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Synthesis of novel cognition enhancers with pyrazolo[5,1-*c*][1,2,4] benzotriazine core acting at γ-aminobutyric acid type A ($GABA_A$) receptor

Gabriella Guerrini [a,*], Giovanna Ciciani [a], Annarella Costanzo [a], Simona Daniele [b], Claudia Martini [b], Carla Ghelardini [c], Lorenzo Di Cesare Mannelli [c], Samuele Ciattini [d]

[a] *Dipartimento di Scienze Farmaceutiche, Laboratorio di Progettazione, Sintesi e Studio di Eterocicli Biologicamente attivi (HeteroBioLab) Università degli Studi di Firenze, Via U. Schiff, 6, 50019 Polo Scientifico, Sesto Fiorentino, Firenze, Italy*
[b] *Dipartimento di Psichiatria, Neurobiologia, Farmacologia e Biotecnologie, Università degli Studi di Pisa, via Bonanno, 6, 56126 Pisa, Italy*
[c] *Dipartimento di Farmacologia Preclinica e Clinica Aiazzi-Mancini, Università degli Studi di Firenze, Viale Pieraccini, 6, 50139 Firenze, Italy*
[d] *Centro di Cristallografia, Università degli Studi di Firenze, Via della Lastruccia, 3, 50019 Polo Scientifico, Sesto Fiorentino, Firenze, Italy*

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ABSTRACT

Memory dysfunction associated with aging, neurodegenerative and psychiatric disorders represents an increasing medical need. Advances in research exploring the biological mechanisms underlying learning and memory have opened new potential approaches for development of memory-enhancing therapies addressed to selective neuronal targets. In this work, we synthesized some derivatives with a pyrazolo[5,1-*c*][1,2,4]benzotriazine core to identify ligands on $GABA_A$ receptors subtype (benzodiazepine site on $GABA_A$-receptor) endowed with the potential of enhancing cognition activity without the side effects usually associated with non-selective $GABA_A$ modulators. In fact, there is much evidence that $GABA_A$-R (γ-aminobutyric acid, type A receptor) subtype ligands have relevance in learning and memory. In vitro and in vivo tests have been performed. Pharmacological data indicate that compounds **7**, **13**, **14** and **22** act as dual functional modulators of $GABA_A$-Rs (promnemonic and anxiolytic agents) while only compounds **3** and **10** stand out as selectively displaying good antiamnesic and procognitive activity (1 and 3 mg/kg, respectively).

1. Introduction

Cognitive dysfunction is one of the most debilitating symptoms of various acute or chronic neurological disorders. It may be a prodromal phase of Alzheimer's disease (AD)[1–3] as well as a cardinal feature in psychiatric conditions.[4–6] Patients with Parkinson's disease[7,8] and Down syndrome[9] also show alterations in learning and memory. Since it is widely demonstrated that various neurotransmitters are involved in the considered diseases, multiple drug therapy could be required. The ever-increasing elderly population and resulting age-related neurological disorders have focused the attention of the pharmaceutical industry on mild cognitive impairments (MCI) which significantly affect the quality of life.

In the last decade a large amount of evidence suggests that the γ-aminobutyric acid type A receptor ($GABA_A$-R) could be a useful target for memory enhancement.[10,11] It is known that γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system (CNS), acting through ionotropic $GABA_A$-Rs[12] and metabotropic (G-protein coupled) $GABA_B$ receptors ($GABA_B$-Rs).

$GABA_A$-Rs, which are members of the ligand-gated ion channels (LGICs) superfamily, are membrane-bound heteropentameric proteins composed principally of α, β and γ subunits. At this time 21 subunits of the $GABA_A$-Rs have been cloned[13] and the most common are two single alpha subunits, two single beta subunits and one gamma subunit $(\alpha)_2(\beta)_2\gamma$.[14] The combination of the isoforms α1–3,5, β2/3 and γ2 form a subset of $GABA_A$-Rs (α1β2γ2, α2β3γ2, α3β3γ2, α5β3γ2) modulated by benzodiazepines (Bzs) and by ligands (also named non-benzodiazepine ligands) belonging to several chemical classes. The Bz binding site is located at the interface of an α and a γ subunit and its pharmacology is

Abbreviations: α7nACh-R, α7nicotinic acetylcoline receptor; AD, Alzheimer's disease; BMS, borane methylsulfide; Bz site/$GABA_A$-R, benzodiazepine site on $GABA_A$ receptor; CMC, carboxymethylcellulose; CNS, central nervous system; DAZ, diazepam, 7-chloro-1-methyl-5-phenyl-3*H*-1,4-benzodiazepin-2(1*H*)-one; Flu, flumazenil (Ro15-1788); GABA, γ-aminobutyric acid; $GABA_A$-R, γ-aminobutyric acid type A receptor; LGCIs, ligand-gated ion channels; MCI, mild cognitive impairments; PTZ, pentylenetetrazole; SAR, structure affinity relationships.

* Corresponding author. Tel.: +39 0554573766; fax: +39 0554573671.
E-mail address: gabriella.guerrini@unifi.it (G. Guerrini).

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mainly determined by the alpha isoform.[15–18] Genetic and pharmacological approaches have identified the function of $GABA_A$-R subtypes. Receptors containing α1β2γ2 subunits mediate sedative, anterograde amnesic and anticonvulsant actions of diazepam.[19–21] The anxiolytic-like effect and muscle relaxant activities are mediated by the α2βγ2 and α3βγ2 receptors; the α3-containing $GABA_A$-Rs may be involved in the inhibitory input for the dopaminergic system[22–24] and the α5βγ2 receptors may influence learning and memory processes.[18–25] The important role of the α5βγ2 $GABA_A$-Rs, mainly localized in the hippocampus[26,27] which is involved in cognitive functions, has been widely studied. The $GABA_A$ α5 receptors' density and function remain relatively intact[28,29] in the learning impairment in Alzheimer's disease while changes in many neurotransmitter receptors and overall cholinergic neurons are depleted during the disease. Several studies[10,30] suggest that an α5-subtype selective inverse agonist could be procognitive without inducing convulsions and/or anxiety and many different chemical classes endowed with selective affinity or selective efficacy have been identified.[31–33] Compounds containing the triazolopyridazine,[34] the triazolophthalazine[35] and the pyrazolotriazine[30] core showed either high affinity or high inverse efficacy at the α5 subtype. During evolution of the triazolophthalazine ligands, several bicyclic[36,37] and tricyclic compounds were synthesized[38] and among them, the ligands α5IA, α5IA-II and MRK-016 emerged.[10,39,40]

Our research group has been extensively involved in the design of derivatives containing a pyrazolo[5,1-*c*][1,2,4]benzotriazine system, with the aim of identifying ligands which bind to the Bz site on the $GABA_A$-R with high affinity and selective pharmacological activity as anxiolytic-like agents or promnemonic agents. In our recent study the 3-iodo-8-(pyridin-4-ylmethoxy)pyrazolo [5,1-*c*][1,2,4]benzotriazine 5-oxide **1**,[41] emerges as the compound that improves mouse memory processes selectively, safely, in a statistically significant manner, and in strict chemical relationship to α5IA, α5IA-II and MRK-016 (Chart 1).

In the present study, lead compound **1** was optimized mainly through classical isosteric replacement at position 8 or through the introduction of 5-membered heterocycles (as triazole, or oxadiazole) in position 3. The binding affinities of the new compounds were investigated and for preliminary pharmacological studies, six compounds among the most representative (bearing different chemical groups) were evaluated in vivo.

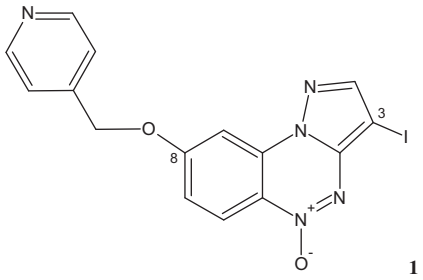
2. Chemistry

All new pyrazolo[5,1-*c*][1,2,4]benzotriazine derivatives **2**–**6**, **8**, **10**–**25** here described are listed in Table 1 (chemical data). The new 3-iodo-8-heteroarylmethylamino derivatives **2**–**5** were prepared from compound **I** 3-iodo-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide[42] treated with an excess of suitable heteroarylalkylamine. The 3-iodo-8-(pyridin-4-ylmethoxy)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide,[41] our lead compound in this work (**1**), was used as starting material for the synthesis of new compound **6**, its *N*-oxide derivative on the pyridine ring useful for SAR (Scheme 1).

Compound **II** (3-carboxy-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide[43]) was used as starting material to obtain the 3-methyl-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine derivative **8** (Scheme 2) using borane dimethylsulfide complex as reducing agent.

This borane complex is reported to reduce the carboxy group to methyl group.[44] Compound **8** was then oxidized to the corresponding N5-oxide **9**, already reported in our previous work, but synthesized with a more complex method and with minor yields.[45] The nucleophilic substitution on compound **9** with 4-pyridylmethanol, 2-chloro-4-hydroxymethylpyridine, and (2-(hydroxymethyl)furan) was realized by phase transfer catalysis (PTC),[41] and the compounds **10**–**12** were, respectively obtained (Scheme 2).

