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Original article

Synthesis, structure and affinity of novel 3-alkoxy-1,2-dihydro-3*H*-1,4-benzodiazepin-2-ones for CNS central and peripheral benzodiazepine receptors

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ABSTRACT

A series of novel 3-alkoxy-1,2-dihydro-3*H*-1,4-benzodiazepin-2-ones (**7-15**) was synthesized and their in vitro affinity for both the central benzodiazepine receptor (CBR) and the peripheral benzodiazepine receptor (PBR) of rat brain was studied. Racemic mixture of 7-bromo-3-(2-methoxy)ethoxy-5-phenyl-1,2-dihydro-3*H*-1,4-benzodiazepin-2-one (**13**) was separated into enantiomers **14, 15** by chiral HPLC. Absolute configuration of R-enantiomer **15** was determined by the method of X-ray diffraction analysis. The affinity of S-enantiomer **14** for CBR ($IC_{50} = 245$ nM) is 20-fold higher than the affinity of R-enantiomer **15** ($IC_{50} = 4930$ nM). A high selectivity for CBR versus PBR (IC_{50} (PBR) > 10000 nM) was shown by all reported compounds. Compound **12** was revealed as a potent ($IC_{50} = 9$ nM) and selective CBR ligand among the synthesized 3-alkoxy-1,2-dihydro-3*H*-1,4-benzodiazepin-2-ones.

1. Introduction

Central benzodiazepine receptors (CBR) [1] and peripheral benzodiazepine receptors (PBR) [2] which differ by their distribution, molecular organization and functions are the main biological targets of benzodiazepines (BD) in the brain.

CBR are presented in the central nervous system (CNS). CBR are part of the heteropentameric $GABA_A$-receptor-associated chloride ion channel, located on postsynaptic membranes of neurons [1]. Ionotropic $GABA_A$ receptors are the members of the Cys-loop family of ligand-gated ion channel (cl-LGIC) [3], which includes muscle and neuronal nicotinic acetylcholine receptors [4], glycine receptors [5], the zinc-activated ion channel [6], as well as the 5-hydroxytryptamine type 3 receptor [7]. These cl-LGIC are pentamers, either composed of five identical subunits (homomers) or different subunits (heteromers) [8,9].

$GABA_A$ receptor subunits represent eight classes with multiple isoforms: α_{1-6}, β_{1-4}, γ_{1-4} δ, ε, π, θ, ρ_{1-3} [10]. The most of $GABA_A$ receptors include two α subunits, two β subunits and one γ subunit. Receptors with different subtype composition are associated with different physiological effects [11,12]. BD requires $\alpha\beta\gamma_2$-type receptors ($\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_2\gamma_2$, $\alpha_3\beta_3\gamma_2$, and $\alpha_5\beta_2\gamma_2$) with the benzodiazepine-binding site located between α and γ_2 subunits [13]. Noteworthy, $\alpha_4\beta\gamma_2$- and $\alpha_6\beta\gamma_2$-type receptors are insensitive to most BD, as well as any receptors containing the δ subunit [14].

PBR are an individual receptor class due to their unique structure, cell localization and physiological functions mediated by them [2,15]. PBR were initially discovered in kidneys [16], later – in different organs and tissues, including the CNS [2]. PBR are 5-transmembrane 169-amino acid polypeptides. These receptors

Abbreviations: CBR, central benzodiazepine receptor; PBR, peripheral benzodiazepine receptor; BD, benzodiazepines; GABA, γ-aminobutiryc acid; cl-LGIC, Cys-loop family of ligand-gated ion channel.

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are localized mainly on the mitochondrial outer membrane of peripheral tissues and CNS glial cells [2,15]. PBR are associated with numerous biological functions including regulation of cellular proliferation, immunomodulation, anion transport, porphyrin transport, heme biosynthesis, apoptosis, regulation of cholesterol transport, steroidogenesis [2,15,17]. In addition, PBR ligands might exert antianxiety effects via activation of neurosteroid synthesis [18]. The ligands binding to PBR induces cholesterol transport into mitochondria and steroid production by glial cells, resulting to the synthesis of pregnane neurosteroids such as allopregnanolone and pregnanolone, which positively modulate the $GABA_A$ receptor functions and provoke anxiolytic effects [18].

The search for novel selective CBR ligands as selective anxiolytics is still interesting for practical medicine and theoretical investigation of molecular mode of action of 1,2-dihydro-3*H*-1,4-benzodiazepin-2-ones.

