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Novel 3-iodo-8-ethoxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide as promising lead for design of α5-inverse agonist useful tools for therapy of mnemonic damage

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Abstract—The synthesis and the binding study of new 3-iodopyrazolo[5,1-*c*][1,2,4] benzotriazine 5-oxides 8-alkyloxy substituted are reported. The replacement at position 3 with an iodine atom, with respect to substituents capable to form a three centered hydrogen bond and/or to form π–π stacking interaction with receptor protein, gave high affinity ligands, independently of the 8-alkyloxy substituent. High-affinity ligands were studied in mice in vivo for their pharmacological effects, considering five potential benzodiazepine actions: anxiolytic-like effects, motor coordination, anticonvulsant action, mouse learning and memory impairment, and ethanol-potentiating action. Compounds **5c** and **5′c** have an inverse agonist profile and for the first time is evidenced a pro-mnemonic activity. These compounds were evaluated also for their binding at Benzodiazepine site on $GABA_A$ receptor complex ($GABA_A$/BzR complex) subtype to evaluate their subtype selectivity.

1. Introduction

Benzodiazepines, widely used in therapy for their anxiolytic, hypnotic, muscle-relaxant, and anticonvulsant activity, allosterically modulate the $GABA_A$ receptors, the largest population of brain inhibitory neurotransmitter receptors. $GABA_A$ receptors belong to the same super-family of pentameric ligand-gated ion channel ($LGIC_S$) as glycine, nicotinic, cholinergic, serotonin-$5HT_3$ and a recently discovered protein ZAC, which is a zinc-activated channel.[1] In $GABA_A$ receptors the binding of two molecules of agonist γ-aminobutyric acid (gaba) is believed to result in a conformational change that leads to the opening of a selective chloride ion channel that causes hyperpolarization of the post-synaptic membrane.

Purification, sequencing, and cloning of $GABA_A$ receptors have led to the identification of 16 different subunits (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, π) among which the α, β, γ subunits are necessary to form a pentameric functional $GABA_A$/BzR complex. Recent studies[2–4] suggest that the principal (60%) $GABA_A$ receptor isoform in the adult brain is $\alpha_1\beta_2\gamma_2$ and subunit stoichiometry is $2\alpha_1 2\beta_2 1\gamma_2$. $GABA_A$ receptors containing α_2 or α_3 subunits constituted an additional 10 to 20% of the total population followed by α_5-containing receptors that are the least abundant and are expressed only in the hippocampus. The benzodiazepine binding site lies at the $\alpha_{1-2,3,5}/\gamma_2$-subunit interface and their pharmacological

Keywords: $GABA_A$ benzodiazepine receptor ligands; Pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxides; Pyrazolo[5,1-*c*][1,2,4]benzotriazine 4-oxides; Inverse agonist; Tricyclic heteroaromatic system.
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effects seem to be associated with the type of α-isoform.[5] Through the use of transgenic mice with point mutations in the α subunit that render the corresponding receptor insensitive to diazepam (while retaining responsivity to gaba), it has been shown that α_1-containing receptors are responsible for mediating the sedative/muscle relaxant activity as well as part of seizure protection and amnesic effect of diazepam. Receptors containing the α_2- and/or α_3-subunit are important for anxiety with their contribution to anxiolysis most probably depending on their receptor occupancy level. The function of α_5-containing receptors is not well understood, even if their relatively high expression in the hippocampus can support a role in cognitive process and/or in neurobehavioral action of alcohol.[6–17]

It is clear that the need to identify subtype selective ligands, either functionally selective or binding selective, may lead to more selective drugs with improved activity and/or fewer side-effects for the treatment of anxiety, sleep disorders, convulsions, and memory deficits.

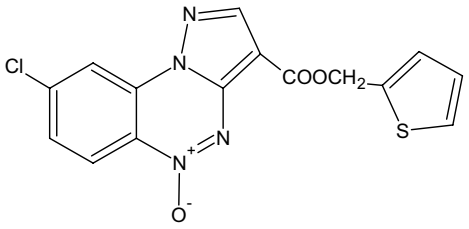
Therefore, the goal of our research was to identify high selective affinity ligands or high selective efficacy ligands pursuing a medicinal chemistry program based on our previous findings.

Recently we reported that within the pyrazolo[5,1-*c*]-[1,2,4]benzotriazine series, the different lipophilic, steric, and electronic features of these substituents seem to influence the in vivo efficacy of ligands.[18–20] From our research it has appeared particularly intriguing that the simple substitution of the chlorine atom ($\sigma = 0.23$) at position 8 with an ethoxy group ($\sigma = -0.24$)[21] in the 3-(2-thienylmethoxycarbonyl)-8-chloropyrazolo-[5,1-*c*] [1,2,4]benzotriazine 5-oxide[20] brings about an overturning of the in vivo profile from agonist to inverse agonist. While the 8-chloroderivative[19] shows a selective anxiolytic-like activity, the 8-ethoxy derivative[20] shows selective anxiogenic properties (see Chart 1).

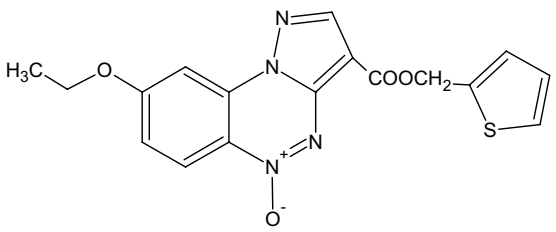
The structure–affinity relationships (SAR) of our pyrazolo[5,1-*c*][1,2,4]benzotriazine derivatives were rationalized assuming that these series of compounds bind the $GABA_A$/BzR complex through N1 and N4 atoms by means of a hydrogen bond involving H_2 and H_1 donor receptor site.[18–20,22] Substituents in position 8 support the hydrophobic interaction of the 3-ligand substituent with receptor protein in the lipophilic pockets (L_1/L_2) influencing the affinity binding. It seems that the electronic nature of the 8-substituent could permit it to interact with 'critical residue'[23,24] on the same pocket of various subtype receptors eliciting different pharmacological effects. Pharmacophoric descriptors, hydrogen-bonding sites (H_1/H_2), lipophilic pockets (L_1/L_2), and steric hindrance areas have been defined in various models that are in accordance with those formulated by other researchers.[25–27]