The compounds bearing the oxadiazole or the triazole ring at position 3 of the pyrazolobenzotriazine system were prepared

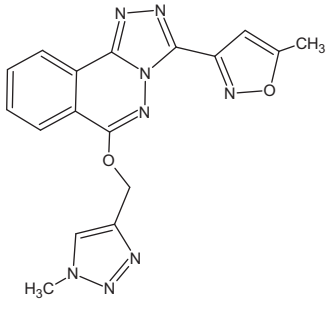


1

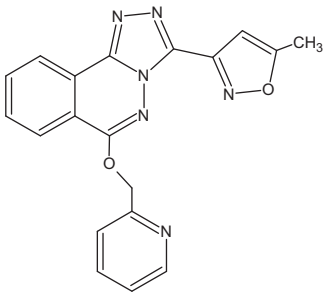
K_i value: 1.3±0.1nM

Promnemonic propertiesat 3 and 10mg/Kg

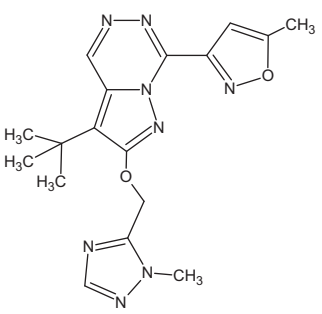
Passive avoidance test



α5IA



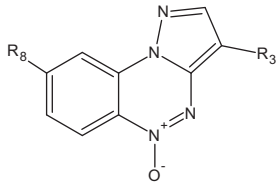
α5IA-II



MRK-016

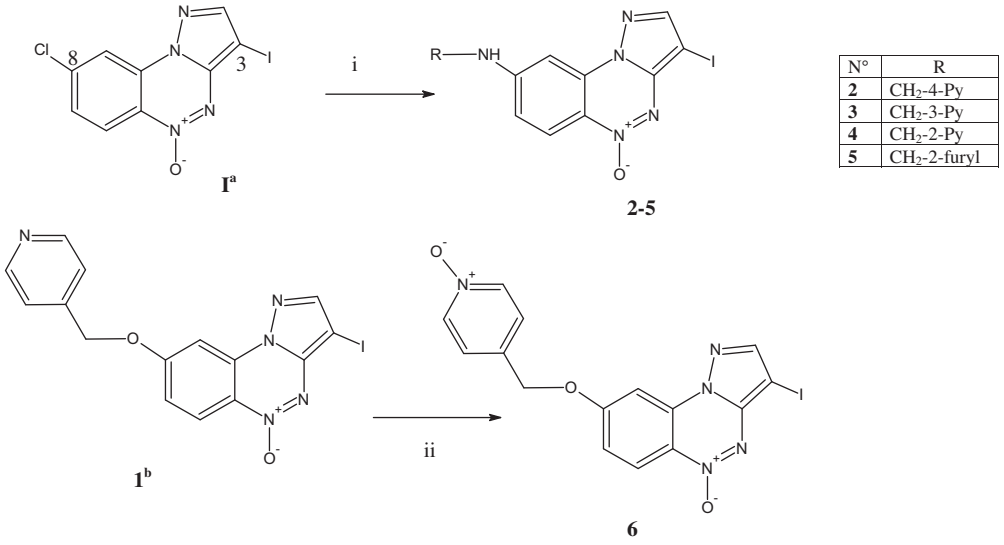
Chart 1.

Table 1
Chemical data for new synthesized compounds



No.	R_3	R_8	Yield (%)	Mp (°C) (recryst. solvent)
1[a]	I	OCH_2-4-Py		—
2	I	$NHCH_2$-4-Py	25	220–222 (Ethanol)
3	I	$NHCH_2$-3-Py	20	230 dec (Ethanol)
4	I	$NHCH_2$-2-Py	19	205 dec (Ethanol)
5	I	$NHCH_2$-2-furyl	75	210–211 (Ethanol)
6	I	OCH_2-4-Py-*N*-oxide	60	236–237 (Methoxyethanol)
7[a]	I	OCH_2-2-thienyl		—
8	Me	Cl	60	156–157 (Ethanol)
9[b]	Me	Cl	23	—
10	Me	OCH_2-4-Py	50	241–242 (Methoxyethanol)
11	Me	OCH_2-2-Cl-pyridin-4-yl	25	276 dec (Ethanol)
12	Me	OCH_2-2-furyl	50	296–297 (Ethanol)
13	1,2,4-Oxadiazol-3-Me-5-yl	OCH_2Ph	79	240–241 (Isopropyl alcohol)
14	1,2,4-Oxadiazol-3-Me-5-yl	OCH_2-4-Py	89	223–224 (Methoxyethanol)
15	1,2,4-Oxadiazol-3-Me-5-yl	OCH_2-2-thienyl	87	240 dec (Methoxyethanol)
16	$CONH_2$	OCH_2Ph	78	239–240 (Methoxyethanol)
17	$CONH_2$	OCH_2-4Py	80	240–241 (Methoxyethanol)
18	$CONH_2$	OCH_2-2-thienyl	87	229–230 (Methoxyethanol)
19	CO–N=CH –$N(Me)_2$	OCH_2Ph	80	209–210 (Toluene)
20	CO–N=CH –$N(Me)_2$	OCH_2-4-Py	88	250–251 (Toluene)
21	CO–N=CH –$N(Me)_2$	OCH_2-2-thienyl	72	235–236 (Toluene)
22	1,2,4-Triazol-3-yl	OCH_2Ph	95	259–260 (Methoxyethanol)
23	1,2,4-Triazol-3-yl	OCH_2-4-Py	80	269–270 (Methoxyethanol)
24	1-Me-1*H*-1,2,4-triazol-5-yl	OCH_2Ph	80	235–236 (Methoxyethanol)
25	1-Me-1*H*-1,2,4-triazol-3-yl	OCH_2Ph	70	255 dec (Ethanol)

[a] See Ref. 41.
[b] See Ref. 45.



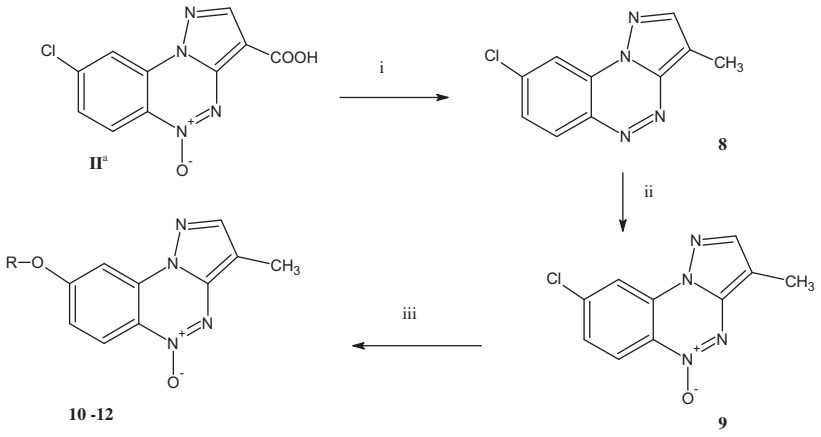
N°	R
2	CH_2-4-Py
3	CH_2-3-Py
4	CH_2-2-Py
5	CH_2-2-furyl

Scheme 1. [a]See Ref. 42; [b]see Ref. 41. Reagents and conditions: (i) EtOH, 50 °C, heteroarylalkylamine; (ii) H_2O_2 30%/AcOH.

according to two synthetic pathways (Scheme 3A and B), depending on the yields or the best reaction work-up for the obtaining of the desired compounds.

To synthesize the derivatives **13**–**15** (route A), the first step was to transform the 3-amide group of compound **III**, 3-carboxamido-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide,[43] into the dimethylaminovinyl derivative **IV**.[46] Then the next cyclization with NH_2OH·HCl gave the corresponding 3-(1,2,4-oxadiazol-3-methyl-5-yl) derivative **V**.[46] In turn, this compound represented the starting material for the nucleophilic substitution of the 8-chlorine atom by PTC. The suitable alcohol is added to a two-phase system (strong aqueous sodium hydroxide solution (40%) and methylene chloride with tetrabutylammonium bromide ($NBu_4^+Br^-$) as catalyst) and compounds **13**–**15** were obtained.

To obtain the 3-(1,2,4-triazol)-3-yl derivatives, we used route B (Scheme 3), in which the nucleophilic substitution of 8-chlorine on



N°	R
10	CH_2-4-Py
11	CH_2- 2 -Cl-Pyrid-4-yl
12	CH_2-2-Furyl

Scheme 2. [a]See Ref. 43. Reagents: (i) $BH_3{\cdot}S(CH_3)_2$ complex; (ii) H_2O_2 30%/AcOH; (iii) R-OH, NaOH 40% solution, $NBu_4^+Br^-/CH_2Cl_2$.

compound **III**[43] was the first step of synthesis and compounds **16**–**18** were obtained. Then the treatment of **16**–**18** with dimethylformamide–dimethylacetal (DMF–DMA) gave the corresponding acylamidines **19**–**21**, key intermediates for the synthesis of 3-triazole derivatives. Only intermediates **19** and **20** cyclized with hydrazine hydrate at 90 °C, gave the corresponding **22** and **23**, while the desired derivative was not obtained from compound **21** even by modifying the reaction conditions.

To better elaborate the structure affinity relationships (SAR), we decided to methylate the 1,2,4-triazole ring of compound **22** with MeI. Street[38] and Del Giudice[47] reported that with NaH as alkaline medium the two regioisomers N_1 and N_2 were prevalently obtained, while the N_4 methylation was not very easy to realize in these conditions.[48,49]

In our synthetic procedure (NaH/DMF/MeI), as expected, a mixture of two isomers in a ratio of about 3/1 (assigned by NCH_3 peaks at 4.051 and 4.153 ppm in ^{1}H NMR spectroscopy) was recovered and only one was obtained as pure product (isomer with the NCH_3 signal at 4.051 ppm) by crystallization. Unfortunately, an X-ray analysis was not performed because unsuitable crystals were obtained and it was impossible to assign the isomer structure using only ^{1}H NMR spectroscopy. Thus, to determine exactly which isomer was obtained, a different synthetic approach and different reaction conditions were used. By reaction of **19** with methylhydrazine (Scheme 4) we obtained only the isomer 3-(1,2,4-triazol-1-methyl-5-yl)-8-benzyloxypyrazolo [5,1-*c*][1,2,4]benzotriazine 5-oxide **24** whose structure was determined by crystallographic data (Fig. 1). Since the NCH_3 peak of **24** falls at 4.153 ppm, compound **24** is not the same isomer obtained by crystallization in the previous reaction. Thus, obviously, we can assign to this latter compound the structure of 3-(1,2,4-triazol-1-methyl-3-yl)-8-benzyloxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide **25**. However, to try to synthesize unambiguously the isomer **25** a literature procedure[48] was followed choosing lithium hydroxide as alkaline medium. Thus **22** was alkylated with MeI in LiOH and only the regioisomer **25** was obtained.

3. Result and discussion

3.1. In vitro binding

The Bz site/$GABA_A$-R binding affinity of newly synthesized compounds was evaluated by their ability to displace [^{3}H]flumazenil (Ro15-1788) from its specific binding in bovine brain membrane and was expressed as K_i value only for those compounds inhibiting radioligand binding by more than 80% at fixed concentrations of 10 μM.