3-Hydroxy-1,2-dihydro-3*H*-1,4-benzodiazepin-2-ones as CBR ligands (7-chloro-3-hydroxy-5-phenyl-1,2-dihydro-3*H*-1,4-benzodiazepin-2-one (oxazepam), 7-chloro-5-(2'-chloro)phenyl-3-hydroxy-1,2-dihydro-3*H*-1,4-benzodiazepin-2-one (lorazepam), 7-chloro-5-(2'-chloro)phenyl-3-hydroxy-1-methyl-1,2-dihydro-3*H*-l,4-benzodiazepin-2-one (lormetazepam), 7-chloro-3-hydroxy-l-methyl-5-phenyl-1,2-dihydro-3*H*-l,4-benzodiazepin-2-one (temazepam)) are applied for treatment of neurological disorders such as convulsions, anxiety, sleep disturbance [19–24]. Novel selective CBR ligand 7-bromo-5-(2'-chloro)phenyl-3-hemisuccinylhydroxy-1,2-dihydro-3*H*-1,4-benzodiazepin-2-one (cinazepam), (K_i 72 nM) [25] is hypnotic and anxiolytic agent with decreased side effects [26,27].

The modulation of the $GABA_A$-receptor-associated channel by 3-subsituted benzodiazepines depends on both the substituent nature and stereocentre configuration. As it has been demonstrated S-enantiomers of 3-hydroxy-1,2-dihydro-3*H*-1,4-benzodiazepine derivatives has higher affinity than R-enantiomers [28]. S-Enantiomer of 7-chloro-3-O-(N,N-dimethylcarbamyl)-1-methyl-5-phenyl-1,2-dihydro-3*H*-1,4-benzodiazepin-2-one (camazepam) exhibits 14-fold higher affinity for benzodiazepine receptors than the R-enantiomer in rat brain [29]. The ratio of IC_{50}(R-enantiomer)/IC_{50}(S-enantiomer) for acetate, hemisuccinate, and α-methyl-β-phenyl-propionate esters of oxazepam is 1.7, 3.8 and 81.2, respectively [30].

Nine novel 3-alkoxy-1,2-dihydro-3*H*-1,4-benzodiazepin-2-ones were synthesized. Structure-activity relationship of the 3-alkoxy derivatives was studied and their in vitro affinity for CBR and PBR of rat brain was evaluated. Racemic mixture of 7-bromo-3-(2-methoxy)ethoxy-5-phenyl-1,2-dihydro-3*H*-1,4-benzodiazepin-2-one (**13**) was separated into enantiomers **14, 15** by chiral HPLC to study influence of stereocentre configuration on affinity for CBR. Absolute configuration of R-enantiomer **15** was determined by the method of X-ray diffraction analysis. S-Enantiomer **14** is 20-fold higher affinity ligand for CBR than R-enantiomer **15**.

2. Chemistry

3-Alkoxy-1,2-dihydro-3*H*-1,4-benzodiazepin-2-ones **7-13** were synthesized by the substitution reaction of 3-chloro-1,2-dihydro-3*H*-1,4-benzodiazepin-2-ones **4-6** with corresponding alcohols to study the structure - affinity for benzodiazepine receptors relationship. 3-Chloro derivatives **4-6** were obtained through the treatment of 3-hydroxy-1,2-dihydro-3*H*-1,4-benzodiazepin-2-ones **1-3** with thionylchloride (Scheme 1).

The structures of 3-alkoxy derivatives **7-13** were identified by the methods of IR spectroscopy, mass spectrometry and NMR spectroscopy.

Compounds **7-13** were obtained as racemic mixtures. Nevertheless, enantiomers of 7-bromo-3-(2-methoxy)ethoxy-5-phenyl-1,2-dihydro-3*H*-1,4-benzodiazepin-2-one (**13**) were separated by HPLC on chiral column. Enantiomeric separation of benzodiazepines, such as oxazepam and temazepam, have been already achieved with chirobiotic stationary phases [31,32]. Therefore, enantioseparation of compounds **14** and **15** was performed with chirobiotic T column using ethanol as mobile phase. The resolution value obtained ($Rs > 4$) was sufficient to realize the enantiomeric semi-preparative separation of **13**. Each enantiomer (**14** or **15**) was obtained with 99% enantiomeric excess (Scheme 2).

