1. Calculations

Conformational analysis of the synthesized compounds and estazolam were performed preliminarily through MMX force field method followed by AM1 calculation implemented in HyperChem8 software (Hypercube, Inc.). The global energy minima conformers of the designed compounds on corresponding conformer of estazolam molecule, a known BZD agonist, were superimposed.

4.2. Materials and instrumentation

Melting points (mp) were determined using the Electrothermal 9100 apparatus and are uncorrected. Infrared spectra were acquired on a Perkin-Elmer 1420 ratio recording spectrometer. A Bruker FT-500 MHz instrument (Bruker Biosciences, USA) was used to obtain ^{1}H-NMR spectra; chloroform-d and DMSO-d_6 were used as solvents. Mass spectra were acquired with a Finnigan TSQ-70 mass spectrometer. Electron-impact ionization was performed at an ionizing energy of 70 eV. HPLC Agilent system and elemental analyser were used to obtain LC Mass spectra and elemental analysis respectively. All chemicals and reagents were obtained commercially from Aldrich or Merck Company and were used without further purification.

4.3. Benzoic acid hydrazide (1)

28ml (222 mmol) of methyl benzoate and 54 ml (1.11 mol) hydrazine hydrate were stirred for 12 h at room temperature in methanol (10 ml).After this time, the solvent was evaporated and white precipitates washed with diethyl ether, to give 25 g (83%) of 1. mp: 103-106°C; IR: (KBr) ν (cm^{-1}) 3316, 3235, 3033, 1667; Mass m/z (%):136 (M^+, 59), 122 (40), 106 (64), 105 (100), 77(100). Anal. Calcd for $C_7H_8N_2O$: C, 61.75; H, 5.92; N, 20.58. Found: C, 61.55; H, 5.98; N, 20.67.

4.4. 2-Amino-5-phenyl-1,3,4-oxadiazole (2)

15 g (110 mmol) of **1** and 13 g (122 mmol) cyanogens bromide were dissolved in methanol (100 mL) and were refluxed for 4h. The solvent was evaporated and the residue recrystallized from isopropyl alcohol and water to give 11 g (62%) of 2. mp: 246-248°C; IR: (KBr) ν (cm^{-1}) 3305, 3123, 1663, 1651; Mass m/z (%):161 (M^+, 100), 118 (98), 105 (74), 103 (70), 91 (61), 77 (87). Anal. Calcd for $C_8H_7N_3O$: C, 59.62; H, 4.38; N, 26.07. Found: C, 59.85; H, 4.40; N, 25.87.

4.5. 7-Methyl-2-phenyl-5*H*-[1,3,4]oxadiazolo-[3,2-a]pyrimidine-5-one (3)

10g (62 mmol) of **2** and 40ml (317 mmol) ethyl acetoacetate were refluxed for 48 h. The excess ethyl acetoacetate was evaporated and the crude product was purified by chromatography on silica gel column. Elution with ethyl acetate/hexane (70:30) gave 4g (30%) of **3**. Mp: 179-180°C; IR: (KBr) ν (cm^{-1})1698, 1632, 1607 ; Mass m/z (%):227 (M^+, 9),

143 (10), 110 (29), 105 (100), 103 (37), 77 (100); 500 MHz 1H-NMR ($CDCl_3$):δ ppm: 2.46 (3H, s, CH_3), 6.32 (1H, s, H-C_6 of oxadiazolopyrimidinone ring), 7.61 (2H, t, J = 7.5 Hz, H_3 & H_5 of phenyl ring), 7.70 (1H, t, J = 7.5 Hz, H_4 of phenyl ring), 8.18 (2H, d, J = 7.5 Hz, H_2 & H_6 of phenyl ring). Anal. Calcd for $C_{12}H_9N_3O_2$: C, 63.43; H, 3.99; N, 18.49. Found: C, 63.30; H, 4.03; N, 18.56.

4.6. General procedure for the synthesis of compounds 4a-4c

500mg (2.2 mmol) of 7-methyl-2-phenyl-5*H*-[1,3,4]oxadiazolo-[3,2-a]pyrimidine-5-one and 4ml (35mmol) substituted amine were refluxed for 5 h. The unreacted amine was evaporated and the remnant was washed with diethyl ether. Finally, the precipitate was filtered and recrystallized from methanol.

4.6.1. 3-Benzyl-5-methyl-2-phenyl-[1,2,4]triazolo[1,5-a]pyrimidin-7(3*H*)-one (4a)

Yield: 68%; mp: 150-152°C; IR: (KBr) ν (cm^{-1}) 1680; Mass m/z (%):316 (M^+, 62), 315 (M^+-1, 21), 212 (32), 110 (67), 91 (100), 77 (14) ;500 MHz ^{1}H-NMR ($CDCl_3$):δ ppm:2.31 (3H, s, CH_3), 5.38 (2H, s, CH_2), 6.07 (1H, s, H-C_6 of triazolopyrimidinone ring), 7.14 (2H, d, J = 6.8 Hz, $H_{2'}$ & $H_{6'}$ of benzyl ring), 7.25-7.30 (3H, m, $H_{3'}$, $H_{4'}$ & $H_{5'}$ of benzyl ring), 7.55 (2H, t, J = 7.5 Hz, H_3 & H_5 of phenyl ring), 7.63 (1H, t, J = 7.5 Hz, H_4 of phenyl ring), 7.69 (2H, d, J = 7.5 Hz, H_2 & H_6 of phenyl ring). Anal. Calcd for $C_{19}H_{16}N_4O$: C, 72.13; H, 5.10; N, 17.71. Found: C, 72.32; H, 5.04; N, 17.64.