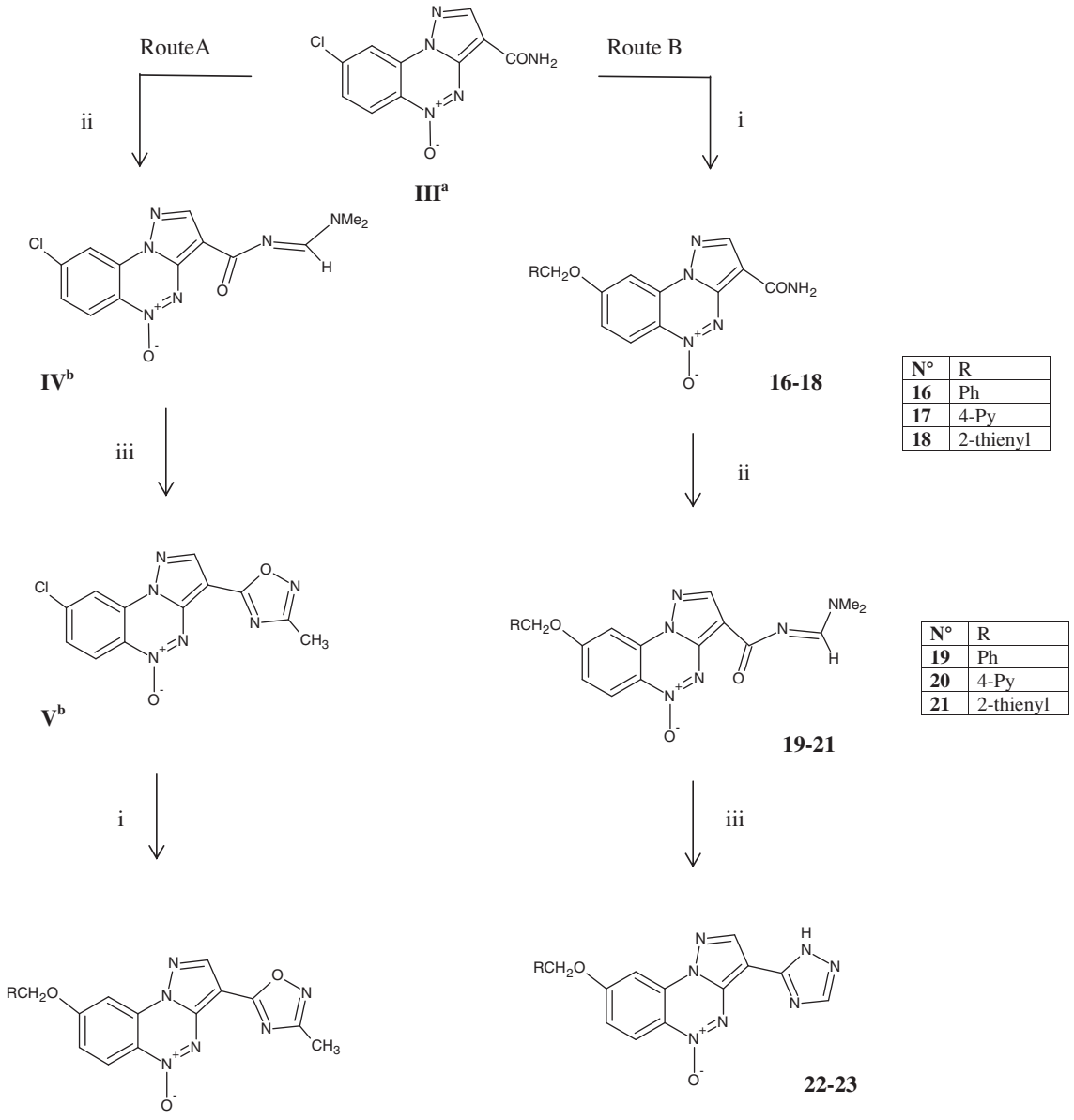
The binding data (Table 2) show that most of the new synthesized compounds have receptor recognition (except **24** and **25**) and that some compounds (**2**–**5**) were able to bind to Bz site/$GABA_A$-R with affinity value (K_i) in a range of 0.2–0.7 nM.

The isosteric substitution O/NH, at 8-position in the lead compound **1**, afforded compound **2** endowed with an excellent affinity value (**2** K_i = 0.331 vs **1**, K_i = 1.3 nM), maintained also in isomeric compounds **3** and **4**, the 3- and 2-pyridil derivatives, (K_i = 0.332 and 0.717 nM, respectively). The substitution of the pyridine ring in compound **4** with a furyl ring gives compound **5** with K_i value = 0.23 nM showing that the introduction of a 5-membered heterocycle is again positive. On the basis of binding data, we observe that compounds **2**–**5** show the best affinity values, confirming previously reported data[45] for the arylalkylamino derivatives. In fact, at position 8, the NH moiety (hydrogen bond donor) versus O (hydrogen bond acceptor) plays a key role in receptor recognition, engaging a hydrogen bond interaction stronger than the oxygen atom (data not reported).

Compound **6**, the *N*-oxide derivative of the lead compound, shows an affinity value reduced fourteen times (K_i = 20.4 nM) with respect to **1**, whereas when the pyridine ring was substituted with a thienyl ring, as in compound **7**, comparable affinity value was maintained (K_i = 1.2 nM).

The substitution on lead compound **1** of the iodine atom with a methyl group gave compound **10** with affinity binding 24-fold lower (**10** K_i = 24.1 nM vs **1** K_i = 1.3 nM), while its 2-chloroderivative **11** has a good affinity value (K_i = 7.4 nM). The substitution of the pyridine ring with a furan moiety gave comparable affinity value to **10** (K_i = 30.9 nM). Despite the reduced ability of the methyl group to form a lipophilic interaction with a receptor, (methyl, π = 0.56 and iodine π = 1.12)[50] the introduction of the heteroaromatic ring at position 8 gave a good hydro/lipophilic balance.

The substitution of iodine, at position 3 in the lead compound, with different 5-membered heterocycles, produced compounds **13**–**15** and **22**–**23** with K_i values in a range of 70.7–1623 nM. The methylation of compound **22** gave the two regioisomers **24** and **25** that are lacking receptor recognition, evidencing the key role



N°	R
16	Ph
17	4-Py
18	2-thienyl

N°	R
19	Ph
20	4-Py
21	2-thienyl

N°	R
13	Ph
14	4-Py
15	2-thienyl

N°	R
22	Ph
23	4-Py

Scheme 3. [a]See Ref. 43; [b]see Ref. 46. Reagents and conditions: (i) R-OH, NaOH 40% solution, $NBu_4^+Br^-/CH_2Cl_2$; (ii) DMF/DMA for **19**–**21** and DMA–DMA for **IV**; (iii) $NH_2NH_2{\cdot}H_2O$, EtOH, 90 °C.

of triazole NH as hydrogen bond donor or the steric hindrance of the methyl group.

We also tested the intermediates **16**–**21** useful for the synthesis of these latter derivatives and they show affinity in the range of 323–1200 nM. Compound **13** results in being the best ligand with a K_i value of 70.7 nM.

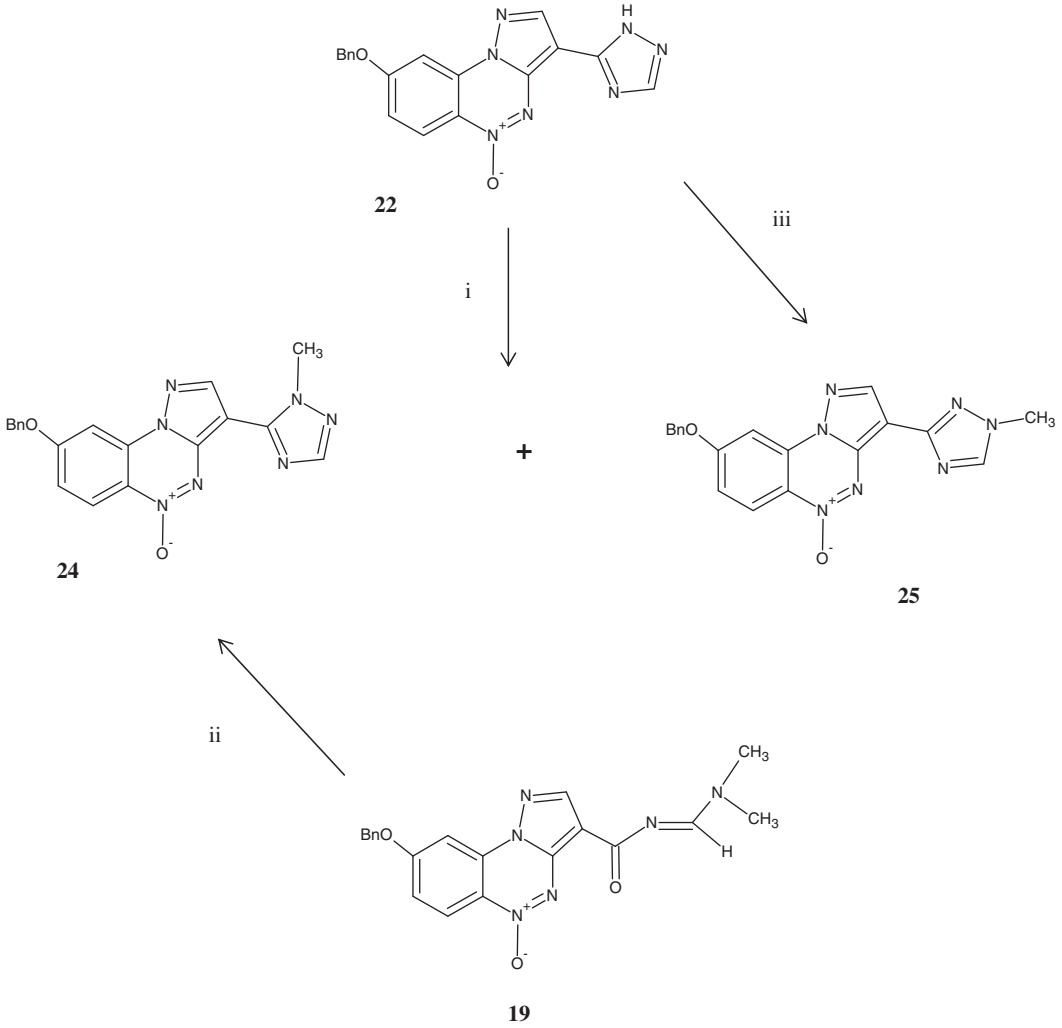
3.2. In vivo activity

Compounds **3**, **7**, **10**, **13**, **14** and **22** were chosen for in vivo tests in animal models, using various rodent species (mice and rats). The effects on learning and memory (passive avoidance test and social learning) and some potential benzodiazepine actions were considered (anticonvulsant activity, potential anxiolytic-like effects and the effect on motor coordination).