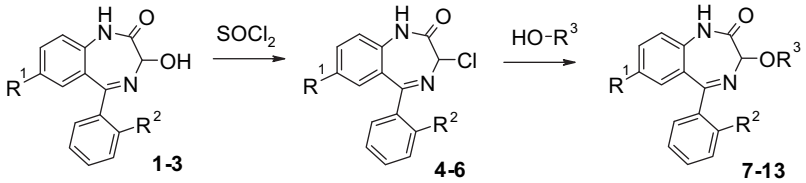
Enantiomers rotate plate of the polarized light and have respective rotation angles: S-enantiomer **14** $[\alpha]_D^{25} + 147.2$ (*c* 0.83, CH_3OH); R-enantiomer **15** $[\alpha]_D^{25} - 148.1$ (*c* 1, CH_3OH).

3. Results and discussion

3.1. Crystal and molecular structure

Absolute configuration of R-enantiomer **15** was determined by the method of X-ray diffraction analysis (Fig. 1).

The main distances between atoms, valence angles and crystal data in 7-member heterocycle **15** are given in the Tables 1 and 2.



1, **4**: $R^1 = Br$, $R^2 = H$;

2, **5**: $R^1 = Br$, $R^2 = Cl$;

3, **6**: $R^1 = CH_3$, $R^2 = Cl$;

7: $R^1 = Br$, $R^2 = H$, $R^3 = C_2H_5$;

8: $R^1 = Br$, $R^2 = Cl$, $R^3 = C_2H_5$;

9: $R^1 = CH_3$, $R^2 = H$, $R^3 = C_2H_5$;

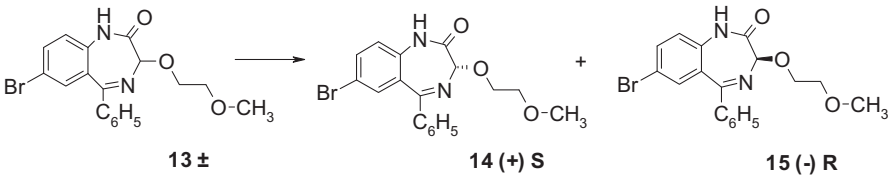
10: $R^1 = Br$, $R^2 = H$, $R^3 = (CH_2)_2OH$;

11: $R^1 = Br$, $R^2 = Cl$, $R^3 = (CH_2)_2OH$;

12: $R^1 = Br$, $R^2 = Cl$, $R^3 = (CH_2)_2OCH_3$;

13: $R^1 = Br$, $R^2 = H$, $R^3 = (CH_2)_2OCH_3$;

Scheme 1.



Scheme 2.

The skeleton of molecule **15** is similar to the 7-bromo-5-(2'-chloro)-phenyl-1,2-dihydro-3*H*-1,4-benzodiazepin-2-one (phenazepam) one [33], its hydroxy derivative [34], lorazepam [35], 7-nitro-5-(2'-chloro)phenyl-1,2-dihydro-3*H*-1,4-benzodiazepin-2-one (clonazepam) [36], 7-chloro-5-(2'-chloro)phenyl-1,2-dihydro-3*H*-1,4-benzodiazepin-2-one [37] and 7-bromo-1,2-dihydro-5-(2-pyridyl)-3*H*-1,4-benzodiazepin-2-one (bromozepam) [38].

Double bond between atoms N(4)–C(5) is of 1.293 Å, and it is similar to above listed compounds. In 7-membered heterocycle delocalization of π-electronic density covers fragment O(2) = C(2)–N(1)–C(10)–C(11)–C(5)–N(4). Bonds C(2)–C(3) = 1.530 Å and C(3)–N(4) = 1.446 Å are not involved in delocalization. 7-Membered cycle has a boat conformation with two-sided angles between base N(1)C(2)N(4)C(5) and fragments C(2)C(3)N(4) and N(1)C(10)C(11)C(5), that are of 117.2° and 33.7°, respectively. Distance C(3)–O(3) is equal to 1.403 Å, and this bond has equatorial location relatively to the cycle. Angle between aromatic fragment and phenyl substituent in the 5th position is of 58.6°, that is close to the found value for phenazepam (75.4°) and for other similar structures [33].

Molecules of R-enantiomer **15** are packed into a chain due to the intermolecular hydrogen bonds between groups N(1)–H···O(2) = 2.958(2) Å (N–H = 0.82(2) Å, O···H = 2.21(2) Å, the angel near H is of 153(2)°). Noteworthy, there is no dimer associates formation, that is not typical for 1,4-benzodiazepin-2-ones derivatives unsubstituted at the first position (Fig. 2).