4.6.2. 3-(4-Fluorobenzyl)-5-methyl-2-phenyl-[1,2,4]triazolo[1,5-a]pyrimidin-7(3*H*)-one (4b)

Yield: 68%; mp: 185-187°C; IR: (KBr) ν (cm^{-1}) 1687; Mass m/z (%):(M^+, 21), 110 (50), 109 (100) ;500 MHz ^{1}H-NMR (DMSO):δ ppm: 2.31 (3H, s, CH_3), 5.35 (2H, s, CH_2),6.06 (1H, s, H-C_6 of triazolopyrimidinone ring), 7.11 (2H, t, J = 8.4 Hz, $H_{3'}$ & $H_{5'}$ of benzyl ring), 7.19-7.21 (2H, m, $H_{2'}$ & $H_{6'}$ of benzyl ring), 7.56 (2H, t, J = 7 Hz, H_3 & H_5 of phenyl ring), 7.61-7.69 (3H, m, H_2, H_4 & H_6 of phenyl ring). Anal. Calcd for $C_{19}H_{15}FN_4O$: C, 68.25; H, 4.52; N, 16.75. Found: C, 68.05; H, 4.56; N, 16.87.

4.6.3. 3-(2-Chlorobenzyl)-5-methyl-2-phenyl-[1,2,4]triazolo[1,5-a]pyrimidin-7(3*H*)-one (4c)

Yield: 69%; mp: 220-222°C; IR: (KBr) ν (cm^{-1}) 1691; Mass m/z (%):350 (M^+, 11), 315(100), 125 (100), 103 (34), 94 (67), 89 (60) ;500 MHz 1H-NMR ($CDCl_3$):δ ppm: 2.40 (3H, s, CH_3),

5.50 (2H, s, CH_2), 6.22 (1H, s, H-C_6 of triazolopyrimidinone ring), 6.98 (1H, d, *J* = 7.6 Hz, $H_{6'}$ of benzyl ring), 7.27 (1H, t, *J* = 7.6 Hz, $H_{5'}$ of benzyl ring), 7.33 (1H, t, *J* = 7.6 Hz, $H_{4'}$ of benzyl ring), 7.46-7.51 (3H, m, $H_{3'}$ of benzyl ring, H_3 & H_5 of phenyl ring), 7.56-7.64 (3H, m, H_2, H_4 & H_6 of phenyl ring). Anal. Calcd for $C_{19}H_{15}ClN_4O$: C, 65.05; H, 4.31; N, 15.97. Found: C, 64.79; H, 4.37; N, 16.08.

4.7. General procedure for the synthesis of compounds 5a-5c

500mg (1.6 mmol) of compounds **4a**-**4c** was added in the mixture of 8 ml hydrazine hydrate and 2 ml water and stirred for 3 h at room temperature. The reaction mixture was cooled at 4°C overnight and recrystallized from ethanol plus a few drops of water to give corresponding final products.

4.7.1. 3-Amino-4-benzyl-5-phenyl-4*H*-1,2,4-triazole (5a)

Yield: 83%; mp: 185-187°C; IR: (KBr) ν (cm^{-1}) 3309, 3126, 1659; Mass m/z (%):273 (M^+ + 23), 251 (M^+ + 1) ;500 MHz 1H-NMR (DMSO):δ ppm: 5.16 (2H, s, CH_2), 6.03 (2H, s, NH_2), 6.96 (2H, d, *J* = 7.6 Hz, $H_{2'}$ & $H_{6'}$ of benzyl ring), 7.24 (1H, t, *J* = 7.6 Hz, $H_{4'}$ of benzyl ring), 7.30 (2H, t, *J* = 7.6 Hz, $H_{3'}$ & $H_{5'}$ of benzyl ring), 7.40-7.42 (3H, m, H_3, H_4 & H_5 of phenyl ring), 7.47-7.49 (2H, m, H_2 & H_6 of phenyl ring). Anal. Calcd for $C_{15}H_{14}N_4$: C, 71.98; H, 5.64; N, 22.38. Found: C, 71.81; H, 5.70; N, 22.49.

4.7.2. 3-Amino-4-(4-fluorobenzyl)-5-phenyl-4*H*-1,2,4-triazole (5b)

Yield: 83%; mp: 177.5-179.5°C; IR: (KBr) ν (cm^{-1}) 3327, 3074, 1644; Mass m/z (%):291(M^+ + 23), 269 (M^+ + 1) ;500 MHz 1H-NMR (DMSO):δ ppm: 5.14 (2H, s, CH_2), 6.06(2H,s, NH_2), 6.99 (2H, dd, *J* = 8.4, 5.5 Hz, $H_{2'}$ & $H_{6'}$ of benzyl ring), 7.14 (2H, t, *J* =8.4 Hz, $H_{3'}$ & $H_{5'}$ of benzyl ring), 7.40-7.50 (5H, m, of phenyl ring). Anal. Calcd for $C_{15}H_{13}FN_4$: C, 67.15; H, 4.88; N, 20.88. Found: C, 67.30; H, 4.85; N, 20.83.