3.3. Passive avoidance test

In order to investigate the effect of compounds **3**, **7**, **10**, **13**, **14** and **22** on learning and memory, mouse performance in the passive avoidance test (an experiment in which the animal learns to avoid a noxious event by suppressing a particular behavior) was evaluated.

Examining the results, we can say that the training session latency values did not differ for the various compounds, while the



Scheme 4. Reagents: (i) NaH/DMF, MeI; (ii) $MeNHNH_2$/AcOH; (iii) LiOH/MeI/EtOH.

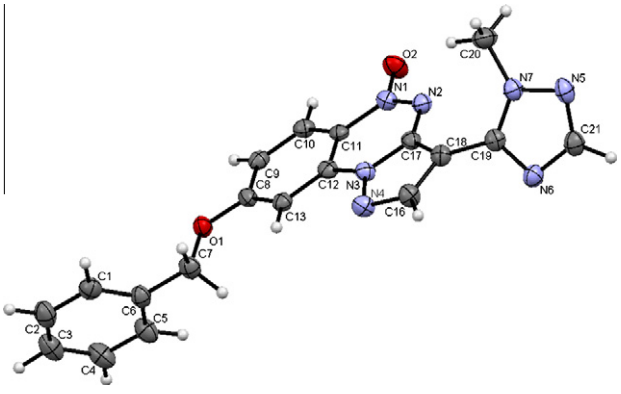


Figure 1. X-ray structure of compound **24**.

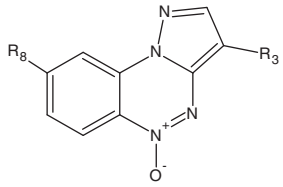
retention latencies differed for some products (depending on the doses). As depicted in Figure 2, mouse performances were tested with compounds **3** (0.1, 1, 3 and 10 mg/kg po), **7** (1, 10, 30 and 100 mg/kg po), **10** (1, 3 and 10 mg/kg po), **13** (1, 3, 10 and 30 mg/kg po), **14** (3, 10 and 30 mg/kg po), **22** (1, 3 and 10 mg/kg po). All tested compounds produced no amnesic effect, but improved mouse memory processes by increasing the retention latency compared to control in a statistically significant manner. The most interesting results were recorded for **3** at 1 mg/kg, for **10** and **22** at 3 mg/kg, and for **13** at 10 mg.

To evaluate the antiamnesic effect of the compounds, (their capability to prevent the amnesic effect of scopolamine (1.5 mg/kg)), we tested them at the dose at which they were completely devoid of promnemonic activity (**7**, **10**, **13** and **22** at 1 mg/kg; **14** at 3 mg/kg and **3** at 0.1 mg/kg) in passive avoidance test with scopolamine (Fig. 2). All compounds were able to completely prevent the amnesic effect of scopolamine.

3.4. Social learning

In the social learning test adult rats were treated with compounds **3** (0.3–10 mg/kg po), **7** (10–100 mg/kg po), **10** (1–10 mg/kg po), **13**, **14**, and **22** (10 mg/kg po) and the duration of exploration of the familiar partner in the second session was shortened in comparison with carboxymethylcellulose (CMC)-treated rats; positive control: piracetam (30 mg/kg po (Table 3). No curtailment was observed if an unknown partner was presented to adult rats (data not shown). No compound modified the duration of active exploration in the first session in comparison with CMC-treated rats. All compounds exhibited comparable efficacy even if the highest potency was exhibited by **3** (1 mg/kg) and the lowest by **7** (30 mg/kg). The shortening of the exploring time of the familiar rat induced by **3**, **7**, **10**, **13**, **14** and **22** in the social learning test indicated procognitive activity in the absence of amnesia.

Table 2
BZR ligand affinity of new compounds



No.	R_3	R_8	*I*%[a] or K_i[b] (nM)
1[c]	I	OCH_2-4-Py	1.30 ± 0.10
2	I	$NHCH_2$-4-Py	0.33 ± 0.08
3	I	$NHCH_2$-3-Py	0.33 ± 0.07
4	I	$NHCH_2$-2-Py	0.72 ± 0.06
5	I	$NHCH_2$-2-furyl	0.23 ± 0.02
6	I	OCH_2-4-Py-*N*-oxide	20.4 ± 2.9
7[c]	I	OCH_2-2-thienyl	1.20 ± 0.10
8	Me	Cl	400.0 ± 11.0
9[d]	Me	Cl	315.0 ± 20.0
10	Me	OCH_2-4-Py	24.1 ± 2.0
11	Me	OCH_2-2-Cl-pyridin-4-yl	7.49 ± 0.19
12	Me	O-CH_2-2-furyl	30.9 ± 3.0
13	1,2,4-Oxadiazol-3Me-5-yl	OCH_2Ph	70.7 ± 5.7
14	1,2,4-Oxadiazol-3Me-5-yl	OCH_2-4-Py	453.2 ± 53.7
15	1,2,4-Oxadiazol-3Me-5-yl	OCH_2-2-thienyl	1623.0 ± 150.9
16	$CONH_2$	OCH_2Ph	323.7 ± 33.0
17	$CONH_2$	OCH_2-4-Py	1200.0 ± 118.0
18	$CONH_2$	OCH_2-2-thienyl	959.6 ± 77.8
19	CO–N=CH –N$(Me)_2$	OCH_2Ph	86.6 ± 13.2
20	CO–N=CH –N$(Me)_2$	OCH_2-4-Py	514.8 ± 78.2
21	CO–N=CH –N$(Me)_2$	OCH_2-2-thienyl	620.0 ± 54.2
22	1,2,4-Triazol-3-yl	OCH_2Ph	325.2 ± 30.0
23	1,2,4-Triazol-3-yl	OCH_2-4-Py	167.9 ± 15.1
24	1-Me-1*H*-1,2,4-triazol-5-yl	OCH_2Ph	46%
25	1-Me-1*H*-1,2,4-triazol-3-yl	OCH_2Ph	50%

[a] Percent of inhibition of specific [^{3}H]Ro15-1788 binding at 10 μM concentration are means ± SEM of five determinations.
[b] K_i value are means ± SEM of five determinations.
[c] See Ref. 41.
[d] See Ref. 45.

3.5. Light–dark box

The effects of compounds **3**, **7**, **10**, **13**, **14** and **22** on mouse anxiety, compared with diazepam (10 mg/kg po), were studied using a light/dark box apparatus. All compounds were tested starting from the lowest dose at which they have procognitive activity: **3** (1 mg/kg po), **7** (30 mg/kg po), **10** (3 and 10 mg/kg po), **13**, **14** and **22** (3, 10 and 30 mg/kg po) (Fig. 3). Compounds **13**, **14** and **22** at the doses of 10 and 30 mg/kg po and **7** at the dose of 30 mg/kg po revealed an anxiolytic effect, comparable to diazepam, in a statistically significant manner. The anxiolytic effect of compounds was completely antagonized by flumazenil (at a dose of 100 mg/kg po), a dose at which flumazenil was able to antagonize the anxiolytic effect of diazepam (data not shown). Compounds **3** and **10** showed no activity in this experimental model.

3.6. Rota-rod test

The effects of compounds **3** and **10** (10 mg/kg po), **7**, **13**, **14** and **22** (30 mg/kg po) were studied in the mouse rota-rod test, in comparison with diazepam (10 mg/kg po), in order to determine any motor incoordination activity. Table 4 reports the pretest and the falls 15, 30, 45, 60 min after the treatment. None of the considered compounds induced any effect on the number of mouse falls from the rota-rod, confirming that mice learned to stay on the rota-rod since the number of falls decreased in a time-dependent manner.

Protection from convulsions was evaluated in mice using pentylentetrazole (PTZ) as chemical convulsant agent. All compounds were tested at the doses of 10 mg/kg po, except **3** that was tested at 1 mg/kg po. Diazepam (10 mg/kg po) completely protected against PTZ-induced shocks and convulsions. These compounds were devoid of any effect on PTZ-induced shocks and convulsions (Table 5).

3.7. Interaction with $GABA_A$ receptor subtypes

Based on the above pharmacological results, we decided to verify the interaction of compounds **3** and **10**, that show selective antiamnesic and promnemonic activity, with $GABA_A$ receptor subtypes (α1, α2, α5). Compound **3** was able to bind the α1- and α5-subtype receptors with an affinity value in the nanomolar range (K_i = 0.449 and 24.9 nM, respectively) and showed a percent of inhibition (*I*% = 37) for the α2-subtype receptor. Surprisingly, compound **10** was able to bind only the α1-subtype receptor (K_i = 22.7 nM) and had no affinity for α2- and α5-subtype receptors (Table 6). Binding to the $GABA_A$-R subtype indicated that compound **3** has subtype affinity for α5 that could explain its in vivo efficacy in memory tasks. Instead, compound **10**, despite its activity in the mouse/rat memory task, has no affinity for the α5-subtype and thus involvement of the GABA system is excluded.

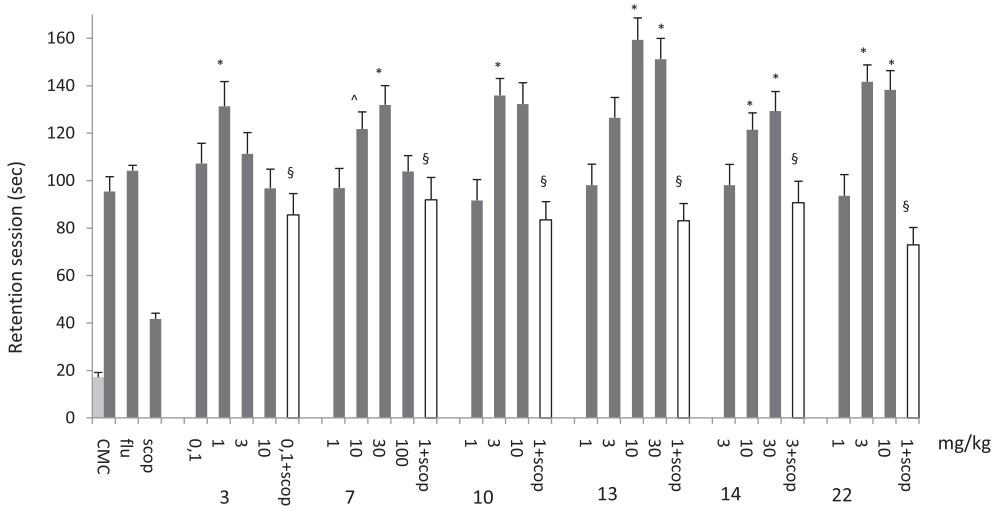


Figure 2. Effect of new compounds in Mouse Passive Avoidance test. All drugs were administered po 30 min before training session with the exception of scopolamine that was injected immediately after punishment. Each value represents the mean of 10–12 mice. The first column represents the training session of CMC-treated group. ^*P* <0.05 and **P* <0.01, versus control mice. §*P* <0.01 versus scopolamine-treated mice.