3.2. Affinity for the central and peripheral benzodiazepine receptors

All synthesized compounds were studied for their in vitro binding affinity for CBR and PBR by means of a binding assay using [^{3}H]flumazenil (Ro15-1788) and [^{3}H]PK11195 as radioligands, respectively. Binding data are expressed as IC_{50} (nM) (Table 3).

All studied compounds demonstrate affinity for the CBR of the rat brain within wide range of values (IC_{50} from 9 nM up to 5000 nM) (Table 3).

Studied compounds **7-15** didn't demonstrate affinity for the PBR of the rat brain. All compounds did not display significant inhibitory effects ($IC_{50} > 1$ μM) on the [^{3}H]PK11195 binding (Table 3).

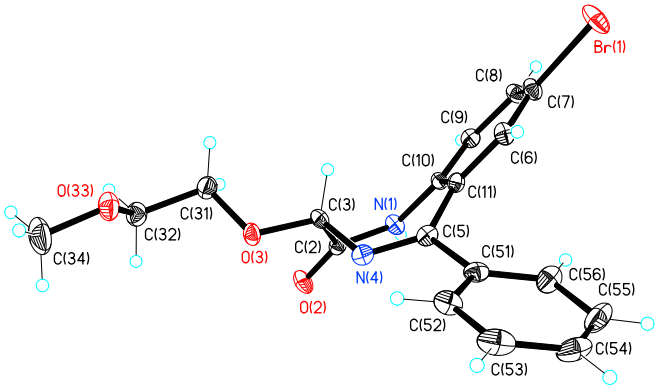


Fig. 1. Molecular structure of R-7-bromo-3-(2-methoxy)ethoxy-5-phenyl-1,2-dihydro-3*H*-1,4-benzodiazepin-2-one (**15**).

Comparison of the compounds pairs containing 5-phenyl and 5-(2'-chloro)phenyl substituents allows to note the increase of affinity for the CBR with of the chlorine atom introduction into ortho-position of 5-phenyl substituent. Affinity increased in 56-fold in the case of compounds **12** and **13**, and in 11-fold in the case compounds of **10** and **11**, that prove previous conclusion [39]. Derivative **12** is the most potent CBR ligand ($IC_{50} = 9 \pm 1$ nM) among the investigated compounds and it is a promising compound for pharmacological research.

Racemic compound **13** has a low affinity for the CBR ($IC_{50} = 510$ nM). S-Enantiomer **14** has 20-fold higher affinity for CBR (IC_{50} 245 nM) as compared to R-enantiomer **15** (IC_{50} 4930 nM) (Fig. 3).

4. Conclusion

Binding to CBR and PBR of synthesized 3-alkoxy derivatives was studied and it was found that all of these compounds are selective CBR ligands. R- and S-enantiomers of 3-(2-methoxy)ethoxy-5-phenyl-1,2-dihydro-3*H*-1,4-benzodiazepin-2-one (**14, 15**) were separated by chiral HPLC. Absolute configuration of R-enantiomer **15** was determined by the method of X-ray diffraction analysis. S-Enantiomer **14** is a most potent ligand of the CBR ($IC_{50} = 245$ nM) as compared to R-enantiomer **15** ($IC_{50} = 4930$ nM). This fact proves that binding to the CBR is stereospecific process. It was found, that 3-alkoxy-5-(2'-chloro)phenyl derivatives demonstrate higher affinity for CBR than 3-alkoxy-5-phenyl-1,4-benzodiazepin-2-ones. The compound **12** demonstrates the highest affinity for CBR.

5. Experimental protocols

5.1. Chemistry

^{1}H-NMR spectra were recorded on a Bruker spectrometer at 300 MHz frequency, in $CDCl_3$ and DMSO-d_6, internal standard TMS, at 25 °C. The IR spectra (in KBr pellets) were recorded on a Specord 75-IR. Mass spectra were obtained using the electron ionization method on mass-spectrometer VG 7070EQ (ionizing voltage is 70 eV, temperature of the ionizing chamber is 200 °C). Values of

Table 1
Bond lengths (Å) between atoms in 7-member heterocycle of compound **15**.

Bond	Length (Å)
O(33)–C(34)	1.406(3)
O(33)–C(32)	1.425(3)
C(2)–O(2)	1.224(2)
C(2)–N(1)	1.362(3)
C(2)–C(3)	1.530(3)
C(5)–N(4)	1.293(3)
C(5)–C(11)	1.478(3)
N(1)–C(10)	1.413(2)
N(1)–H(1N1)	0.82(2)
O(3)–C(3)	1.403(21)
O(3)–C(31)	1.434(2)
C(34)–H(12A)	1.395(3)
C(31)–C(32)	1.446(3)
C(31)–H(10A)	1.496(3)

Table 2
Valence angles (ω°) in 7-member heterocycle of compound **15**.