4.7.3. 3-Amino-4-(2-chlorobenzyl)-5-phenyl-4*H*-1,2,4-triazole (5c)

Yield: 84%; mp: 180.5-182°C; IR: (KBr) ν (cm^{-1}) 3465, 3389, 3318, 1626; Mass m/z (%):307 (M^+ + 23), 285 (M^+ + 1); 500 MHz 1H-NMR ($CDCl_3$):δ ppm: 4.65 (2H, s, CH_2), 5.16 (2H, s, NH_2),6.99 (1H, dd, *J* = 8.0, 1.7 Hz, $H_{6'}$ of benzyl ring), 7.31-7.39 (2H, m, $H_{4'}$ & $H_{5'}$ of benzyl ring), 7.41-7.47 (3H, m, $H_{3'}$ of benzyl ring, H_3 & H_5 of phenyl ring), 7.49-7.54 (3H, m, H_2, H_4 & H_6 of phenyl ring). Anal. Calcd for $C_{15}H_{13}ClN_4$: C, 63.27; H, 4.60; N, 19.68. Found: C, 63.46; H, 4.56; N, 19.57.

4.8. Radioligand receptor binding assay

All of the binding assays were done in triplicates. The radioligand receptor binding studies were done as previously described.[21-22] Briefly, the cortical membrane tissue of Male Sprague-Dawley rats weighing 300-350g (Pasteur Institute, Tehran, Iran) was homogenized in 20 mL ice-cold Tris-HCl buffer (30 mM, pH 7.4) using a Silent S homogenizer (Heidolph, Germany) at medium speed. The homogenate was centrifuged at 600 g for 10 min using a Beckman Coulter L90K centrifuge. The resulting supernatant was centrifuged at 27000 g for 15 min followed by three centrifugations and resuspensions cycles. The washed pellet was suspended in 20 mL buffer, incubated at 37°C for 30 min and then centrifuged for 10 min at 27000 g. The pellet was washed once, and the final pellet was re-suspended in 30 mL Tris-HCl buffer (50 mM, pH 7.4). All of the centrifugation was performed at 4°C. [23-25] The amount of protein was estimated in the membrane preparation by the Bradford method using bovine serum albumin (BSA) as a standard. [26] The membrane preparation was stored at -20°C until it was used 1-15 days later. For binding studies, 100 µg of frozen membrane was thawed, resuspended into Tris-HCl buffer (50 mM, pH 7.4), and incubated with 7 different concentrations of [^{3}H]-flumazenil at 30°C for 35 min. The incubation was terminated by the centrifugation of reaction mixture at 1500 g for 4 min at 4°C. The total binding (TB) (receptor + radioligand), non-specific binding (NSB) (receptor + radioligand + excess diazepam), and specific binding (SB) (TB-NSB) were measured at various radioligand concentrations. The amount of radioligand required to saturate the receptors was used to determine the receptor binding affinity of [^{3}H]-flumazenil (K_d) and the BZD receptor density (B_{max}) based on non-linear regression analysis of the saturation curve data.[27] The K_d and B_{max} for [^{3}H]-flumazenil are 1.35 ± o.316nM and 0.638 ± 0099 Pmol/mg respectively. In competition studies, 100 µg of membrane protein in Tris-HCl buffer (50 mM, pH 7.4) was incubated with 8.6×10^{-5}nmol [^{3}H]-flumazenil and increasing amount of newly synthesized ligands in a final volume 0.5mL at 30°C for 35 min.[24] After incubation, the assay was terminated by centrifugation (1500 g, 4C, 5 min). Binding (receptor + radioligand) and NSB were measured at various concentrations of unlabeled ligand. TB is determined in the absence of any added competitor (non-radioactive ligand). The concentration of non-radioactive ligand that inhibits the binding of [^{3}H]-flumazenil by 50% is IC_{50} value.[27-29] All of experiments were done in triplicates. Nonspecific binding (NSB) was determined in parallel assays performed in the presence of 100 µM diazepam.

4.9. Docking studies

The homology model of the Diazepam bound $GABA_A$ receptor (α1β2ϒ2) developed by Ernst et al. was retrieved the supplementary material of their published paper.[30] The structures of compounds were investigated using the Lamarckian genetic algorithm search method implemented in AutoDock 4.2.3 software (http://autodock.scripps.edu). The receptors were kept rigid, and ligands were allowed to be flexible. Polar hydrogens and Kollman united atom partial charges were added to the individual protein atoms. Each structure was energy minimized under MM+ method in HyperChem8 software and converted to pdbqt format file using AutoDockTools version1.5.6rc3 (http://mgltools.scripps.edu). A docking grid box was built with 40, 40 and 40 points in 42.820, 44.4360 and 6.8690 directions and the number of generations and maximum number of energy evaluations was set to 100 and 2,700,000, respectively. Docking results were clustered with a root mean square deviation (RMSD) of 0.5 Å and evaluated by Pymol software version 1.5.0.1 (http://pymol.findmysoft.com).

4.10. Pharmacological evaluations

4.10.1. Animals and drugs

Male NMRI albino mice (weighing 18-22 g; 12 weeks old) were used for pharmacological evaluations of the novel compound (**4c**). Animals were purchased from Pasteur Institute, Iran. They were transferred to the animal house of School of Pharmacy 7 days before the experiments and housed in a 12 hours light/dark condition with a controlled temperature and humidity. During the housing, animals had free access to standard food and water. For all the experiments, the mice were transferred to the lab 60 minutes before starting the tests to get habituated to the lab environment. Animals were divided to experimental groups randomly and each mouse was used only one time for the experiments. Before starting the experiments, proposal of the experiments were sent to institutional animal care and use committee of Shahid Beheshti University of Medical Sciences and approval of the committee was acquired. All the experiments were performed in accordance with National Institute of Health (NIH) Principles of Laboratory Animal Care (NIH publication #85-23). In all experiments the minimum numbers of mice were used and sufficient attentions were considered to reduce pain or discomfort of animals. The novel compound **4c**, diazepam, as reference BZD agonist, and flumazenil, as antagonist of BZD receptors, were dissolved in mixture of DMSO and water (1:10) and administered 5 ml/kg; i.p.; 30 minutes before the experiments. PTZ, pentobarbital,

and midazolam were dissolved in distilled water and injected i.p. (volume of injection was 5 ml/kg).