In the attempt to induce the inverse-agonist efficacy toward the α_5-subtype receptor, to obtain ligands useful either in therapy as cognition enhancers in Alzheimer's disease and related dementias[14] or in the pathogenesis of hepatic encephalopathy,[28] we decided to synthesize a new series of pyrazolo[5,1-*c*][1,2,4]benzotriazine derivatives. The substituent at position 8 was maintained able to confer high affinity (as halogen, methyl, ethoxy, methoxy, methylthio),[20] while at position 3 a lipophilic substituent (e.g., halogen atom) was introduced. This is unable to form a three-centered hydrogen bond, as hypothesized for 3-ester derivatives[19,29,30] and unable to form a π–π stacking interaction, as hypothesized for 3-pentatomic heteroaryl derivatives.[18]

As a first step, the introduction of a halogen atom (Cl, Br, I) at position 3 was realized, also considering that some 3-bromopyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide derivatives previously reported[31] resulted as good affinity ligands. Among the 3-halogen derivatives, the 3-iodo substituted compounds showed the best affinity value, and in particular 3-iodo-8-ethoxypyrazolo[5,1-*c*]-[1,2,4]benzotriazine 5-oxide (**5c**). Then, using the 3-iodopyrazolo[5,1-*c*][1,2,4]benzotriazine as core lead, we tried to optimize this structure with introduction of various ethereal groups at position 8- with a longer alkylic chain, or more hindered or functionalized than



Anxiolytic-like properties[19]



Lead

Anxiogenic properties[20]

Chart 1.

the ethoxy group; also the introduction at the same position of a hydroxy group, highly hydrophilic ($\pi = -0.67$) and able to form a hydrogen bond, was realized.

Evaluation of the importance of the N-oxide group and the influence of its position (4 or 5) in the molecule on the affinity and efficacy was realized with the synthesis of 3-iodo-8-ethoxypyrazolo[5,1-*c*][1,2,4]benzotriazine, **5cR** and 3-iodo-8-ethoxypyrazolo[5,1-*c*][1,2,4] benzotriazine 4-oxide, **5c′**. From a previous study[32] it stands out that some pyrazolo[5,1-*c*][1,2,4] benzotriazine 4-oxides were good affinity ligands at the $GABA_A$/BzR complex endowed with GR (gaba ratio) < 1 and then potentially displaying an inverse agonist profile.

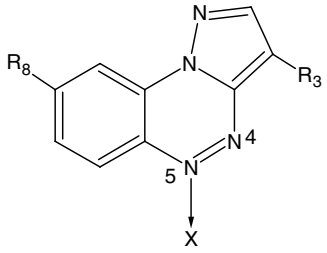
2. Chemistry

The starting material for the synthesis of 3-haloderivatives was the corresponding 3-unsubstituted pyrazolo [5,1-*c*][1,2,4]benzotriazine 5-oxide variously substituted at position 8: **1**,[33] **2**, **3**, **4**,[31] and **5**.[33] All compounds described here are listed in Tables 1 and 2.

Cyclization in basic conditions of 1-(2-nitro-5-bromophenyl)- **I** and 1-(2-nitro-5-iodophenyl)-5-aminopyrazole **II**, obtained from acid hydrolyses of corresponding ethyl 5-aminopyrazole-4-carboxylate,[20] gave new compounds **2** and **3**, following the described method for **1**, **4**, and **5**.[31,33] The treatment of **1**–**5** with *N*-chlorosuccinimide (NCS), bromine and iodine monochloride (ICl) in chloroform in the classical halogenation reactions (see Scheme 1) gave the corresponding 3-chloro- **1a**–**5a**, the 3-bromo- **1b** and **4b**,[31] **2b**, **3b**, and **5b**[33] and the 3-iodo derivatives **1c**, **4c** and **5c**,[20] **2c**, **3c**.

To evaluate the importance of the N-oxide group, its position in the molecule (4 or 5), and the possibility to enhance the inverse agonist profile that seems to be conferred by the ethoxy group in position 8,[20] compounds **5cR** and **5′c** the deoxyderivative and 4-oxide isomer of **5c**, respectively, were synthesized. The 8-ethoxypyrazolo[5,1-*c*][1,2,4]benzotriazine, **5R**[32] was useful starting material for the synthesis of 4-oxide derivative **5′**, as previously described;[32] either **5R** or **5′** was halogenated with ICl yielding the 3-iodo-8-ethoxypyrazolo[5,1-*c*]-[1,2,4]benzotriazine, **5cR** and 3-iodo-8-ethoxypyrazolo-[5,1-*c*][1,2,4] benzotriazine 4-oxide, **5′c** (see Scheme 2).

Table 1. Chemical data for pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxides

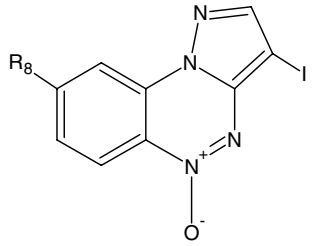


Compound	R_3	R_8	X	MF (M_W)	Yield (%)	Mp °C (recryst. solvent)
1	H	Cl	O			
2	**H**	**Br**	O	$C_9H_5ON_4Br$ (264.97)	55	224–225 °C (ethanol)
3	**H**	**I**	O	$C_9H_5ON_4I$ (312.06)	90	235–236 °C (ethanol)
4	H	CH_3	O			
5	H	OCH_2CH_3	O			
5R	H	OCH_2CH_3	—			
5′	H	OCH_2CH_3	O[a]			
1a	**Cl**	**Cl**	O	$C_9H_4ON_4Cl_2$ (255.0)	70	240–241 °C (ethanol)
2a	**Cl**	**Br**	O	$C_9H_4ON_4BrCl$ (299.51)	60	247–248 °C (ethanol)
3a	**Cl**	**I**	O	$C_9H_4ON_4ClI$ (346.51)	75	249–250 °C (ethanol)
4a	**Cl**	**CH_3**	O	$C_{10}H_7ON_4Cl$ (234.56)	70	195–196°C (methoxyethanol)
5a	**Cl**	**OCH_2CH_3**	O	$C_{11}H_9O_2N_4Cl$ (264.67)	90	232–233 °C (methoxyethanol)
1b	Br	Cl	O			
2b	**Br**	**Br**	O	$C_9H_4ON_4ClBr$ (299.51)	67	259–260 °C (ethanol 80%)
3b	**Br**	**I**	O	$C_9H_4ON_4BrI$ (390.86)	90	267–268 °C (ethanol 80%)
4b	Br	CH_3	O			
5b	Br	OCH_2CH_3	O			
1c	I	Cl				
2c	**I**	**Br**	O	$C_9H_4ON_4BrI$ (390.86)	82	251–252 °C (methoxyethanol)
3c	**I**	**I**	O	$C_9H_4ON_4I_2$ (437.95)	65	276–277 °C (ethanol)
4c	I	CH_3	O			
5c	I	OCH_2CH_3	O			
5cR	**I**	**OCH_2CH_3**	—	$C_{11}H_9ON_4I$ (340.10)	45	188–190 °C (ethanol)
5′c	**I**	**OCH_2CH_3**	O[a]	$C_{11}H_9O_2N_4I$ (356.10)	40	198–199 °C (2-propanol)

In bold new compounds.