Table 3
Effect of compounds **3**, **7**, **10**, **13**, **14**, **22** in rat social learning test

Compounds	Dose (mg/kg) po	First session	Second session	Δ
Treatment				
CMC 1%	0.1 ml	63.1 ± 6.1	35.3 ± 3.3	27.6
3	0.3	58.3 ± 8.4	28.9 ± 6.1	29.4
	1	65.3 ± 3.1	21.6 ± 4.2^	43.7
	10	63.4 ± 7.5	20.9 ± 3.7^	42.5
7	10	68.3 ± 8.5	35.8 ± 3.2	32.5
	30	65.9 ± 7.3	23.6 ± 4.1^	42.3
	100	67.7 ± 6.6	25.8 ± 4.9^	41.9
10	1	62.7 ± 7.3	29.5 ± 5.5	33.2
	3	63.4 ± 5.2	20.2 ± 4.3^	43.2
	10	65.1 ± 7.3	23.7 ± 4.4^	41.4
13	10	69.8 ± 6.6	26.7 ± 3.8^	43.1
14	10	66.3 ± 7.7	21.7 ± 3.6^	44.6
22	10	65.8 ± 7.1	25.5 ± 4.3^	40.3
Piracetam	30	68.2 ± 7.3	21.9 ± 5.1^	46.3

^ P <0.05 versus control rats. Tests was performed 30' after drug administrations. Each value represents the mean of six rats.

Table 4
Effect of **3**, **7**, **10**, **13**, **14**, **22** in the mouse rota-rod test

Treatment		Falls from rotating rod after treatment			
Compounds	Dose (mg/kg) po	Pre-test	15 min	30 min	45 min
CMC 1%	0.1 mL	6.1 ± 0.5	5.5 ± 0.5	3.2 ± 0.4	2.5 ± 0.4
3	10	6.3 ± 0.5	4.9 ± 0.4	3.1 ± 0.3	2.4 ± 0.4
7	30	5.8 ± 0.4	5.1 ± 0.3	3.0 ± 0.2	2.8 ± 0.3
10	10	5.9 ± 0.5	5.2 ± 0.4	3.0 ± 0.4	2.8 ± 0.4
13	30	5.9 ± 0.5	5.4 ± 0.5	3.4 ± 0.3	2.7 ± 0.4
14	30	6.3 ± 0.6	5.3 ± 0.5	3.6 ± 0.4	2.4 ± 0.5
22	30	6.0 ± 0.4	5.3 ± 0.4	3.4 ± 0.4	2.9 ± 0.3
Diazepam	10	6.3 ± 0.3	6.4 ± 0.3^	6.4 ± 0.5*	5.6 ± 0.4*

Each value represents the mean of five mice. ^P <0.05, *P <0.01 in comparison with CMC treated mice.

3.8. Effect on ACh release

As a preliminary study, the release of ACh was evaluated and, as shown in Figure 4, a statistically significant increase of ACh release in freely moving rats was induced by administration of compound **10** (10 mg/kg ip), which peaked 60–80 min after administration and returned to basal values within 120 min.

4. Conclusion

A series of new 3,8 disubstituted pyrazolo[5,1-*c*][1,2,4]benzotriazine derivatives were designed and synthesized to identify ligands to the Bz site on $GABA_A$-R endowed with high affinity and/or selective efficacy as promnemonic agents. All new compounds were studied in in vitro tests and six compounds, with opportune features in their substituents, were chosen for preliminary in vivo evaluation of their potential effect on learning, memory and anxiety. The modification (O/NH at position 8 and I/Me at position 3) on lead compound **1** allowed us to obtain compounds **3** and **10** that have a selective promnemonic effect at 1 and 3 mg/kg. The selectivity of these two compounds was assessed in recombinant subtypes $GABA_A$-R ($\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_2\gamma_2$, $\alpha_5\beta_3\gamma_2$.): compound **3** was able to bind the α1- and α5-subtype receptors with an affinity value in the nanomolar range (K_i = 0.449 and 24.9 nM, respectively) and shows an inhibition percent (I% = 37) to α2-subtype receptor. Compound **10**, surprisingly, was able to bind only α1-subtype receptors (K_i = 22.7 nM) and shows no affinity for α2- and α5-subtype receptor. From these biological results, compound **3** has subtype affinity for α5 that could explain its in vivo efficacy on memory tasks. Instead, **10**, despite its activity on mouse/rat memory tasks, has no affinity for α5-subtype and thus another neurotransmitter, rather than GABA, could be involved. A preliminary study showed that **10** enhances the percentage of ACh release and the maximum effect was reached 60–80 min after the administration. This aspect will be the object of our next research.

Another important result was obtained for compounds **13**, **14** and **22**, which possess both promnemonic- and anxiolytic-like effects in the range of 10–30 mg/kg. Elucidation of this dual profile could be an attractive research starting point for neurological dysfunctions where both cognitive enhancement and anxiolysis are required.

5. Experimental section

5.1. Chemistry

Melting points were determined with a Gallenkamp apparatus and were uncorrected. Silica gel plates (Merk F_{254}) and silica gel

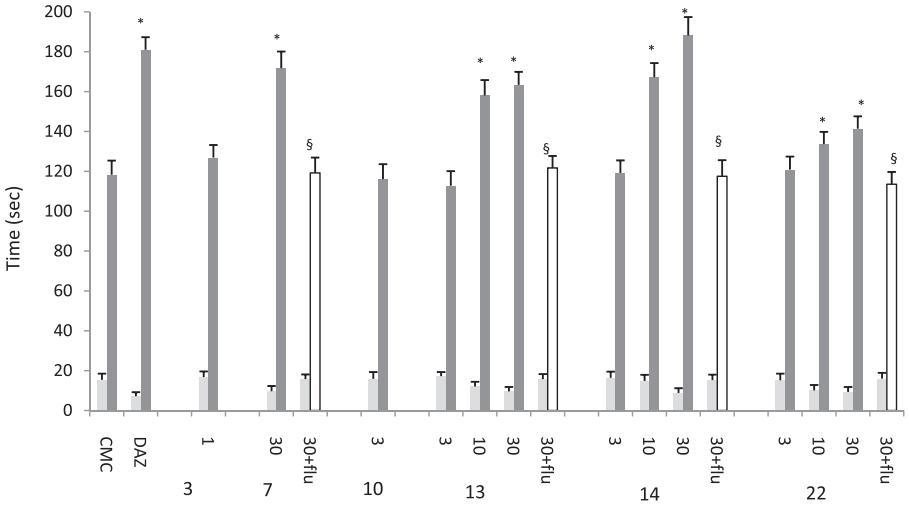


Figure 3. Effect of compounds **3**, **7**, **10**, **13**, **14** and **22** in light–dark box test. All drugs were administered po 30 min before the test. Each value represents the mean of eight mice. First column represents the number of transfer in 5 min, and the second column represents the time spent in light. *P <0.01 versus control mice. §P <0.01 versus flumazenil-treated mice.

Table 5
Lack of anticonvulsant activity of **3**, **7**, **10**, **13**, **14**, **22** in comparison with diazepam in PTZ treated mice

Compounds	Treatment (mg/kg) po	% Protection on PTZ induced convulsions
CMC 1%	0.1 mL	7.1
Diazepam	10	100*
3	1	0
7	10	0
10	10	0
13	10	10
14	10	10
22	10	0

Pentylenetetrazole (PTZ) 90 mg kg^{-1} ip. All compounds were administered 30 min before test. Each value represents the mean of 10 mice.
* P <0.01 versus control mice.

Table 6
Affinity value at recombinant $\alpha_{1,2,5}\beta_2\gamma_2$ $GABA_A$/Bz complex subtypes

No.	$I\%$[a] or K_i[b] (nM)			
	Cortex	α_1	α_2 (%)	α_5
3	0.33 ± 0.07	0.45 ± 0.04	37	24.9 ± 2.0
10	24.1 ± 2.0	22.7 ± 2.0	5	36%

[a] Percent of inhibition of specific [^{3}H]Ro15-1788 binding at 10 μM concentration are means ± SEM of five determinations.
[b] K_i value are means ± SEM of five determinations.

60 (Merk 70–230 mesh) were used for analytical and column chromatography, respectively. The structures of all compounds were supported by their IR spectra (KBr pellets in nujol mulls, Perkin–Elmer 1420 spectrophotometer) and ^{1}H NMR data (measured with a Bruker 400 MHz). Chemical shifts were expressed in δ ppm, using DMSO-d_6 or $CDCl_3$ as solvent. The chemical and physical data of new compounds are shown in Table 1; all new compounds possess a purity ⩾95%: microanalyses were performed with a Perkin–Elmer 260 analyzer for C, H, N. The crystal structure of compound **25** was solved by means of single-crystal X-ray diffraction.

5.1.1. General procedure for the synthesis of 2–5

To the starting material 3-iodo-8-chloro pyrazolo [5,1-*c*][1,2,4]benzotriazine 5-oxide **I**[42] (0.150 mmol) in EtOH (5 mL) was added an excess of suitable amine (2 mL) and was kept under vigorous stirring at 50 °C. TLC monitored the reaction and, when the starting material disappeared, ice was added to obtain a precipitate. Compounds **2** and **5** were purified by column chromatography (toluene/ethyl acetate 8/2)

5.1.2. 3-Iodo-8-(pyridin-4-ylmethylamino)pyrazolo[5,1-*c*][1,2,4] benzotriazine 5-oxide (2)

Yellow crystals; TLC eluent: toluene/ethyl acetate 8:2 v/v; IR ν cm^{-1} 3271; ^{1}H NMR (DMSO-d_6) δ 8.54 (d, 2H, H-2′ and H-6′, J = 5.5 Hz); 8.37 (m, 1H, NH, exch.); 8.23 (s, 1H, H-2); 8.14 (d, 1H, H-6, J = 8.9 Hz); 7.38 (d, 2H, H-3′ and H-5′, J = 5.5 Hz); 7.03 (m, 2H, H-7 and H-9); 4.55 (d, 2H, CH_2-N, J = 5.2 Hz). Anal. C, H, N.