4.10.2. PTZ-induced lethal convulsion test and MES tests

The anticonvulsant activity of the novel compound was determined in two models of seizure, PTZ and MES induced seizure.[23, 31] In PTZ model, the ability of the novel compound to protect mice against lethal dose of PTZ (100 mg/kg) was evaluated. Animals were closely observed for 30 minutes after injection of PTZ and the number of dead mice was reported. In MES test, the ability of the novel compound to prevent MES induced seizure in mice was evaluated. In this test, occurrence of hind limb tonic extension (HLTE) in mice following applying MES (60 Hz, 37.2 mA and 0.25 s) was assessed. The electrical current was applied through ear electrodes and mice were observed for 30 seconds for incidence of HLTE.

4.10.3. Passive avoidance test

Step throw passive avoidance test was used for evaluation of anterograde amnesia induced by the novel compound **4c** and midazolam (1mg/kg, i.p.), a BZD with amnestic effects.[1] The passive avoidance cage had two different compartments separated by a sliding door. One compartment was black and dark and another one was white and lighted. On training day, mouse was placed in the white compartment, facing away from sliding door for 30 seconds. Then, the sliding door was opened and mouse was able to enter the dark compartment. When the mouse entered the dark compartment with four paws, mouse got a foot shock (0.5 mA, 2 seconds). On the testing day (after 24 hours), mouse was placed in lighted compartment and the latency to enter the dark compartment was reported.

4.10.4. Potentiation of pentobarbital sleeping time

Pentobarbital (65 mg/ kg i.p.) was injected 30 min after administration of the novel compound **4c** and diazepam (1 mg/kg). The duration of a loss of the righting reflex was considered as the sleep time. Mice were placed on an electric blanket during the experiment to prevent hypothermia.[32, 33]

4.10.5. Preliminary evaluation of toxicity

For evaluation of toxicity of the novel compound **4c**, 10 mice were selected randomly. Doses of 50 and 150 mg/kg, which are 10 and 30 times more than the ED_{50} of hypnotic dose, were used in this study and animals were observed for 24 hours after injection of the compound **4c.**

4.11. Data Analysis

The binding parameters of [^{3}H]-flumazenil (K_d and B_{max}) and newly synthesized ligands were calculated from non-linear regression analysis of the saturation and competitive curve data by using the activity base software package (Program Prism, Graph Pad, San Diego, CA). The IC_{50}s of the novel compounds in binding studies were analyzed using one way ANOVA with Tukey's HSD post-hoc test. In PTZ and MES tests, probit-regression method and SPSS software (Chicago, IL; version 13) was used to determine ED_{50} of the novel compound **4c** and diazepam. Fisher's exact probability test was used to analyze the difference between the ED_{50} of the novel compound and diazepam. In PTZ and MES tests, the data were presented as mean with 95% confidence intervals and in the rest of the pharmacological evaluations, one-way ANOVA with Tukey's HSD post-hoc test was used and all the data were presented as mean + SEM. $P<0.05$ considered statistically significant.

5. Acknowledgment

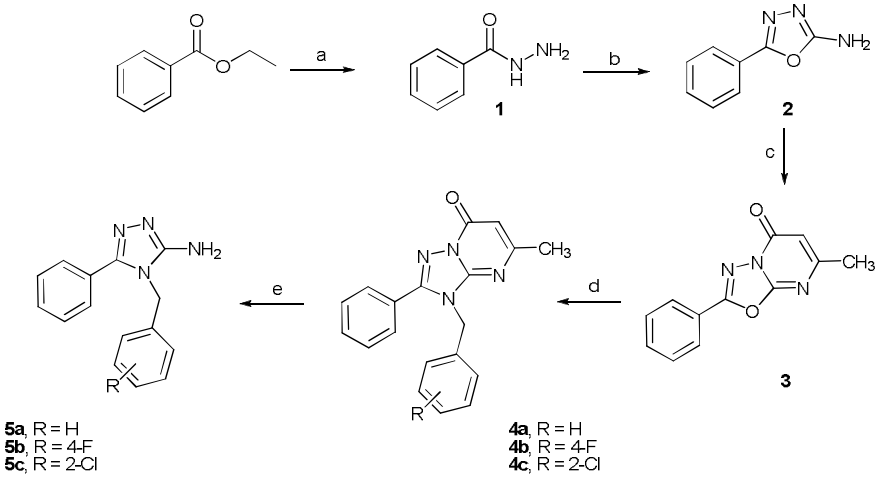
This work was supported by grant from the Research Council of Shahid Beheshti University of Medical Sciences and Iran National Science Foundation (INSF). We would like to acknowledge the Radiopharmacy Laboratory, School of Pharmacy, Shahid Beheshti University of Medical Sciences for radioligand receptor binding studies.