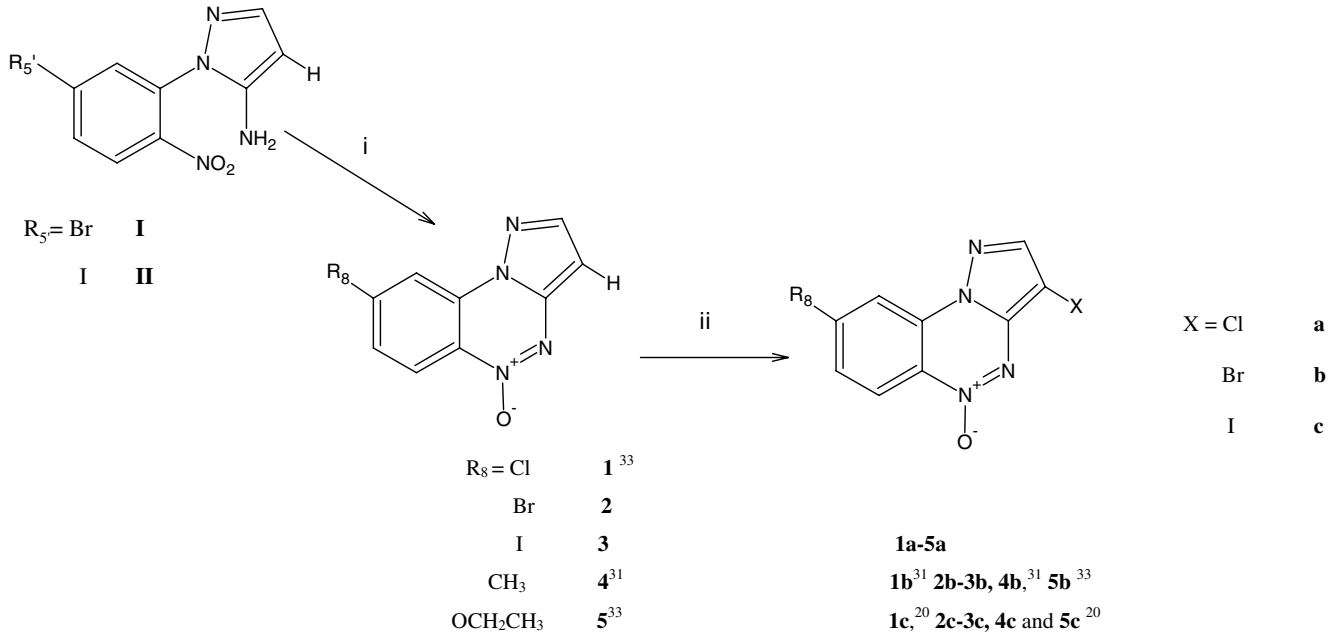
[a] N4-oxide isomer. For compounds **1**, **5**, and **5b** see 33. For compounds **4**, **1b**, and **4b** see Ref. 31. For compounds **5R** and **5′** see Ref. 32. For compound **1c** see Ref. 35. For compounds **4c** and **5c** see Ref. 20.

Table 2. Chemical data for new 3-iodo-8-alkyloxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxides



Compound	R_8	MF (M_W)	Yield (%)	Mp, °C (recryst. solvent)
6	OH	$C_9H_5O_2N_4$ I (328.06)	55	300 °C dec (ethanol 80%)
9	$OCH(CH_3)_2$	$C_{12}H_{11}O_2N_4I$ (370.14)	40	156–157 °C (ethanol)
10	$O(CH_2)_2CH_3$	$C_{12}H_{11}O_2N_4I$ (370.14)	45	204–205 °C (ethanol)
11	$O(CH_2)_3CH_3$	$C_{13}H_{13}O_2N_4I$ (384.17)	55	161–163 °C (ethanol)
12	$O(CH_2)_4CH_3$	$C_{14}H_{15}O_2N_4I$ (398.23)	65	144–145 °C (ethanol)
13	OCH_2-cyclopropyl	$C_{13}H_{11}O_2N_4I$ (382.15)	50	229–230 °C (ethanol)
14	$O(CH_2)_2OCH_3$	$C_{12}H_{11}O_3N_4I$ (386.15)	35	207–209 °C (ethanol/water)
15	$O(CH_2)_2N(CH_3)_2$	$C_{13}H_{14}O_2N_5I$ (399.18)	55	182–183 °C (ethanol, 80%)
16	$O(CH_2)_2Cl$	$C_{11}H_8O_2N_4ICl$ (390.56)	53	219–220 °C (2-propanol)
17	$OCH_2C{\equiv}CH$	$C_{12}H_7O_2N_4I$ (366.11)	40	219–220 °C (ethanol)

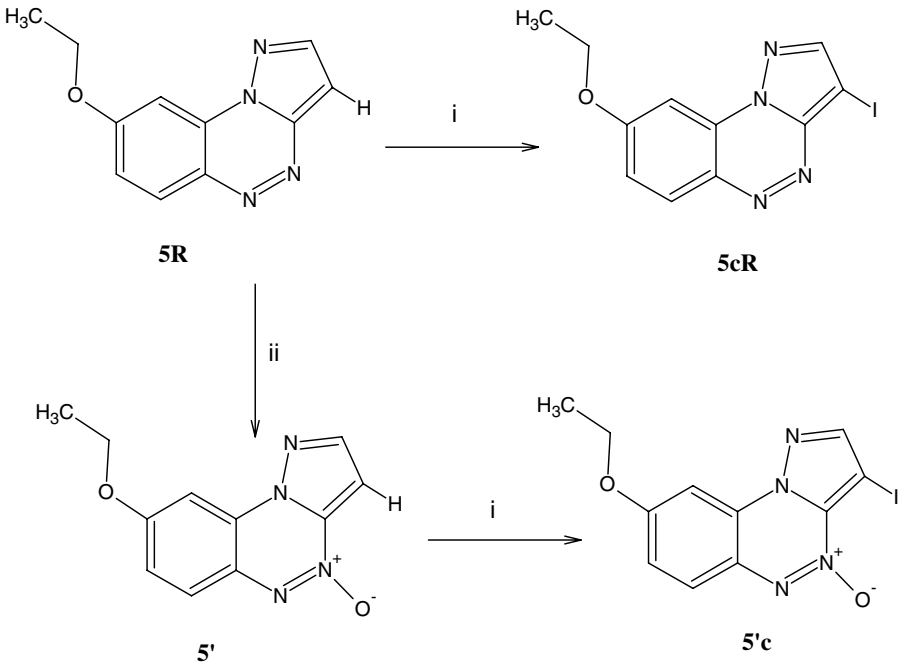
For compounds **7** and **8** see Ref. 20.