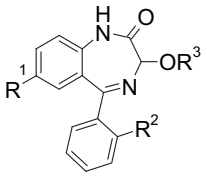
Valence angles	ω°
C(34)–O(33)–C(32)	111.36(16)
O(2)–C(2)–N(1)	122.17(19)
O(2)–C(2)–C(3)	123.6(2)
N(1)–C(2)–C(3)	114.25(16)
N(4)–C(5)–C(11)	124.42(17)
N(4)–C(5)–C(51)	117.3(2)
C(11)–C(5)–C(51)	118.32(19)
C(2)–N(1)–C(10)	126.09(16)
C(2)–N(1)–H(1N1)	114.4(16)
C(10)–N(1)–H(1N1)	119.0(16)
C(3)–O(3)–C(31)	112.31(14)
C(11)–C(10)–N(1)	122.19(18)
C(5)–N(4)–C(3)	116.95(18)
O(3)–C(31)–C(32)	108.54(17)
C(10)–C(11)–C(5)	122.31(18)
O(3)–C(3)–N(4)	106.74(15)
O(3)–C(3)–C(2)	111.00(16)
N(4)–C(3)–C(2)	107.37(14)
O(33)–C(32)–C(31)	109.79(17)

exact molecular ions masses were measured on mass-spectrometer VG 7070EQ with resolution 10 000 using the peak matching method. Thin layer chromatography was performed on plates Silufol UV-254, in acetonitrile–chloroform–hexane (1:1:3) system and in benzene–acetonitrile–hexane–methanol (25:15:5:1) system, using UV-light at λ = 254 nm. Semi-preparative purification and analytical separation were performed on a Varian 9010 HPLC pump coupled to a Varian 9050 UV detector. Analytical Chiral HPLC separation: stationary phase: CHIROBIOTIC T (150 mm × 4.6 mm; 5 μm) purchased from Interchim (Monluçon, France), mobile phase: 100% EtOH at 1 mL/min, UV detector: λ = 254 nm, compound **14** tr = 2.56 min, compound **15** tr = 5.14 min. Semi-preparative purification: stationary phase: CHIROBIOTIC T (250 mm × 10 mm; 5 μm) purchased from Interchim (Monluçon, France), mobile phase: 100% EtOH at 4 mL/min, UV detector: λ = 254 nm. The collected fractions were evaporated to dryness under vacuum.

5.2. General procedure for the synthesis of the 3-chloro derivative (4-6)

A well pounded 3-hydroxy-1,4-benzodiazepine **1-3** (1.5 mmol) and thionylchloride (10 ml) were added in round-bottomed flask. Reaction mixture was reflux for 15 min after effervescence finished and left for 12 h at room temperature. Thionylchloride was evaporated under reduced pressure. Anhydrous chloroform (10 ml) was added to residue and evaporated. This operation was twice repeated. Obtained residue was used in next stage without purification.

Table 3
In vitro binding affinity (IC_{50}, nM) for CBR and PBR of compounds **7-15**.



No	R^1	R^2	R^3	IC_{50}, nM	
				CBR[a]	PBR[b]
7 (R,S)	Br	H	C_2H_5	>1000	>1000
8 (R,S)	Br	Cl	C_2H_5	52 ± 3	>1000
9 (R,S)	CH_3	H	C_2H_5	>1000	>1000
10 (R,S)	Br	H	$(CH_2)_2OH$	540 ± 15	>1000
11 (R,S)	Br	Cl	$(CH_2)_2OH$	48 ± 2	>1000
12 (R,S)	Br	Cl	$(CH_2)_2OCH_3$	9 ± 1	>1000
13 (R,S)	Br	H	$(CH_2)_2OCH_3$	510 ± 40	>1000
14 (S)	Br	H	$(CH_2)_2OCH_3$	245 ± 21	>1000
15 (R)	Br	H	$(CH_2)_2OCH_3$	4 930 ± 58	>1000

[a] The concentration of tested compounds that inhibited [^{3}H]flumazenil specific binding to rat brain synaptosomal membranes (IC_{50}) by 50% was determined with eight concentrations of the displacers, each performed in triplicate. IC_{50} values are the mean ± SEM of three determinations.
[b] [^{3}H]PK11195 was incubated in the presence 1 μM of the compounds examined.