References

1. Guerrini, G.; Ciciani, G.; Cambi, G.; Bruni, F.; Selleri, S.; Besnard, F.; Montali, M.; Martini, C.; Ghelardini, C.; Galeotti, N.; Costanzo, A. *Bioorg. Med. Chem.* **2007**, 15, 2573.
2. Zarghi, A.; Faizi, M.; Shafaghi, B.; Ahadian, A.; Khojastehpoor, H. R.; Zanganeh, V.; Tabatabai, S. A.; Shafiee, A. *Bioorg. Med. Chem. Lett.* **2005**, 15, 3126.
3. Huh, K.-H.; Endo, S.; Olsen, R. W. Eur. *J. Pharmacol.* **1996**, 310, 225.
4. Smith, G. B.; Olsen, R. W. *Trends Pharmacolo. Sci.* **1995**, 16, 162.

5. Carotti, A.; Altomare, C.; Savini, L.; Chiasserini, L.; Pellerano, C.; Mascia, M. P.; Maciocco, E.; Busonero, F.; Mameli, M.; Biggio, G.; Sanna, E. *Bioorg. Med. Chem.* **2003**, 11, 5259.
6. Akbarzadeh, T.; Tabatabai, S. A.; Khoshnoud, M. J.; Shafaghi, B.; Shafiee, A. *Bioorg. Med. Chem.* **2003**, 11, 769.
7. Fryer, R. I; In Comprehensive Medicinal Chemistry; Hansch, C., Ed.; Pergamon: Oxford, **1990**, 3, 539.
8. Almasirad, A.; Tabatabai, S. A.; Faizi, M.; Kebriaeezadeh, A.; Mehrabi, N.; Dalvandi, A ;.Shafiee, A. *Bioorg. Med. Chem. Lett.* **2004**, 14, 6057.
9. Zarghi, A.; Tabatabai, S. A.; Faizi, M.; Ahadian, A.; Navabi, P.; Zanganeh, V.; Shafiee, A. *Bioorg. Med. Chem. Lett.* **2005**, 15, 1863.
10. Almasirad, A.; Vousooghi, N ;.Tabatabai, S. A.; Kebriaeezadeh, A.; Shafiee, A. *Acta Chim. Slovenica.* **2007,** 54, 317.
11. Foroumadi, A.; Sheibani, V.; Sakhteman, A.; Rameshk, M.; Abbasi, M.; Farazifard, R.; Tabatabai, S.; Shafiee, A. *Daru J. Pharm. Sci.* **2007**, 15.
12. Zarghi, A.; Hajimahdi, Z.; Mohebbi, S.; Rashidi, H.; Mozaffari, S.; Sarraf, S.; Faizi, M.; Tabatabai, S. A.; Shafiee, A. *Chem. Pharm. Bull.* **2008,** 56, 509.
13. Zarghi, A.; Hamedi, S.; Tootooni, F.; Amini, B.; Sharifi, B.; Faizi, M.; Tabatabai, S. A.; Shafiee, A. *Sci. Pharm.* **2008,** 76, 185.
14. Mahdavi, M.; Akbarzadeh, T.; Sheibani, V.; Abbasi, M.; Firoozpour, L.; Tabatabai, S. A.; Shafiee, A.; Foroumadi, A. *Iranian J. Pharm. Res.* **2010,** 9, 265.
15. Faizi, M.; Ahangar, N.; Shafaghi. B.; Shafiee, A.; Tabatabai. S. A. *Iranian J. Pharm. Res.* **2012**, 11, 83.
16. Tabatabai, S. A.; Barghi Lashkari, S.; Zarrindast, M. R.; Gholibeikian, M.; Shafiee, A. *Iranian J. Pharm. Res.* **2013,** 12, 103.
17. Gehlen, H.; Simon, B. *Arch. Pharm.* **1969**, 303, 501.
18. Davidson, J. S.; Dhami, S. S. *Chem. Ind.* **1978**, 3, 92.
19. Haefely, W.; Kyburz, E.; Gerecke, M.; Mohler, H. *Adv. Drug Res.* **1985**, 14, 165.
20. Victor, C.; Loren, M.; Erin, E.; John , K.; Howard, M.; Franco, T.; John, R.; John, M.; Amit, B.; Neil, C.; Keith,W.; Beverley, O. *J. Neurosci.* **2006**, 26, 5024.
21. Ahmadi, F.; Faizi, M.; Tabatabai, S. A.; Beiki, D.; Shahhosseini, S. *Nucl. Med. Biol.* **2013**, 40, 896.

22. Ahmadi, F.; Dabirian, S.; Faizi, M.; Tabatabai, S. A.; Beiki, D.; Shahhosseini, S. *Iranian J. Pharm. Res*. **2013**, 13, 79.
23. Guerrini, G.; Ciciani, G.; Cambi, G.; Bruni, F.; Selleri, S.; Melani, F.; Montali, M.; Martini, C.; Ghelardini, C.; Norcini, M. *Bioorg*. *Med. chem*. **2008,** 16, 4471.
24. Kahnberg, P.; Lager, E.; Rosenberg ,C.; Schougaard, J.; Camet, L.; Sterner, O.; Nielsen, E. Q.; Nielsen, M.; Liljefors, T. *J. Med. Chem*. **2002,** 45, 4188.
25. Martini, C.; Lucacchini, A.; Ronca, G.; Hrelia, S.; Rossi, C. *J. Neurochem.* **1982,** *38*, 15.
26. Bradford ,M. M. *Anal. Biochem*. **1976**, 72, 248.
27. Motulsky, H.; Neubig, R. The GraphPad guide to analysing radioligand binding data. 1995-96 by GraphPad software, Inc. 1-19.
28. Bigott-Hennkens, H.; Dannoon, S.; Lewis, M.; Jurisson, S. *Q. J. Nucl. Med. Mol. Imaging*. **2008**, 52, 245.
29. Bennet, J. P. Methods in binding studies. In: H. l. Yamamura, Enna, S. J.; Kuhar, M. J. (Eds.) Neurotransmitter Receptor Binding. New York: Raven Press, **1978**,.57.
30. Richter, L.; de Graaf, C.; Sieghart, W.; Varagic, Z.; Mörzinger, M.; de Esch, I. J.; Ecker, G. F.; Ernst, M. *Nat. Chem. Biol*. **2012**, 8, 455.
31. Lankau, H.-J.; Unverferth, K.; Grunwald, C.; Hartenhauer, H.; Heinecke, K.; Bernöster, K.; Dost, R.; Egerland, U.; Rundfeldt ,C. Eur. *J. Med. Chem*. **2007,** 42, 873.
32. Raihan, M.; Habib, M.; Brishti, A.; Rahman, M.; Saleheen, M.; Manna, M. *Drug Discovery Ther.* **2011**, *5*, 185.
33. Suleyman, H.; Guvenalp, Z.; Kizilkaya, M.; Demirezer, L. O. Yakugaku zasshi. *Pharm. Soc. Jpn*. **2007**, 127, 1263.