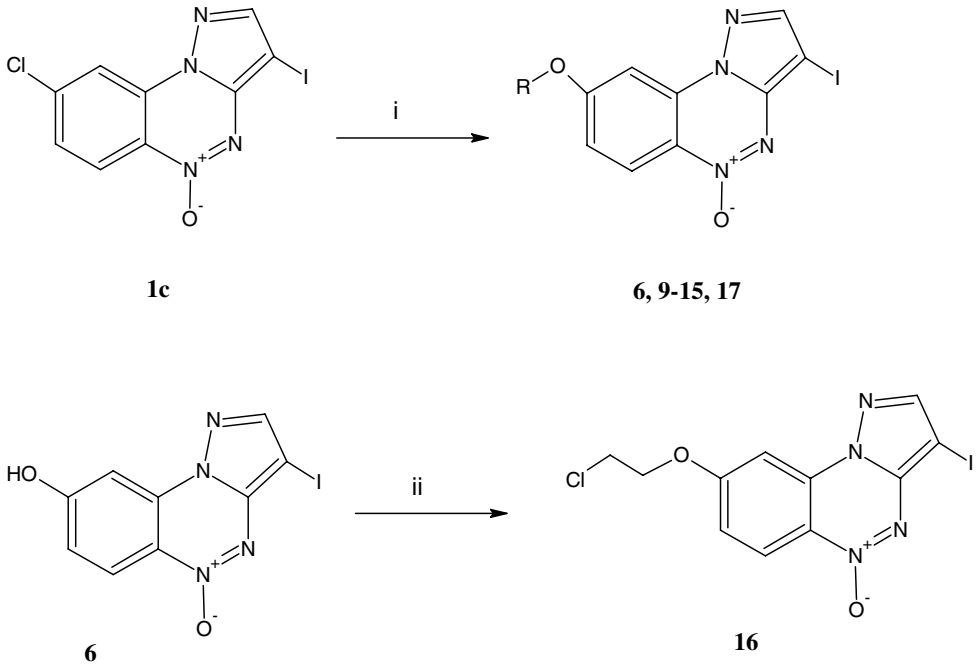
Scheme 1. Reagents: (i) 10% sodium hydroxide solution. (ii) NCS or Br_2 or ICl in $CHCl_3$.

To better explore the role of the ethereal group at position 8, new compounds, **6**, **9**–**17** bearing at position 3 the iodine atom (the best 3-haloderivative in terms of binding data), were synthesized. Compounds **7** (8-methoxyderivative) and **8** (8-thiomethylderivative)[20] in this report are useful tools for structure–affinity relationships (SAR), see Table 2.

The starting material to obtain all new 3-iodo-8-alkyloxy derivatives was compound **1c**, 3-iodo-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide. Treatment of **1c** with a 40% sodium hydroxide solution and a few milliliters of diethylene glycol dimethyl ether (diglyme) yielded the 8-hydroxy derivative **6**, after dilution and acidification, as orange crystals. When the 40% sodium hydroxide solution was used as catalyst and *N*,*N*-dimethylaminoethanol as reagent/solvent, compound **15** was achieved. Products **9**–**14**, **17** were obtained exploiting the phase transfer catalyzed (PTC)[34] chlorine displacement at position 8 of the benzotriazine system. In this procedure the suitable alcohol was added to a two-phase system consisting of a strong aqueous sodium hydroxide solution (40%), catalyst (tetrabutylammonium bromide, $Bu_4N^+Br^-$) and a methylene chloride solution of **1c** (see Scheme 3). The same PTC system was followed for compound **16** using as starting material the 3-iodo-8-hydroxyderivative, **6**, and chlorobromoethane as reagent.



Scheme 2. Reagents: (i) ICl in $CHCl_3$. (ii) H_2O_2/AcOH/Ac_2O as in Ref. 32, see the same reference also for compounds **5R** and **5′**.



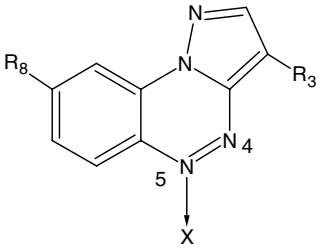
Scheme 3. Reaction conditions: (i) 40% sodium hydroxide solution for **6**; tetrabutylammonium bromide NBu_4^+Br, ROH, CH_2Cl_2 for **9**–**14**, **17**; *N*,*N*-dimethylethanolamine/40% sodium solution as catalyst for **15**. (ii) 40% sodium hydroxide solution, tetrabutylammonio bromide $NBu_4^+Br^-$, CH_2Cl_2, $ClCH_2CH_2Br$.

3. Biological results

The $GABA_A$/Bz receptor complex binding affinity of new 3-halogen derivatives 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. Binding data for 3-halogen derivatives (**1a**–**5a**, **2b**–**3b**, **1c**–**5c**), 5-deoxy and 4-oxide derivatives (**5cR** and **5c′**), 3-iodo-8-alkoxy derivatives (**6**–**17**), and for reference compounds useful for the SAR discussion are reported in Tables 3 and 4.