5.1.3. 3-Iodo-8-(pyridin-3-ylmethylamino)pyrazolo[5,1-*c*][1,2,4] benzotriazine 5-oxide (3)

Yellow crystals; TLC eluent: toluene/ethyl acetate 8:2 v/v; IR ν cm^{-1} 3270; ^{1}H NMR (DMSO-d_6) δ 8.67 (s, 1H, H-2′); 8.50 (d, 1H, H-6′, J = 4.9 Hz); 8.42 (t, 1H, NH, exch., J = 5.2 Hz); 8.25 (s, 1H, H-2); 8.15 (d, 1H, H-6, J = 8.9 Hz); 7.80 (d, 1H, H-4′, J = 7.8 Hz); 7.40 (dd, 1H, H-5′, J = 7.8, 4.9 Hz); 7.05 (m, 2H, H-7 and H-9); 4.60 (d, 2H, CH_2-N, J = 5.2 Hz). Anal. C, H, N.

5.1.4. 3-Iodo-8-(pyridin-2-ylmethylamino)pyrazolo[5,1-*c*][1,2,4] benzotriazine 5-oxide (4)

Yellow crystals; TLC eluent: toluene/ethyl acetate 8:2 v/v; IR ν cm^{-1} 3270; ^{1}H NMR (DMSO-d_6) δ 8.58 (d, 1H, H-6′, J = 5.0 Hz); 8.40 (t, 1H, NH, exch., J = 5.3 Hz); 8.23 (s, 1H, H-2); 8.12 (d, 1H, H-6, J = 8.9 Hz); 7.79 (dt, 1H, H-4′, J = 8.0, 2.1 Hz); 7.42 (d, 1H, H-3′, J = 8.0 Hz); 7.32 (dt, 1H, H-5′, J = 7.7, 1.2 Hz); 7.06 (m, 2H, H-7 and H-9); 4.65 (d, 2H, CH_2-N, J = 5.3 Hz). Anal. C, H, N.

5.1.5. 3-Iodo-8-(fur-2-ylmethylamino)pyrazolo[5,1-*c*][1,2,4] benzotriazine 5-oxide (5)

Yellow crystals; TLC eluent: toluene/ethyl acetate 8:2v/v; IR ν cm^{-1} 3272; ^{1}H NMR (DMSO-d_6) δ 8.28 (s, 1H, H-2); 8.22 (t, 1H, NH, exch., J = 5.3 Hz); 8.14 (d, 1H, H-6, J = 8.9 Hz); 7.65 (s, 1H, H-5′); 7.18 (d, 1H, H-9, J = 2.7 Hz); 7.03 (dd, 1H, H-7, J = 8.9, 2.7 Hz); 6.43 (m, 2H, H-3′ and H-4′); 4.55 (d, 2H, CH_2-N, J = 5.3 Hz). Anal. C, H, N.

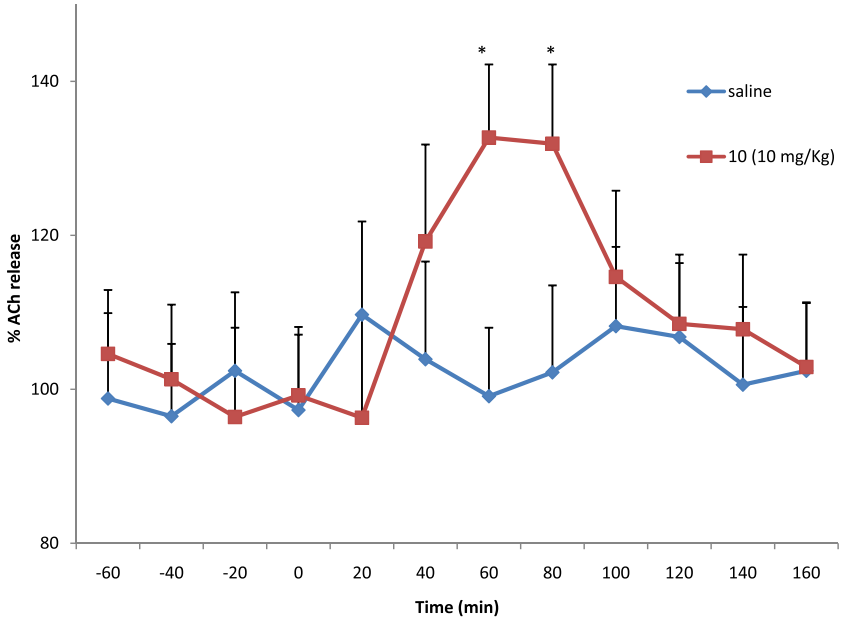


Figure 4. Release of ACh of **10**. Saline and compound **10** were injected at time '0', ip number of rat (Wistar): 6. *P <0.01 versus saline.

5.1.6. 3-Iodo-8-(1-oxidopyridin-4-ylmethoxy)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (6)

An aqueous solution of hydrogen peroxide (30%, 3 mL) in acetic acid (17 mL) was added to 1[41] (210 mg, 0.5 mmol) and the mixture was stirred at 90 °C for 3 h. The mixture was cooled, and a second portion of H_2O_2 (1 mL) was added. The mixture was stirred for another 15 h at 90 °C monitoring by TLC (eluent: dichloromethane/methanol 10:1 v/v). The pH was adjusted to 6 with aqueous solution of sodium hydroxide (10%). The yellow crude precipitate was filtered off and was recrystallyzed by methoxyethanol. Orange crystals; IR ν cm^{-1} 1538; ^1H NMR (DMSO-d_6) δ 8.40 (d, 1H, H-6, J = 8.9 Hz); 8.38 (s, 1H, H-2); 8.27 (d, 2H, H-2′ and H-6′, J = 5.5 Hz); 7.77 (d, 1H, H-9, J = 2.7 Hz); 7.55 (d, 2H, H-3′ and H-5′, J = 5.5 Hz); 7.38 (dd, 1H, H-7, J = 8.9, 2.7 Hz); 5.45 (s, 2H, CH_2O). Anal. C, H, N.

5.1.7. 3-Methyl-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine (8)

To 1 mmol of compound **II**[43] in dry THF (100 mL) a solution of 2.5 mmol borane dimetilsulphide complex (BMS) was added. The temperature was maintained approximately at 0° during the addition. The solution was then brought to reflux and maintained there for 1 h. Then, when the temperature was about 0 °C, the reaction mixture was quenched by the slow addiction of 10 mL of methanol. HCl conc. was then added slowly (pH 2), refluxed for 1 h and then, when the temperature was 0 °C, sodium hydroxide solution (40%) were added. The aqueous phase was extracted with ether (three times with a total of 100 mL). After drying with sodium sulfate, a precipitate was recovered. Yellow crystals; TLC eluent: toluene/ethyl acetate 8:2v/v; ^1H NMR ($CDCl_3$) δ 8.55 (d, 1H, H-6, J = 8.9 Hz); 8.43 (d, 1H, H-9, J = 2.7 Hz); 8.09 (s, 1H, H-2); 7.67 (dd, 1H, H-7, J = 8.9, 2.7 Hz); 2.70 (s, 3H, CH_3). Anal. C, H, N.

5.1.8. 3-Methyl-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (9)

To a solution of **8** (0.1 mmol) in acetic acid (30 mL) were added hydrogen peroxide 30% (6 mL).

The solution was refluxed for 8 h and the precipitate was filtered and recrystallized obtaining the same product reported in our previously paper.[45]

Yellow crystals; TLC eluent: isopropyl ether/cyclohexane 8:3 v/v; ^1H NMR ($CDCl_3$) δ 8.45 (d, 1H, H-6, J = 8.9 Hz); 8.35 (d, 1H, H-9, J = 2.7 Hz); 7.90 (s, 1H, H-2); 7.55 (dd, 1H, H-7, J = 8.9, 2.7 Hz); 2.40 (s, 3H, CH_3). Anal. C, H, N.

5.1.9. General procedure for the synthesis of 10–18

The reaction was carried out in 10 mL of dichloromethane to which was added the starting material **9**, **III**,[43] **V**,[46] (0.3 mmol), 5 mL of 40% sodium hydroxide solution, 0.5 mmol of tetrabutylammonium bromide (PTC), and the suitable alcohol in large excess (ratio with starting material 1:15) under vigorous stirring. The reaction was monitored by TLC, and when the starting material disappeared, the organic layer was separated and the aqueous layer extracted twice with 10 mL of dichloromethane. The combined organic extracts were evaporated and the residue was recovered with isopropil ether and then recrystallized by suitable solvent.

5.1.10. 3-Methyl-8-(pyridin-4-ylmethyloxy)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (10)

From **9** yellow crystals; TLC eluent: dichloromethane/methanol 10:1 v/v; ^1H NMR (DMSO-d_6) δ 8.62 (d, 2H, H-2′ and H-6′, J = 5.5 Hz)); 8.38 (d, 1H, H-6, J = 8.9 Hz); 8.14 (s, 1H, H-2); 7.74 (d, 1H, H-9, J = 2.7 Hz); 7.52 (d, 2H, H-3′ and H-5′, J = 5.5 Hz); 7.36 (dd, 1H, H-7, J = 8.9, 2.7 Hz); 5.52 (s, 2H, CH_2-O); 2.26 (s, 3H, CH_3). Anal. C, H, N.

5.1.11. 3-Methyl-8-(2-chloropyridin-4-ylmethyloxy)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (11)

From **9** yellow crystals; TLC eluent: toluene/ethyl acetate 8:2: v/v; ^1H NMR (DMSO-d_6) δ 8.48 (d, 1H, H-6′, J = 5.5 Hz)); 8.38 (d 1H, H-6, J = 8.9 Hz); 8.14 (s, 1H, H-2); 7.76 (d, 1H, H-9, J = 2.7 Hz); 7.68 (s, 1H, H-3′); 7.56 (d, 1H, H-5′, J = 5.5 Hz); 7.38 (dd, 1H, H-7, J = 8.9, 2.7 Hz); 5.50 (s, 2H, CH_2-O); 2.26 (s, 3H, CH_3). Anal. C, H, N.