Scheme 1. Reagents and conditions: (a): $NH_2NH_2.H_2O$, Methanol, rt, 12 h; (b): BrCN, Methanol, reflux, 4 h; (c): CH_3COCH_2COOEt, reflux, 48 h; (d): substituted amine, reflux, 4 h; (e): $NH_2NH_2.H_2O$, H_2O/Dioxane, reflux, 6 h.

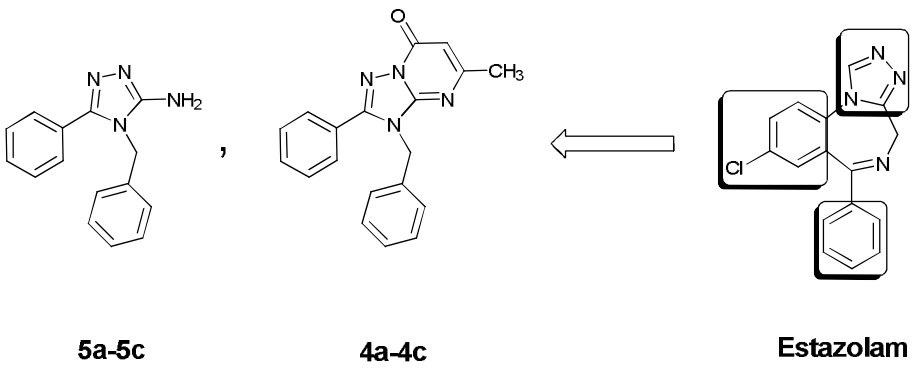


Figure 1. The structure of the designed compounds and estazolam. The main pharmacophores have been conserved.

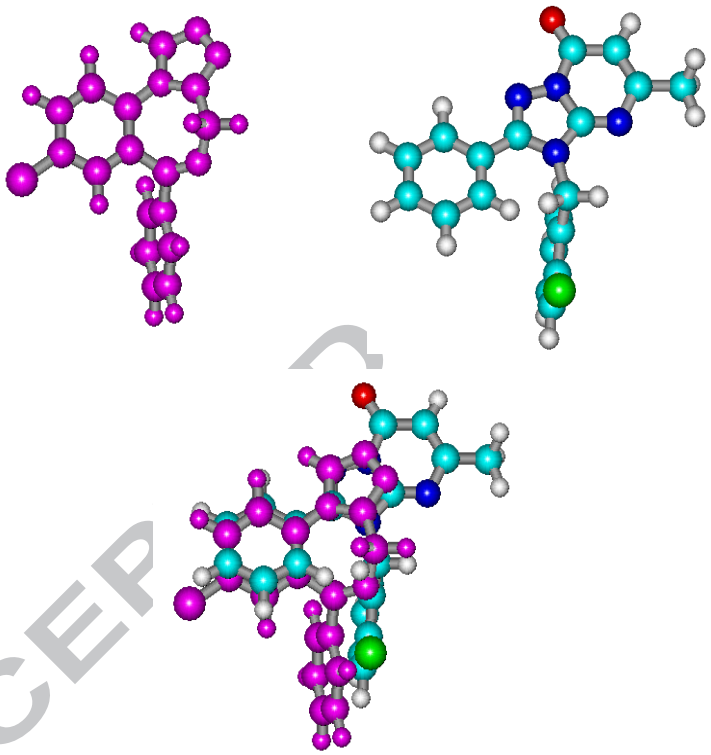


Figure 2. Stereo view of the superimposition of the energy minima conformers of estazolam (top left, violet) and compound **4c** (top right).