It is noteworthy that within the 3-halogen series (Cl, Br, I) (see Table 3), the best 3-substituent is iodine independently from 8-substituent; in fact, the affinity K_i range was 374–160 nM for 3-chloro derivatives, 120–36.9 nM

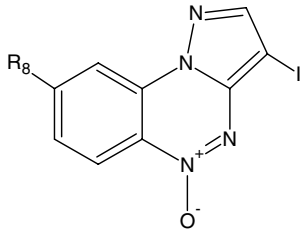
Table 3. $GABA_A/BzR$[a] Ligand affinity of pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxides 3-halogeno derivatives



Compound	R_3	R_8	X	K_i[b]
1a	Cl	Cl	O	374 ± 30
2a	Cl	Br	O	332 ± 30
3a	Cl	I	O	202 ± 13
4a	Cl	Me	O	196 ± 16
5a	Cl	OEt	O	160 ± 11
1b[c]	Br	Cl	O	120
2b	Br	Br	O	73 ± 8
3b	Br	I	O	81 ± 6
4b[c]	Br	Me	O	53.2
5b[d]	Br	OEt	O	36.9
5′b[e]	Br	OEt	O[f]	15.6
1c	I	Cl	O	22.8 ± 1.3
2c	I	Br	O	14 ± 2
3c	I	I	O	14 ± 2
4c	I	Me	O	12 ± 1
5c	I	OEt	O	4.8 ± 0.5
5cR	I	OEt	—	18.0
5′c	I	OEt	O[f]	1.7

[a] From bovine cortical membranes.
[b] K_i values are means ± SEM of five determinations.
[c] See Ref. 31.
[d] See Ref. 33.
[e] See Ref. 32.
[f] N4-oxide isomer.

Table 4. $GABA_A/BzR$[a] ligand affinity of 3-iodo-8-alkyloxypyrazolo-[5,1-*c*][1,2,4]benzotriazine 5-oxides



Compound	R_8	K_i[b] (nM)
5c	OCH_2CH_3	4.8
6	OH	1700
7	OCH_3	13.5
8	SCH_3	15.8
9	$OCH(CH_3)_2$	42
10	$O(CH_2)_2CH_3$	12
11	$O(CH_2)_3CH_3$	5.4
12	$O(CH_2)_4CH_3$	6.5
13	OCH_2-cyclopropyl	7.6
14	$O(CH_2)_2OCH_3$	37
15	$O(CH_2)_2N(CH_3)_2$	233
16	$O(CH_2)_2Cl$	62
17	$OCH_2C{\equiv}CH$	**0.8**

[a] From bovine cortical membranes.
[b] K_i values are means ± SEM of five determinations.

for 3-bromo derivatives, and 22.8–4.8 nM for 3-iodo derivatives. On the other hand, the electronic features of the 8-substituent also influenced the affinity: the most electron-donating group, in this case the ethoxy group ($\sigma = -0.24$), was the best substituent, and this trend was observed within every 3-halogen series (3-chlorine, 3-bromine, 3-iodine). Then, the more lipophilic atom at position 3, iodine ($\pi = 1.43$), is synergic with a more electron-donating atom at position 8 (see compound **5c**, $K_i = 4.8$ nM).

When the 3-iodopyrazolo[5,1-*c*][1,2,4]benzotriazine scaffold was substituted at position 8 with a series of etheric chains, a trend was evidenced (see Table 4).

From compounds **6**–**17** binding data emerge that the decreased affinity of compound **6** ($K_i = 1700$ nM), the 8-hydroxy derivative, shows that a hydrophilic group at position 8 is detrimental for receptor affinity as previously reported.[31] For the other compounds (**7**–**17**), an evaluation on the basis of the type of etheric chain in position 8 can be made.

As regards compounds **7**, **8** and **10**, a reduced value of affinity of about threefold, with respect to the 'new lead' compound 8-ethoxy derivative **5c** ($K_i = 4.8$ nM), is shown: $K_i = 13.5$ nM, $K_i = 15.8$ nM, and $K_i = 12.0$ nM, respectively. In the case of the 8-thiomethoxy derivative, **8**, the presence of the sulfur atom instead of oxygen, as in **7**, is negligible.

Compounds **9**, **14**, **15**, and **16**, bearing an isopropoxy-, methoxyethoxy-, *N*,*N*-dimethylaminoethoxy- and 2-chloroethoxy group at position 8, show the lowest binding affinity: **9**, $K_i = 42$ nM; **14**, $K_i = 37$ nM; **15**, $K_i = 233$; **14**, $K_i = 62$. In all cases this reduction of affinity is probably due to steric hindrance, a much enhanced feature in compound **15**. The substitution of the oxygen atom of the methoxy group of **14** with a methylene group, as in the *n*-butoxy derivative **11**, enhanced the binding affinity by about fivefold: **11** $K_i = 5.4$ nM versus **14**, $K_i = 37$ nM. Surprisingly, the insertion of another methylene group as in compound **12**, (8-pentyloxy derivative) or the presence of a semi-rigid residue, as in the 8-methylcyclopropyloxy derivative **13**, maintains the affinity value comparable to that of compound **11**; the K_i values are 6.5 and 7.6 nM, respectively.

The best affinity value is shown by compound **17**, *O*-propinyl derivative, with a K_i value in the sub-nanomolar range (0.8 nM): probably the presence of a triple bond, capable of forming π–π stacking interaction, is responsible for this high affinity. Moreover, this 8-substituent presents the same carbon number as compound **10** but in this latter case the presence of simple bonds permits the chain to have many conformations, while in compound **17** the geometry of the substituent is linear and this fact can permit a better receptor protein interaction.

As far as compounds **5cR** and **5′c** are concerned, either the lack of the 5-oxide group as in **5cR**, or its shift to

4-position as in **5′c**, maintained $GABA_A$/BzR complex affinity in the nanomolar range. In particular, the deoxyderivative **5cR** follows the trend of *N*-deoxy compounds[31] which, with respect to the 5-oxide derivatives, have lower affinity values (**5cR**, K_i = 18.0 nM vs **5c** K_i = 4.8nM). In the case of the 4-oxide compound, **5′c**, the affinity value is very good (K_i = 1.7 nM) showing that even the position of the N-oxide group is important. In fact, if the N-oxide group is in 4-position it will be capable per se of directly interacting with the donor site (H_2/H_1) on the receptor protein; this fact is evidenced also by the compound couple **5b** and **5b′** (see Table 3).[32] Thus, we can hypothesize that in the 5-oxide series the N-oxide group reinforces the interaction of the $N4/H_1$ donor site by means of a three-centered hydrogen bond, while in the 4-oxide series the same group becomes protagonist, as evidenced in jointed compounds **5b/5b′** and **5c/5c′**.

On the other hand, as stated previously, the presence at the position 3 of an iodine atom confers good affinity. In fact comparing compounds **5′c** and **5′b**, 3-bromo-8-ethoxypyrazolo[5,1-*c*][1,2,4]benzotriazine 4-oxide[32] (Table 3), a tenfold lower value of affinity is shown by compound **5′b** with respect to compound **5′c** (K_i = 15.6 nM vs K_i = 1.7 nM).