5.1.12. 3-Methyl-8-(fur-2-ylmethyloxy)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (12)

From **9** yellow crystals; TLC eluent: toluene/ethyl acetate 8:2 v/v; ^1H NMR (DMSO-d_6) δ 8.35 (d, 1H, H-6, J = 8.9 Hz); 8.15 (s, 1H, H-2); 7.85 (d, 1H, H-9, J = 2.7 Hz); 7.75 (m, 1H, H-3′); 7.30 (dd, 1H, H-7, J = 8.9, 2.7 Hz); 6.70 (m, 1H, H-5′); 6.53 (m, 1H, H-4′); 5.40 (s, 2H, CH_2-O); 2.25(s, 3H, CH_3). Anal. C, H, N.

5.1.13. 3-(1,2,4-Oxadiazol-3-methyl-5-yl)-8-benzyloxypyrazolo[5,1-*c*][1,2,4]benzotriazine5-oxide (13)

From **V**[46] yellow crystals; TLC eluent: dichloromethane/methanol 10:1 v/v; ^1H NMR (DMSO-d_6) δ 8.91 (s, 1H, H-2); 8.44 (d, 1H, H-6, J = 8.9 Hz); δ 7.86 (d, 1H, H-9, J = 2.7 Hz); 7.55 (m, 2H, H2′ and H6′); 7.44 (m, 4H, H-7, H-3′, H-4′ and H-5′); 5.49 (s, 2H, CH_2O); 2.43 (s, 3H, CH_3). Anal. C, H, N.

5.1.14. 3-(1,2,4-Oxadiazol-3-methyl-5-yl)-8-(pyridin-4-ylmethoxy)pyrazolo[5,1-*c*][1,2,4] benzotriazine5-oxide (14)

From **V**[46] yellow crystals; TLC eluent: dichloromethane/methanol 10:1 v/v; ^1H NMR (DMSO-d_6) δ 8.92 (s, 1H, H-2); 8.67 (d, 2H, H-2′ and H-6′, J = 5.5 Hz)); 8.47 (d, 1H, H-6, J = 8.9, 2.7 Hz); δ 7.87 (d, 1H, H-9, J = 2.7 Hz); 7.61 (d, 2H, H-3′ and H-5′, J = 5.5 Hz)); 7.52 (dd, 1H, H-7, J = 8.9, 2.7 Hz); 5.61 (s, 2H, CH_2O); 2.43 (s, 3H, CH_3). Anal. C, H, N.

5.1.15. 3-(1,2,4-Oxadiazol-3-methyl-5-yl)-8-(thien-2-ylmethoxy)pyrazolo[5,1-*c*][1,2,4]benzo triazine5-oxide (15)

From **V**[46] yellow crystals; TLC eluent: dichloromethane/methanol 10:1 v/v; ^1H NMR (DMSO-d_6) δ 8.92 (s, 1H, H-2); 8.43 (d, 1H, H-6, J = 8.9 Hz); δ 7.91(d, 1H, H-9, J = 2.7 Hz); 7.63 (d, 1H, H-5′, J = 4.8 Hz); 7.45 (dd, 1H, H-7, J = 8.9, 2.7 Hz); 7.35 (d, 1H, H-3′, J = 3.5 Hz); 7.10 (dt, 1H, H-4′, J = 4.8, 3.5 Hz); 5.70 (s, 2H, CH_2-O); 2.44 (s, 3H, CH_3). Anal. C, H, N.

5.1.16. 3-Carbamoyl-8-benzyloxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (16)

From **III**[43] yellow crystals; TLC eluent: toluene/ethyl acetate 8:2 v/v; IR ν cm^{-1} 3340, 3290, 1684; ^1H NMR (DMSO-d_6) δ 8.56 (s, 1H, H-2); 8.40 (d 1H, H-6, J = 8.9 Hz); 7.82 (d, 1H, H-9, J = 2.7 Hz); 7.58 (br s, 1H, exch., NH); 7.54 (m, 2H, H-2′ and H-6′); 7.42 (m, 4H, H-7, H-3′,H-4′ and H-5′); 7.12 (br s, 1H, exch., NH); 5.47 (s, 2H, CH_2O). Anal. C, H, N.

5.1.17. 3-Carbamoyl-8-(pyridin-4-ylmethoxy)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (17)

From **III**[43] yellow crystals; TLC eluent: dichloromethane/methanol 10:1 v/v; IR ν cm^{-1} 3347, 3200, 1685; ^1H NMR (DMSO-d_6) δ 8.63 (d, 2H, H-2′ and H-6′, J = 5.5 Hz); 8.57 (s, 1H, H-2); 8.43 (d 1H, H-6, J = 8.9 Hz); 7.82 (d, 1H, H-9, J = 2.7 Hz); 7.58 (br s, 1H, exch., NH); 7.53 (d, 2H, H-3′ and H-5′, J = 5.5 Hz); 7.47 (dd, 1H, H-7, J = 8.9, 2.7 Hz); 7.12 (br s, 1H, exch., NH); 5.56 (s, 2H, CH_2-O). Anal. C, H, N.

5.1.18. 3-Carbamoyl-8-(thien-2-ylmethoxy)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (18)

From **III**[43] yellow crystals; TLC eluent: toluene/ethyl acetate 8:2 v/v; IR ν cm^{-1} 3445, 3315, 1670; ^1H NMR (DMSO-d_6) δ 8.58 (s, 1H,

H-2); 8.39 (d, 1H, H-6, J = 8.9 Hz); 7.87 (d, 1H, H-9, J = 2.7 Hz); 7.63 (d, 1H, H-5′, J = 4.8 Hz)); 7.55 (br s, 1H, exch., NH); 7.42 (dd, 1H, H-7, J = 8.9, 2.7 Hz); 7.39 (d, 1H, H-3′, J = 3.5 Hz); 7.09 (m, 2H, exch., NH and H-4′); 5.56 (s, 2H, CH_2-O). Anal. C, H, N.

5.1.19. General procedure for the synthesis of 19–21

To compounds **16**–**18** (1.9 mmol) in anhydrous toluene (10 mL) and anhydrous DMF (1 mL) was added dimethylformamide–dimethylacetale (DMF–DMA) to obtain the intermediates **19**–**21**. The reaction mixture was stirred at 100–110 °C for 2 h and after cooling, the residue was filtered and washed with diethylether.

5.1.20. 3-(*N*-(Dimethylaminomethylene)carbamoyl)-8-benzyloxypyrazolo[5,1-*c*][1,2,4] benzotriazine 5-oxide (19)

From **16** yellow crystals; TLC eluent: dichloromethane/methanol 10:1 v/v; IR ν cm^{-1} 1638; ^{1}H NMR (DMSO-d_6) δ 8.63 (s, 1H, H-2); 8.61 (s, 1H, =CH); 8.38 (d 1H, H-6, J = 8.9 Hz); 7.82 (d, 1H, H-9, J = 2.7 Hz); 7.54 (m, 2H, H-2′ and H-6′); 7.42 (m, 4H, H-7, H-3′, H-4′ and H-5′); 5.46 (s, 2H, CH_2O); 3.20 (s, 3H, N-CH_3); 3.17 (s, 3H, N-CH_3). Anal. C, H, N.

5.1.21. 3-(*N*-(Dimethylaminomethylene)carbamoyl)-8-(pyridin-4-ylmethoxy)pirazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (20)

From **17** yellow crystals; TLC eluent: dichloromethane/methanol 10:1 v/v; IR ν cm^{-1} 1641; ^{1}H NMR (DMSO-d_6) δ 8.62 (m, 4H, H-2′, H-6′; H-2 and =CH); 8.41 (d, 1H, H-6, J = 8.9 Hz); 7.81 (d, 1H, H-9, J = 2.7 Hz); 7.54 (m, 2H, H-3′ and H-5′); 7.44 (dd, 1H, H-7, J = 8.9, 2.7 Hz); 5.55 (s, 2H, CH_2-O); 3.21 (s, 3H, N-CH_3); 3.17 (s, 3H, N-CH_3). Anal. C, H, N.

5.1.22. 3-(*N*-(Dimethylaminomethylene)carbamoyl)-8-(thien-2-ylmethoxy)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (21)

From **18** yellow crystals; TLC eluent: dichloromethane/methanol 10:1 v/v; IR ν cm^{-1} 1640; ^{1}H NMR (DMSO-d_6) δ 8.64 (s, 1H, H-2); 8.60 (s, 1H, =CH); 8.38 (d, 1H, H-6, J = 8.9 Hz); 7.86 (d, 1H, H-9, J = 2.7 Hz); 7.62 (d, 1H, H-5′, J = 4.8 Hz); 7.38 (dd, 1H, H-7, J = 8.9, 2.7 Hz); 7.34 (d, 1H, H-3′, J = 3.5 Hz); 7.09 (dt, 1H, H-4′, J = 4.8, 3.5 Hz); 5.66 (s, 2H, CH_2-O); 3.20 (s, 3H, N-CH_3); 3.19 (s, 3H, N-CH_3). Anal. C, H, N.

5.1.23. General procedure for the synthesis of 22–23

To a suspension of **19** (0.30 mmol) in AcOH (10 mL) was added hydrazine hydrate (0.30 mmol, 0.05 mL). The reaction was kept at 90–100 °C for 2 h. The residue was filtered and recrystallized from a suitable solvent.

5.1.24. 3-(1,2,4-Triazol-3-yl)-8-benzyloxypyrazolo[5,1-*c*][1,2,4] benzotriazine 5-oxide (22)

From **19** yellow crystals; TLC eluent: toluene/ethyl acetate 8:2 v/v; IR ν cm^{-1} 3230; ^{1}H NMR (DMSO-d_6) δ 11.7 (br s, 1H, exch., NH); 8.66 (s, 1H, H-2); 8.50 (s, 1H, CH triazole); 8.40 (d, 1H, H-6, J = 8.9 Hz); 7.83 (d, 1H, H-9, J = 2.7 Hz); 7.55(m, 2H, H-2′ and H-6′); 7.42 (m, 4H, H-7, H-3′, H-4′ and H-5′); 5.47 (s, 2H, CH_2O). Anal. C, H, N.