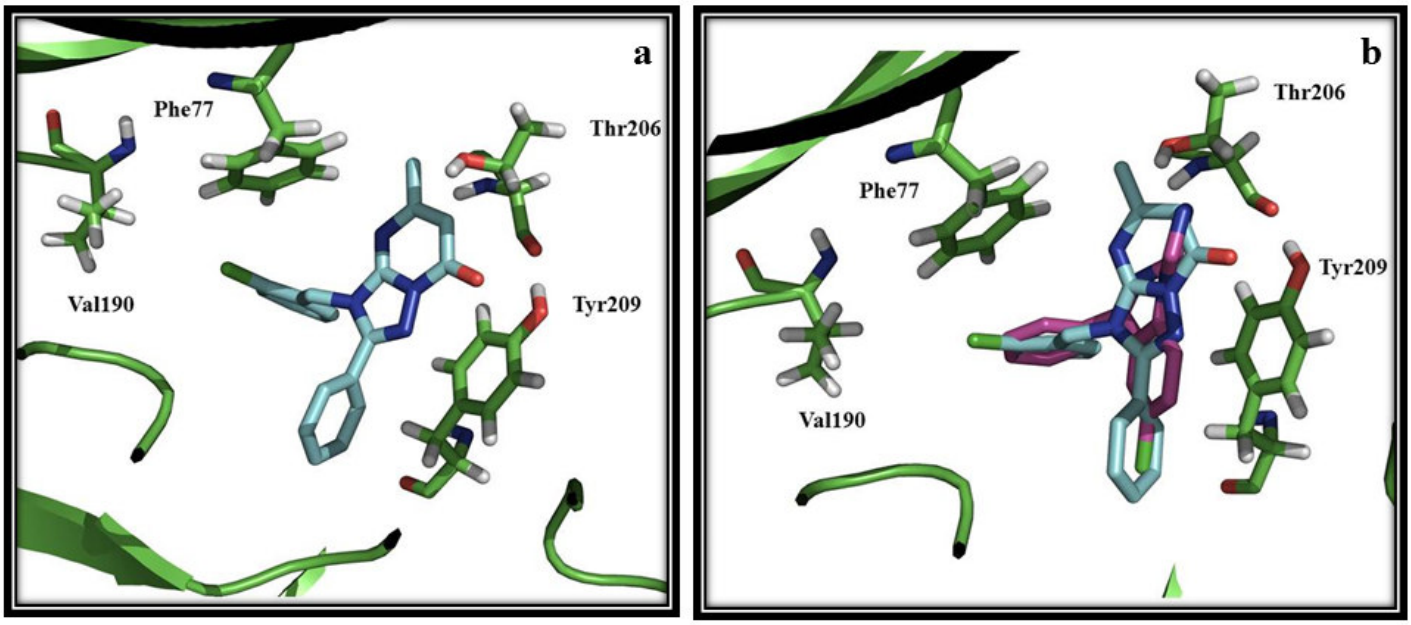


Figure 3. (**a**) Interaction of **4c** with amino acids of diazepam-binding pocket of $GABA_A$ receptor (α1β2Υ2). (**b**) Overlay of **4c** (blue) and estazolam (magenta) in pocket of $GABA_A$ receptor (α1β2Υ2).

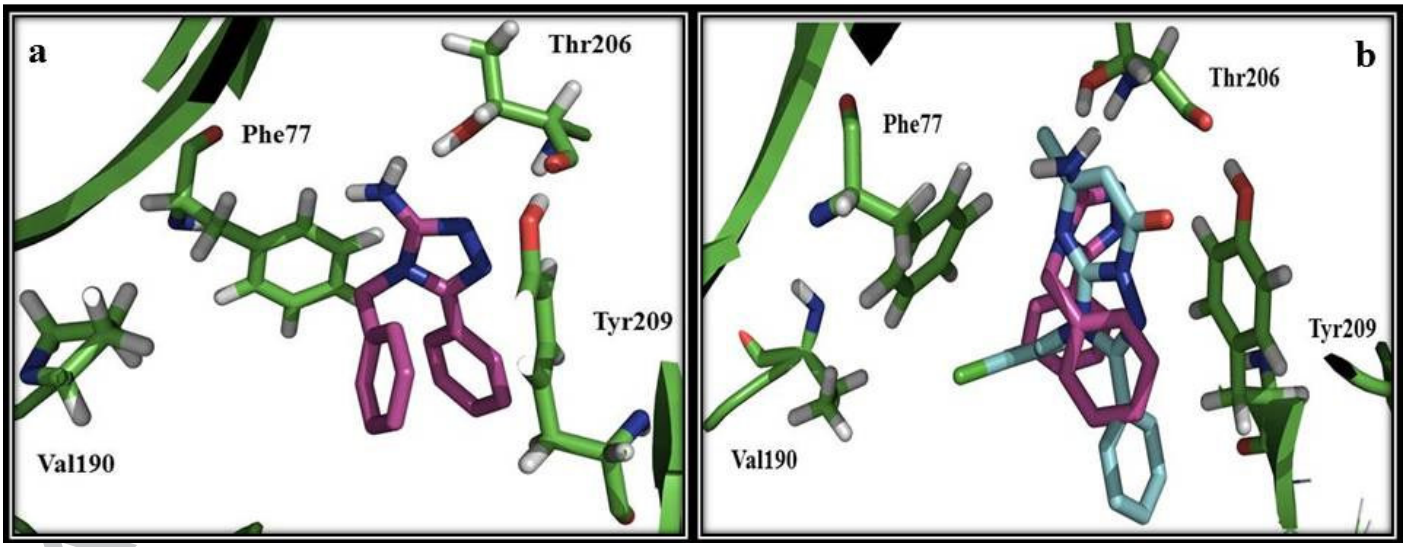


Figure 4. (**a**) Interaction of **5a** with amino acids of diazepam-binding pocket of $GABA_A$ receptor (α1β2Υ2). (**b**) Overlay of **5a** (magenta) and **4c** (blue) in pocket of $GABA_A$ receptor (α1β2Υ2).