3.1. Binding of selected compounds at $\alpha_{x(1,3,5)}\beta_{2/3}\gamma_2$ $GABA_A$/BzR complex subtype

Compounds **5c**, **5cR**, and **5′c** were chosen to evaluate on recombinant rat $\alpha_{x(1,3,5)}\beta_{2/3}\gamma_2$ $GABA_A$/BzR complex receptor subtype, which are stably expressed in human embryonic kidney cells (HEK293), their ability to displace [^{3}H]Ro15-1788, in comparison with diazepam (full agonist) (Table 5).

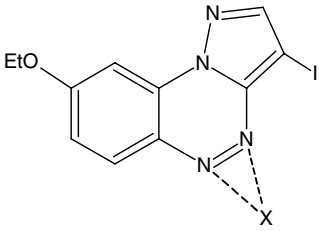
4. Pharmacological results

The same compounds which were studied at recombinant receptor **5c**, **5cR**, and **5′c** were studied in mice in vivo for their pharmacological effects. Five potential benzodiazepine actions were considered: potential anxiolytic-like effects were screened using light/dark choice test, motor coordination with the rota-rod test; the anticonvulsant action was evaluated using the new drugs against pentylenetetrazole-induced convulsions; mouse learning and memory impairment was evaluated by passive avoidance test; and finally, the drugs were tested also for their ethanol-potentiating action. Diazepam was used as the positive reference molecule (see Table 6 and Figs. 1–3).

4.1. Effect on motor coordination

The effects of compounds **5c**, **5cR**, and **5′c** on animal motor coordination were investigated, using the mouse rota-rod test as screening method, to discover any ataxic effect as compared to diazepam (Table 6). As expected, the reference compound dose-dependently (0.3, 1 and 3 mg/kg po) increased the number of falls from the rotating rod, reaching statistical significance at a dose of 3 mg/kg in comparison with the vehicle-treated group of mice (Table 6). None of the newly synthesized substances induced any effect on the number of mice falls from the rota-rod, as reported in Table 6.

Table 5. Affinity value at recombinant $\alpha_{x(1,3,5)}\beta_2\gamma_2$ $GABA_A$/BzR subtypes



Compound	X	K_i[a] (nM)			
		Cortex	α1	α3	α5
5c	5-O	4.8	4.1	34	99
5cR	—	18.0	13.8	133	95
5′c	4-O	1.7	2.6	12.3	70
Diazepam[b]		10	12	14	9.80

[a] K_i values represent the means ± SEM derived from three independent experiments, conducted in triplicate.
[b] See Ref. 18.

4.2. Effect on mouse anxiety

Effects on mouse anxiety of newly synthesized molecules and diazepam were studied using a light/dark box apparatus. In our experiments, compound **5cR** showed a weak anxiolytic-like effect at doses of 3-10 mg/kg (Fig. 1) but it was not antagonized by flumazenil at a dose of 100 mg/kg ip, a dose at which flumazenil was able to antagonize the anxiolytic effect of diazepam.[20] This finding could be explained by hypothesizing that the anxiolytic-like activity of **5cR** is due to activation of other receptor systems involved in anxiety. In the opposite manner, compounds **5c** and **5′c** decreased the time spent in the light compartment of the light/dark box apparatus in a statistically significant way (Fig. 1), showing anxiogenic-like effects. Compound **5c** at 10 mg/kg also diminished the number of transfers from one compartment to the other in a statistically significant manner (Fig. 1). This parameter often seems to indicate an anxiogenic-like activity.[36,37] The anxiogenic-like effects of **5c** and **5′c** were completely antagonized by the nonselective benzodiazepine antagonist flumazenil (Fig. 1), confirming the inverse agonist profile at the $GABA_A$/BzR complex of the newly synthesized ligands. Recently it has been proposed that not only the activation of the α_2- but also of the α3-subtype is 'responsible' for the anxiolytic-like effect of benzodiazepine ligands.[9,38–40]

4.3. Effect against chemically induced convulsions

Anticonvulsant activity was studied using pentylenetetrazole (PTZ) as chemical convulsant stimuli. Diazepam (0.3, 1, 3 mg/kg po) dose-dependently and significantly

Table 6. Motor coordination and anticonvulsant effects of new compounds in comparison with diazepam

Treatment[a]	mg/kg po	Motor coordination rota-rod test		Anticonvulsant activity		
		n	*n* of falls in 30 s	*n*	Latency (s)	Against PTZ-induced attacks
CMC 1%[b]	0.1 ml	9	0.8 ± 0.4	11	289.1 ± 63.1	0%
Diazepam	0.3	10	0.5 ± 0.27	10		70%***
	1	15	0.6 ± 0.21	15		100%***
	3	6	1.2 ± 0.4*	6		100%***
5c	3	9	0.2 ± 0.1	10		10%
	10	10	0.2 ± 0.1	10		0%
	30	10	0.2 ± 0.1	10		0%
5cR	3	9	0.3 ± 0.2	7	237.7 ± 40.2	0%
	10	8	0.7 ± 0.3	8		0% (1)[c]
5cR+ diazepam	3			8	1210.6 ± 364.8*	12.5%
	0.3					
5′c	3			8	293.4 ± 67.5	0%
	10	8	0.4 ± 0.2	8		12.5% (3)[c]
	30	8	0.6 ± 0.3			
5′c+ diazepam	3			8	613.0 ± 147.3	0%
	0.3					

[a] Treatment with new compounds and diazepam (po) was performed 30 min and flumazenil (ip) 40 min before the test. *$P < 0.05$, ***$P < 0.001$ versus control mice.
[b] Carboxymethylcellulose 1%.
[c] Number of dead mice.