5.3. General procedure for the synthesis of the 3-alkoxy-1,2-dihydro-3H-1,4-benzodiazepin-2-ones (7-13)

To the suspension of 3-chloro derivatives **4-6** (1.4 mmol) in anhydrous chloroform (10 ml) appropriate alcohol (7.5 mmol) was added. The result mixture was refluxed and left for 24 h. Reaction mixture was washed with water until pH 7. Solvent was evaporated under reduced pressure. The resulting oil was crystallized from acetonitrile or ethanol.

5.3.1. Compound *7*

Yield: 52 %; m.p. = 218–222 °C; EI–MS *m/z* 358 [M^+]; HRMS calcd for $C_{17}H_{15}BrN_2O_2$ [M]$^+$ m/z, 358,031; found, 358,033; ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 10.04 (s, 1H), 7.60–7.18 (m, 8H), 4.77 (s, 1H), 4.08–3.98 (m, 1H), 3.76–3.66 (m, 1H), 1.39 (t, 3H); IR (KBr): ν (cm^{-1}): 3173, 3106, 2929, 1702, 1604.

5.3.2. Compound *8*

Yield: 46 %; m.p. = 205–208 °C; EI–MS *m/z* 391 [M^+]; HRMS calcd for $C_{17}H_{14}BrClN_2O_2$ [M]$^+$ m/z, 391,992; found, 391,994; ^{1}H

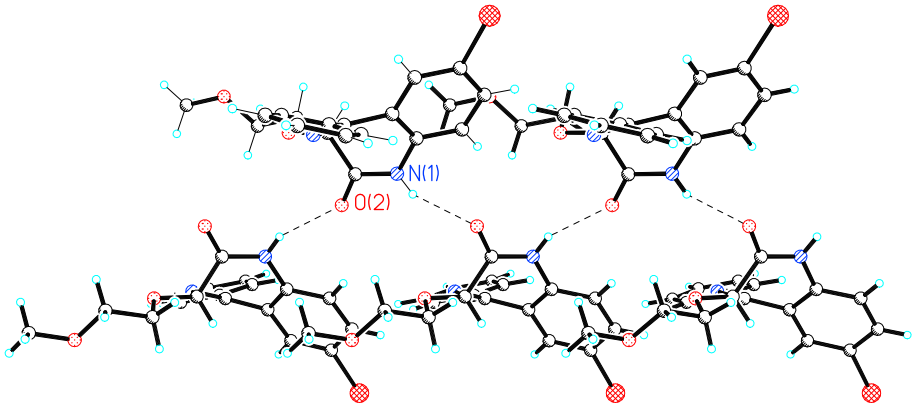


Fig. 2. Crystal structure of the R-7-bromo-3-(2-methoxy)ethoxy-5-phenyl-1,2-dihydro-3*H*-1,4-benzodiazepine-2-one (**15**).

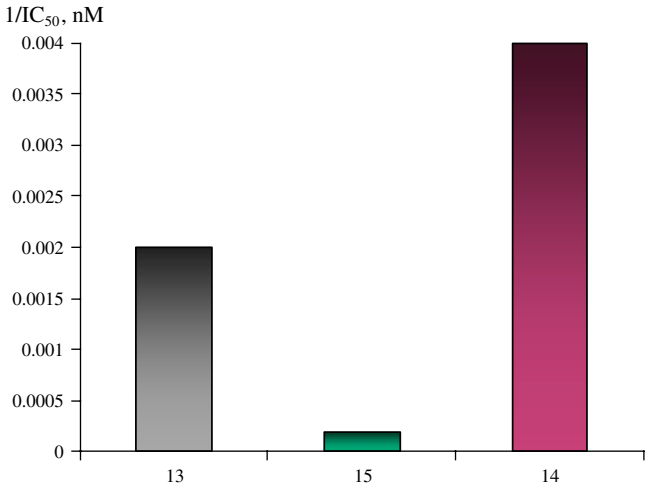


Fig. 3. The affinity for CBR of CNS for racemate **13**, R-enantiomer **15** and S-enantiomer **14**.

NMR ($CDCl_3$, 300 MHz) δ: 9.78 (s, 1H), 7.59–7.15 (m, 7H), 4.82 (s, 1H), 4.11–4.00 (m, 1H), 3.75–3.65 (m, 1H),1.38 (t, 3H); IR (KBr): ν (cm^{-1}): 3216, 3155, 2924, 2862, 1718, 1691, 1605.