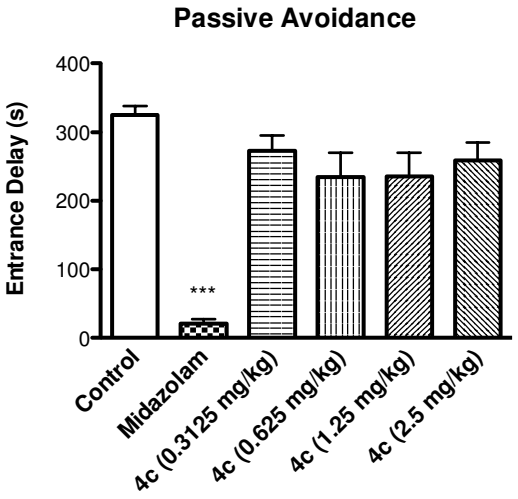


Figure 5. Effect of the novel compound on anterograde memory in passive avoidance test: The delay time to entrance to the dark compartment in testing day is shown. Data are presented as mean + SEM. In all groups n=8. *** represents $P<0.001$ compared to control. 1 mg/kg of midazolam was used in this experiment.

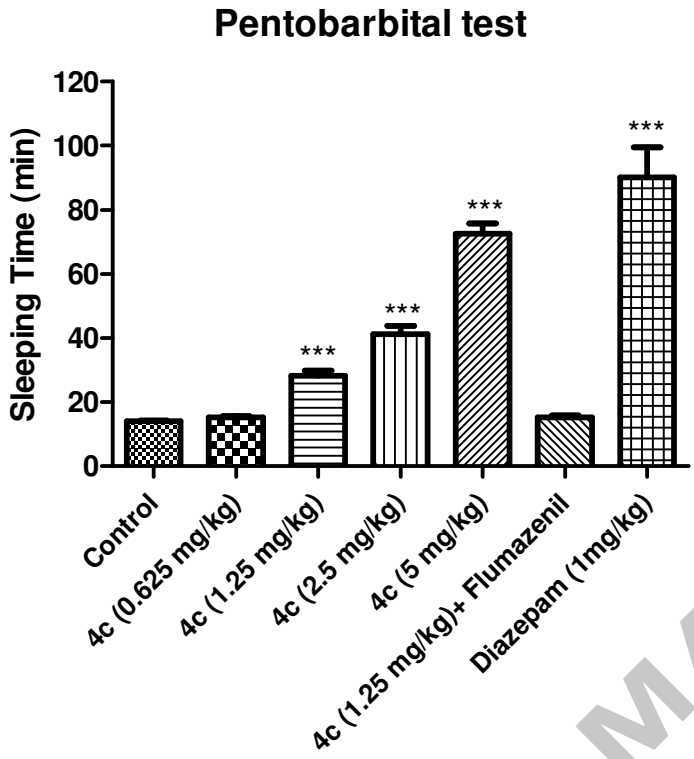


Figure 6. Hypnotic effect of the novel compound in pentobarbital test: The sleeping time (duration of loss of righting reflex) is shown. Data are presented as mean + SEM. In all groups n=8. *** represents $P<0.001$ compared to control. 10 mg/kg of flumazenil was used in this experiment.

Table 1. In vitro binding affinities of the novel compounds tested on ^{3}H-flumazenil binding to benzodiazepine receptor.

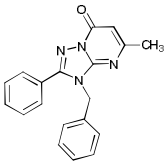
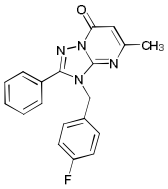
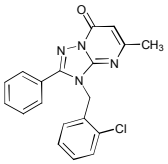
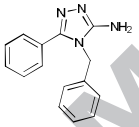
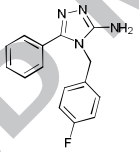
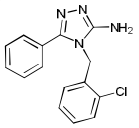
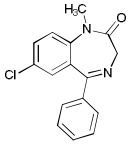
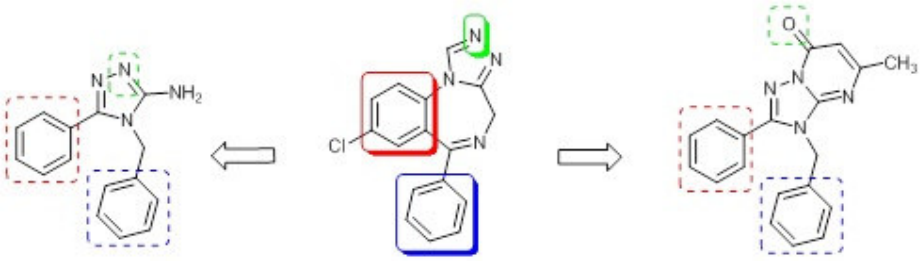
Compound	Structure	K_i (nM)	IC_{50} ± SEM (nM)
4a		1.46	2.39 ± 0.99
4b		0.97	1.59 ± 0.103
4c		0.42	0.68 ± 0.14
5a		0.53	0.87 ± 0.11
5b		2.73	4.45 ± 0.034
5c		1.37	2.24 ± 0.108
Diazepam		1.53	2.5 ± 0.066

Table 2. PTZ-induced lethal convulsion test and MES tests of the compound **4c**

Compound	ED50 mg/kg [a]	
	PTZ	MES
4**c**	56.57 (32.23 - 99.28)	15.01 (8.21 - 30.02)
Diazepam	0.75 (0.56 - 0.96)	0.74 (0.45 - 1.07)

[a] n = 10, 95% Confidence limits in parentheses



Based on SAR of benzodiazepine receptor agonists, novel scaffold are introduced as hypnotic and anticonvulsant agents without any impairing effect on memory and learning.