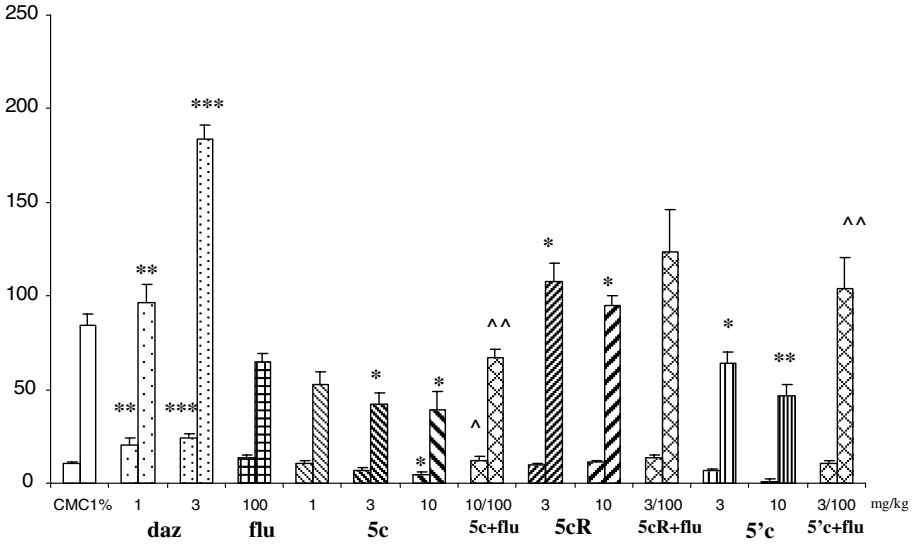


Figure 1. Effect of compounds **5c**, **5cR**, and **5′c** on light–dark box test in mice in comparison with that of **daz** (diazepam) (po). Each column represents the number of transfers in 5 min and the time spent in light time (s). Treatment with compounds and diazepam (ip) was performed 30 min and with **flu** (flumazenil) (ip) 40 min before the test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus controls (ANOVA, Fischer's test). ^$P < 0.05$, ^^$P < 0.01$ versus **5c**-, **5cR**-, and **5′c**-treated mice.

protected mice from PTZ-induced convulsions (Table 6), while effects caused by newly synthesized compounds, **5c**, **5cR**, and **5′c**, did not differ significantly from those of controls. On the other hand, **5′c** was able to prevent the protective effect of diazepam on PTZ-induced convulsions (Table 6). None of the other molecules demonstrated any anticonvulsant action, nor were they able to prevent the protective effect of diazepam on PTZ-induced convulsions. It has been reported that the anticonvulsant effect is principally due to activation of the α_1-subtype: the antagonism exerted by **5′c** against diazepam-induced protection was probably due to an antagonism on α_1-subtype $GABA_A$/Bz receptor complex.

4.4. Effects on mouse learning and short-term memory

To investigate the effect that newly synthesized compounds have on learning and memory, mice performance on passive avoidance test, in which the punishment consisted in a fall (40 cm) into cold water (10 °C)[41] instead of a painful electric foot shock, was investigated. In this assay, the difference in time (s)

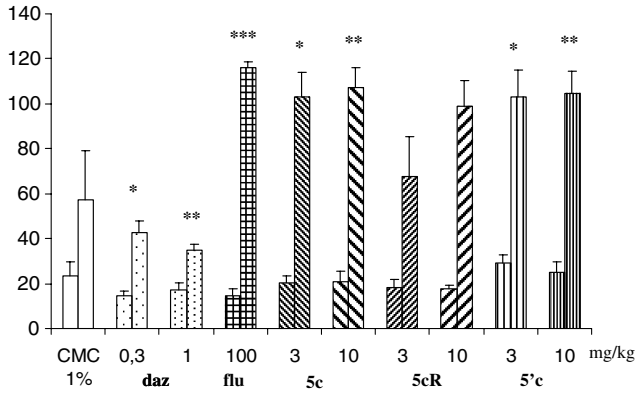


Figure 2. Effect of compounds **5c**, **5cR**, and **5′c** on learning and memory performance on passive avoidance test, in comparison with those of **daz** (diazepam, po) and **flu** (flumazenil, ip). Mice were treated 30 min before the training test (first column). The retention test was performed 24 h later (second columns). Each column represents means ± SEM of 9–10 mice. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ versus controls (ANOVA, Fischer's test).

taken to enter the dark compartment on training and retention test is used as an index of how the mouse has remembered the fall into cold water. As can be seen in Figure 2, compounds **5c** and**5′c** enhanced, in a statistically significant manner at all tested doses (3, 10 mg/kg), the mouse performance. The enhanced cognition, therefore, might be associated to an inverse agonist efficacy to α_5-subtype receptor.[14,15]

4.5. Effect on ethanol-induced sleeping time

The potency of diazepam to affect the ethanol-induced sleeping time is well-known and alcohol intake may compromise the daytime use of anxiolytics. Ethanol-reinforced actions are often connected to the activation of the α_5-subtype receptor, and high affinity ligands endowed with inverse agonism profile at this subtype receptor could be very useful to attenuate the neurobehavioral actions of alcohol.[16,17]

Ligand **5c**, at the highest anxiogenic-like doses (3, 10 mg/kg), significantly cut down the ethanol-induced sleeping time, with respect to the controls; the effect of ligands **5′c** and **5cR**, instead, did not differ from that of the controls (Fig. 3). As regards the α_5 subunit, compounds **5c**, **5′c** and **5cR** bind to it with an intermediate affinity value (K_i = 99 nM, K_i = 95 nM and K_i = 70 nM, respectively). If the ethanol additive effects are assumed to be due to activation of this subunit with agonist profile, in our case compound **5c** might behave on this subunit as inverse-agonist.

5. Conclusion

Taken together, the biological and pharmacological data reported in this work represent an effective development with respect to our previously reported studies.[19,20] As far as the SAR data are concerned, the replacement at position 3 with an iodine atom, a lipophilic substituent unable to form a three-centered hydrogen bond and/or unable to form a π–π stacking interaction with receptor protein[18,20,30] yielded high affinity ligands, independently of the 8-alkyloxy substituent.

A preliminary evaluation of the lipophilic pocket size, where the 8-substituent could fit, can be evidenced: when the 8-alkyloxychain is lengthened up to five carbon atoms (**10**–**12**), high affinity ligands have been obtained; a partial steric hindrance of the 8-substituent is however tolerated, as in compounds **14**–**16** that have only a reduced affinity value.

The surprising high affinity of compound **17**, the 8-propenyloxy derivative, suggests that in position 8 the π–π interaction with receptor protein is highly required. This latter finding is worthy of further chemical and biological /pharmacological investigation.