5.3.3. Compound **9**

Yield: 57 %; m.p. = 223–225 °C; EI–MS *m/z* 294 [M^+]; HRMS calcd for $C_{18}H_{18}N_2O_2$ [M]$^+$ m/z, 294,136; found, 294,139; ^{1}H NMR (CDCl3, 300 MHz) δ: 9.65 (s, 1H), 7.49–7.05 (m, 8H), 4.76 (s, 1H), 4.11–3.65 (m, 2H), 2.55 (s, 3H), 1.38 (t, 3H); IR (KBr): ν (cm^{-1}): 3266, 3150, 1670, 1605.

5.3.4. Compound **10**

Yield: 81 %; m.p. = 224–229 °C; EI–MS *m/z* 374 [M^+]; HRMS calcd for $C_{17}H_{15}BrN_2O_3$ [M]$^+$ m/z, 374,026; found, 374,028;^{1}H NMR (DMSO-d6, 300 MHz) δ: 10.82 (s, 1H), 7.80–7.21 (m, 8H), 4.81 (s, 1H), 4.65 (br. s, 1H), 3.83–3.77 (m, 1H), 3.7–3.67 (m, 1H), 3.64–3.61 (m, 2H); IR (KBr): ν (cm^{-1}): 3411, 3191, 3030, 2911, 2851, 1701, 1605.

5.3.5. Compound **11**

Yield: 44 %; m.p. = 120–124 °C; EI–MS *m/z* 408 [M^+]; HRMS calcd for $C_{17}H_{14}BrClN_2O_3$ [M]$^+$ m/z, 407,987; found, 407,989;^{1}H NMR (DMSO-d6, 300 MHz) δ: 10.93 (s, 1H), 8.31 (s, 1H), 7.75–7.07 (m, 7H), 4.86 (s, 1H), 3.84–3.78 (m, 1H), 3.69–3.65 (m, 1H), 3.62–3.58 (m, 2H); IR (KBr): ν (cm^{-1}): 3340, 3175, 3102, 2933, 1683, 1610.

5.3.6. Compound **12**

Yield: 27%; m.p. = 179–188 °C; EI–MS *m/z* 422 [M^+]; HRMS calcd for $C_{18}H_{16}BrClN_2O_3$ [M]$^+$ m/z, 422,003; found, 422,006;^{1}H NMR ($CDCl_3$, 300 MHz) δ: 9.62 (s, 1H), 7.59–7.13 (m, 7H), 4.94 (s, 1H), 4.18–4.12 (m, 1H), 3.97–3.90 (m, 1H), 3.97–3.64 (m, 2H), 3.36 (s, 3H); IR (KBr): ν (cm^{-1}): 3418, 3209, 2883, 1710, 1672, 1628.

5.3.7. Compound **13**

Yield: 42 %; m.p. = 204–208 °C; EI–MS *m/z* 388 [M^+]; HRMS calcd for $C_{18}H_{17}BrN_2O_3$ [M]$^+$ m/z, 388,042; found, 388,044;^{1}H NMR ($CDCl_3$, 300 MHz) δ: 9.81 (s, 1H), 7.61–7.15 (m, 8H), 4.89 (s, 1H), 4.12–4.10 (m, 1H), 3.98–3.94 (m, 1H), 3.77-3.70(m, 2H), 3.37 (s, 3H); IR (KBr): ν (cm^{-1}): 3210, 2916, 2874, 1706, 1671, 1607.

5.4. Crystal structure determination of compound **15**

Crystal **15** monoclinic, space group $P2_1$, $a = 9.5342(3)$, $b = 8.2524(3)$, $c = 11.1086(5)$ Å, $\beta = 97.23(3)°$, $V = 867.08(6)$ Å3, $Z = 2$, $\rho_{calc} = 1.491$gcm-3. (130 K).

The structure was solved by direct methods (3007 reflections with $F(hkl) \geq 2\sigma$ (F), $R(int) = 0.0169$, final $R_1 = 0.0202$, $wR_2 = 0.0447$) and was refined by full-matrix least-squares techniques based on F^2. The non-H atoms were refined with anisotropic displacement parameters. In all cases, positions of H-atoms on carbon atoms were calculated and were allowed to ride. Other hydrogen atoms were found from difference Fourier maps and refined isotropically. Calculation was performed using SHELX-97 [40] crystallographic software package. Molecules of the **15** have R-configuration with Flack parameter – 0.0033 (56). CCDC reference number is 705472.