5.1.25. 3-(1,2,4-Triazol-3-yl)-8-(pyridin-4-ylmethoxy) pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (23)

From **20** yellow crystals; TLC eluent: dichloromethane/methanol 10:1 v/v; IR ν cm^{-1} 3390; ^{1}H NMR (DMSO-d_6) δ 11.9 (br s, 1H, exch., NH); 8.63 (m, 4H, H-2′, H-6′; H-2 and CH triazole); 8.43 (d 1H, H-6, J = 8.9 Hz); 7.83 (d, 1H, H-9, J = 2.7 Hz); 7.53 (d, 2H, H-3′ and H-5′, J = 5.5 Hz); 7.43 (dd, 1H, H-7, J = 8.9, 2.7 Hz); 5.56 (s, 2H, CH_2-O). Anal. C, H, N.

5.1.26. 3-(1,2,4-Triazol-1-methyl-5-yl)-8-benzyloxypyrazolo [5,1-*c*][1,2,4]benzotriazine 5-oxide (24)

To a suspension of **19** (0.30 mmol) in AcOH (10 mL) was added methylhydrazine (0.05 mL). The reaction was kept at 90–100 °C for 2 h. The residue was filtered and recrystallized from a suitable solvent. Yellow crystals; TLC eluent: dichloromethane/methanol 10:1 v/v; ^{1}H NMR ($CDCl_3$) δ 8.50 (m, 2H, H-2 and H-6); 8.01 (s, 1H, CH triazole); 7.88 (d, 1H, H-9, J = 2.7 Hz); 7.46 (m, 5H, phenyl); 7.31 (dd, 1H, H-7, J = 8.9, 2.7 Hz); 5.36 (s, 2H, CH_2O); 4.16 (s, 3H, N-CH_3); Crystal data for compound **24** are reported in the Supplementary data; Anal. C, H, N.

5.1.27. 3-(1,2,4-Triazol-1-methyl-3-yl)-8-benzyloxypyrazolo [5,1-*c*][1,2,4]benzotriazine 5-oxide (25)

Method a: To a solution of (0.35 mmol) **22** in anhydrous DMF (15 mL) was added a suspension of 50% NaH (0.76 mmol, 18.2 mg) and an excess of CH_3I (0.05 mL).The reaction was kept at 50–60 °C for 18 h. The final solution was evaporated and the mixture of two isomers (3:1) was purified by recrystallization with ethanol obtaining compound **25**.

Method b: A regioselective alkylation was realized using LiOH as base and **25** as pure product was obtained. To a solution of **22** (0.035 mmol) in EtOH 96% (15 mL) was added a solution of LiOH 0.25 M (5 mL) and CH_3I (0.035 mL). Then the reaction mixture was refluxed for 20 h and the formed precipitate was filtered and purified by recrystallization.

Yellow crystals; TLC eluent: dichloromethane/methanol 10:1 v/v; ^{1}H NMR ($CDCl_3$) δ 8.62 (s, 1H, H-2); 8.50 (d, 1H, H-6, J = 8.9 Hz); 8.14 (s, 1H, CH triazole); 7.85 (d, 1H, H-9, J = 2.7 Hz); 7.46 (m, 5H, phenyl); 7.24 (dd, 1H, H-7, J = 8.9, 2.7 Hz); 5.33 (s, 2H, CH_2O); 4.05 (s, 3H, N-CH_3). Anal. C, H, N.

5.2. Radioligand binding assay on bovine cerebral cortex membranes and on $GABA_A$ receptor subtypes

[^{3}H]Ro15-1788 (specific activity 70.8 Ci/mmol) was obtained from NEN Life Sciences products. All the other chemicals, which were of reagent grade, were obtained from commercial suppliers. Bovine cerebral cortex membranes were prepared as previously described.[51] The membrane preparations were diluted with 50 mM Tris–citrate buffer, pH 7.4, and used in the binding assay. Protein concentration was assayed using the method of Lowry.[52] [^{3}H]Ro15-1788 binding studies were performed as previously reported. Clonal mammalian cell lines, expressing relatively high levels of $GABA_A$ receptor subtypes ($\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_2\gamma_2$, $\alpha_5\beta_3\gamma_2$), were maintained as previously described in minimum essential medium Eagle's with EBSS, supplemented with 10% fetal calf serum, L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2/95% air at 37 °C. After removal, the cells were harvested by centrifugation at 500×g. The crude membranes were prepared after homogenization in 10 mM potassium phosphate, pH 7.4, and differential centrifugation at 48,000 g for 30 min at 4 °C. The pellets were washed twice in this manner before final resuspension in 10 mM potassium phosphate, pH 7.4, that contained 100 mM potassium chloride [^{3}H]Ro15-1788 binding assays to transfected cell membranes was carried out as previously described.

In brief, the cell line membranes were incubated in a volume of 500 μl, which contained [^{3}H]Ro15-1788 at a concentration of 1–2 nM and test compound in the 10^{-9}–10^{-5} M range. Nonspecific binding was defined by 10^{-5} M diazepam. Assays were incubated to equilibrium for 1 h at 4 °C. The compounds were dissolved in DMSO, the level of which did not exceed 1% and which was maintained constant in all tubes. At least six different concentrations of

each compound were used. The data of $n = 5$ experiments carried out in duplicate were analyzed by means of an iterative curve-fitting procedure (program Prism, GraphPad, San Diego, CA), which provided IC_{50}, K_i, and SEM values for tested compounds, the K_i values being calculated from the Cheng and Prusoff equation.[53]

5.3. Pharmacological methods

The experiments were carried out in accordance with the Animal Protection Law of the Republic of Italy, DL No. 116/1992, based on the European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize animal suffering and to reduce the number of animals involved. Male CD-1 albino mice (22–24 g) and male Wistar rats (180–200 g) (Harlan Italy) were used. Twelve mice and three rats were housed per cage and fed a standard laboratory diet, with tap water ad libitum for 12h light/dark cycles (lights on at 7 am). The cages were brought into the experimental room the day before the experiment, for acclimatization purposes. All experiments were performed between 10:00 am and 15:00 pm.

5.3.1. Passive-avoidance test

The test was performed according to the step-through method described by Jarvik.[54] The apparatus consisted of a two-compartment acrylic box with a lighted compartment connected to a darkened one by a guillotine door. As soon as the mouse entered the dark compartment, it received a thermal shock punishment. The latency times for entering the dark compartment were measured in the training test and after 24 h in the retention test.

5.3.2. Social learning test

The social learning test was performed according to Dantzer,[55] Juvenile male rats (80–100 g) were used as social stimuli for studying adult rats (220–240 g). On the first day of the experiment, a juvenile rat was introduced into the adult male's cage and the time spent in social-investigatory behaviour by the adult male within a 5 min fixed interval was recorded. After 24 h, the same juvenile rat was placed again into the adult male's cage and social-investigatory behaviour was recorded during a 5 min interval. On the same day, the social-investigatory behaviour towards a second unfamiliar juvenile rat was also recorded.

5.3.3. Mouse light/dark box test

The apparatus (50 cm long, 20 cm wide, and 20 cm high) consisted of two equal acrylic compartments, one dark and one light, illuminated by a 60 W bulb lamp and separated by a divider with a 10×3-cm opening at floor level. Each mouse was tested by placing it in the center of the lighted area, facing away from the dark one, and allowing it to explore the novel environment for 5 min. The number of transfers from one compartment to the other and the time spent in the illuminated side were measured. This test exploited the conflict between the animal's tendency to explore a new environment and its fear of bright light.

5.3.4. Rota-rod test

The integrity of the animals' motor coordination was assessed using a rota-rod apparatus (Ugo Basile, Varese, Italy) at a rotating speed of 24 rpm. The number of falls from the rod in 30 s were counted 15, 30, 45 and 60 min after drug administration.

5.3.5. Pentylenetetrazole (PTZ)-induced seizure

PTZ (90 mg/kg sc) was injected 30 min after the administration of drugs. The frequency of the occurrence of clonic generalized convulsions was noted over a period of 30 min.

5.3.6. Determination of ACh release by cerebral microdialysis

Microdialysis was performed in the rat parietal cortex according to Giovannini.[56] The coordinates used for implantation of the horizontal microdialysis tube (AN 69 membrane, molecular weight cut off >15 kDa, Dasco, Italy) were AP 0.5 mm and H 2.3 mm from the bregma.[57] One day after surgery, the microdialysis tube was perfused at a constant flow rate (2 pl/min) with Ringer solution (NaCl 147, KCl 4.0, $CaCl_2$ 1.2 mM) containing 7 pM physostigmine sulphate. After a 1 h settling period, the perfusate was collected at 20 min intervals in test tubes containing 5 pl of 0.5 mM HC1 to prevent ACh hydrolysis. The samples were then assayed for ACh content, either immediately or after freezing. ACh was detected and quantified by high performance liquid chromatography (HPLC) with an electrochemical detector, as described by Damsma.[58]

5.4. Drugs

Diazepam (Valium 10—Roche), flumazenil (Roche), pentylenetetrazole (PTZ) (Sigma), scopolamine (Sigma) were used. All drugs except PTZ were suspended in 1% carboxymethylcellulose sodium salt and sonicated immediately before use. PTZ was dissolved in isotonic (NaCl 0.9%) saline solution. Drug concentrations were prepared in such a way that the necessary dose could be administered in a volume of 10 ml/kg by the po, ip or sc routes.

5.5. Statistical analysis

Results are given as the mean ± SEM. Statistical analysis was performed by means of ANOVA, followed by Scheffe's post-hoc test. Student's two-tailed t-test was used to verify the significance between two means. Data were analyzed using a computer program (Number Cruncher Statistical System, Version 5.03 9/92). For percentage values, Chi-Square analysis was used in accordance with Tallarida and Murray. $P < 0.05$ were considered significant.

5.6. Crystallographic analyses

The data were collected at 150(2)K on Xcalibur3 CCD 4-circle diffractometer using a graphite monochromator, Mo Ka radiation. A reference frame was monitored every 50 frames to control the stability of the crystal and the system revealed no intensity decay. The data set was corrected for Lorentz, polarization effects, absorption corrections were performed by the ABSPACK multiscan procedure of the CrysAlis[59] data reduction package. The structure was solved using direct method with SHELXS-97 software, and the refinement was carried out using the SHELXL-97[60] software package. All non-hydrogen atoms were located from the initial solution or from subsequent electron density difference maps during the initial course of the refinement. After locating the non-hydrogen atoms, the models were refined against F^2, first using isotropic and finally anisotropic thermal displacement parameters. Finally, the hydrogen atoms were located from the electron density difference maps and isotropically refined. Programs used in the crystallographic calculations included WinGX[61] and ORTEP for graphics.[62] Crystal structural data are available from the Cambridge Crystallographic Data Center CCDC No. 846436

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.02.027.

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