5.5. Biological methods

5.5.1. Materials

Adult male Wistar rats with a body weight of 180–220 g were maintained under an artificial 12-h-light/dark cycle (light on 08.00–20.00 h). Food and water were freely available until the time of the experiment. Animal care and handling throughout the experimental procedures were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). The experimental protocol were approved by the Animal Ethical Committee of the University of Cagliari.

5.5.2. In vitro receptor binding assays

5.5.2.1. [^{3}H]Flumazenil binding. Affinity of compounds **7-15** for CBR of rat brain was determined by modified method [41] and values of IC_{50} were evaluated.

Animals were anesthetized and decapitated, the cerebral cortex was quickly extracted and homogenized in 30 ml of 0.05 M ice-cold citrate buffer (pH 7.1 at 4 °C) with a Dounce homogenizer. The homogenate was centrifuged at 20 000 g for 15 min at 4 °C. The pellet was resuspended in initial volume of the same buffer and centrifuged again under the same conditions. The process of homogenization and centrifugation was repeated for 4 times. Supernatant was decanted, residue was resuspended in 0.05 M of ice-cold citrate buffer (pH 7.1 at 4 °C) to obtain the suspension with wet membranes concentration of 50 mg/ml.

Binding of [^{3}H]flumazenil (Ro15-1788) was carried out in the final incubation volume of 0.5 ml, containing 0.1 ml of radioligand ([^{3}H]flumazenil) at concentration of 1 nM, 0.1 ml of tested compound at concentration of 1 μM, 0.1 ml of 0.05 M citrate buffer (pH 7.1 at 4 °C), and 0.2 ml of membrane suspension.

All assay tubes were incubated for 60 min at 4 °C. The incubation was quenched by the addition of 6 ml of 0.05 M ice-cold citrate buffer (pH 7.1 at 4 °C) to samples and rapid filtration on a 12-position harvester using Whatman GF/C filters. The filters were washed with 6 ml of the same buffer. The dried filters were placed into the scintillation vials. The vials were filled with 10 ml of standard Optifase scintillator (LKB, Sweden), kept for 24 h at 20 °C, and then the radioactivity was counted with a scintillation counter Rackbeta 1219 Spectral.

To determine the non-specific binding of radioligand, the incubation was carried out in the presence of 10 μM of unlabeled flumazenil. Specific binding was determined as a difference between general and non-specific bindings. The value of non-specific radioligand binding (SB_0) was less than 10% from total value. IC_{50} values of the different compounds were determined by testing a series of eight increasing concentrations (0.1 nM–10 μM).

5.5.3. In vitro receptor binding assays

5.5.3.1. [^{3}H]PK11195 binding. Binding assays were determined by modified method [42].

After sacrifice the brain was rapidly removed, the cerebral cortex was dissected, and tissues were used for the assay. The tissues were

homogenized in 30 ml of ice-cold Tris-HCl buffer (pH 7.4 at 4 °C) with a Dounce homogenizer. The homogenate was centrifuged at 20 000 g for 30 min, and the pellet was resuspended in 30 ml of ice-cold Tris-HCl buffer (pH 7.4 at 4 °C) and recentrifuged. The new pellet was resuspended in 30 ml of ice-cold Tris-HCl buffer (pH 7.4 at 4 °C) and used for the assay. [^{3}H]PK11195 binding was determined in a final volume of 0.5 ml, containing 0.1 ml of radioligand ([^{3}H]PK11195) at concentration of 1 nM, 0.1 ml of tested compound at concentration of 1 μM, 0.1 ml of 0.05 M Tris-HCl buffer (pH 7.4 at 4 °C), and 0.2 ml of membrane suspension. Incubations (4 °C) were initiated by addition of membranes and were terminated 120 min. The incubation was quenched by the addition of 6 ml of 0.05 M Tris-HCl buffer (pH 7.4 at 4 °C) to samples and rapid filtration on a 12-position harvester using Whatman GF/C filters. The filters were washed with 6 ml of the same buffer. The dried filters were placed into the scintillation vials. The vials were filled with 10 ml of standard Optifase scintillator (LKB, Sweden), kept for 24 h at 20 °C, and then the radioactivity was counted with a scintillation counter Rackbeta 1219 Spectral.

Nonspecific binding was defined as binding in the presence of 10 μM unlabeled PK11195, and specific binding was determined as a difference between general and non-specific bindings. The value of non-specific radioligand binding (SB_0) was less than 20% from total value.

Supplementary data

The supplementary crystallographic data for the structure analysis of compound **15** have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC 705472. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB21EZ, UK (fax: +44 1223 336 033; e-mail: deposit@ccdc.cam.ac.uk).

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