In vivo pharmacological studies, in particular on 8-ethoxy derivatives **5c** and **5′c**, show for these ligands an inverse-agonist/antagonist profile. Both compounds

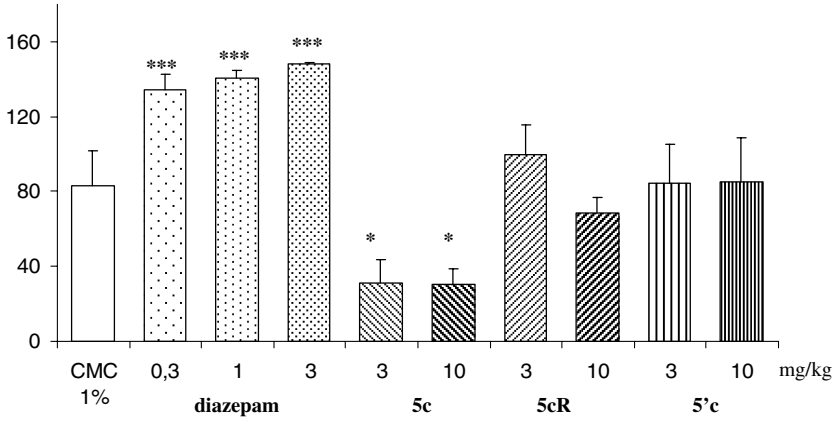


Figure 3. Effect of compounds **5c**, **5cR**, and **5′c** on ethanol-induced sleeping time, in comparison with that of diazepam (po). Each column represents means ± SEM of 5–16 mice. Substances were administered 30 min before ethanol (4 g/kg ip). $*P < 0.05$, and $***P < 0.001$ versus controls (ANOVA, Fischer's test).

have neither anticonvulsant nor motor coordination effects, suggesting an antagonist profile at α1-subtype receptor. As regards the light–dark box test, the same compounds, at 3 and 10 mg/kg, have an anxiogenic effect due, probably, to an inverse-agonist profile to α2/α3-subtype receptors, analogously to the lead compound (see Chart 1). The innovation is that compounds **5c** and **5′c** (N5- and N4-oxide isomers, respectively) with respect to the lead compound have, in a statistical manner, a pro-mnemonic activity and, only for compound **5c**, the ability to cut down the ethanol-induced sleeping time showing a full inverse-agonist profile at α5-subtype receptor.

In summary, the introduction at position 3 of an iodine atom on the 8-ethoxypyrazolo[5,1-*c*] [1,2,4]benzotriazine core enables obtainment of ligands which, with respect to the lead compound, maintain high affinity at α1-, α3-subtype receptor and, for the first time, display good affinity at α5-subtype receptor. Moreover, they have enlarged the efficacy spectrum. The electron-donating group at position 8 confers to the ligand inverse-agonist properties, as previously stated.[20]

The shift of the N-oxide group from position 5 to position 4 (**5c** and **5′c**) gives ligands endowed with anxiogenic properties and endowed with pro-mnemonic activity, while the effect on ethanol-induced sleep time is maintained only in compound **5c**.

With the aim of obtaining useful ligands for therapy of mnemonic damage, compound **5′c** and in particular compound **5c** became the new leads on which to design compounds with enhanced α5-inverse agonist profile. Further studies are in progress to eliminate the anxiogenic undesirable activity.

6. Experimental

6.1. Chemistry

Melting points were determined with a Gallenkamp apparatus and are uncorrected. Silica gel plates (Merk F_{254}) and silica gel 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 coupling constant values ($J_{H6–H7,\ H7–H6}$; $J_{H7–H9,\ H9–H7}$) were in agreement with the assigned structure. The chemical and physical data of new compounds are shown in Tables 1 and 2; microanalyses were performed with a Perkin-Elmer 260 analyzer for C, H, N, and the results were within ±0.4% of the theoretical value.

6.1.1. General procedure for the synthesis of I and II. A suspension of ethyl 1-(2-nitro-5-bromophenyl)- and ethyl 1-(2-nitro-5-iodophenyl)-5-aminopyrazolo-4-carboxylate (5.0 mmol) in 15 mL concd hydrochloric acid was refluxed for 8 h. After cooling at room temperature the respective hydrochlorides were filtered. Upon dissolution in water/ice and neutralization with conc. ammonia, the 5-aminopyrazoles **I** and **II** were filtered and characterized.

6.1.1.1. 1-(2-Nitro-5-bromophenyl)-5-aminopyrazole I. Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 3401, 3324; ^{1}H NMR ($CDCl_3$) δ 7.88 (d, 1H, H-4′); 7.85 (d, 1H, H-6′); 7.70 (dd, 1H, H-4′); 7.48 (d, 1H, H-3); 5.70 (d, 1H, H-4); 4.20 (br s, exch. 2H, NH_2). Anal C, H, N.

6.1.1.2. 1-(2-Nitro-5-iodophenyl)-5-aminopyrazole II. Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 3407, 3327; ^{1}H NMR (DMSO-d_6) δ 8.00 (m, 2H, H-3′ and H-6′); 7.76 (dd, 1H, H-4′); 7.28 (d, 1H, H-3); 5.50 (bs, exch. 2H, NH_2); 5.42 (d, 1H, H-4);. Anal C, H, N.

6.1.2. General procedure for the synthesis of 2 and 3. A suspension of **I** and **II** (0.5 mmol) in 25 mL of 10% sodium hydroxide was kept at 40 °C for 3 h. The yellow precipitates were filtered and recrystallized from ethanol.

6.1.2.1. 8-Bromopyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 2. From **I**. Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1590; ^{1}H NMR (DMSO-d_6) δ 8.48 (d, 1H, H-9); 8.32 (d, 1H, H-6); 8.28 (d, 1H, H-2); 7.86 (dd, 1H, H-7); 6.92 (d, 1H, H-3). Anal C, H, N.

6.1.2.2. 8-Iodopyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 3. From **II**. Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1584; ^{1}H NMR (DMSO-d_6) 8.62 (d, 1H, H-9); 8.26 (d, 1H, H-2); 8.12 (d, 1H, H-6); 8.02 (dd, 1H, H-7); 6.90 (d, 1H, H-3). Anal C, H, N.

6.1.3. General procedure for the synthesis of 3-halogen derivatives 1a–5a, 1b–5b, 1c–5c, 5cR, and 5′c. A solution of suitable 8-substituted pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide **1**, **2**, **3**, **4**, **5** (0.50 mmol) in chloroform (10 mL) was added of *N*-chlorosuccinimide (NCS) (100 mg) and a catalytic amount of benzoyl peroxide to obtain compounds **1a**–**5a**; of an excess of bromine (1.0 mL) to obtain compounds **1b**, **2b**–**3b**, **4b** and **5b**; of a iodine monochloride (1:2) chloroform solution to obtain compounds **1c**, **2c**–**3c**, **4c**, **5c** and the reaction was monitored by TLC. Even compounds **5R** and **5′** were treated with ICl in the same above conditions to obtain **5cR** and **5′c**. The final solution was evaporated to dryness and the crude residue was recrystallized by suitable solvent.

6.1.3.1. 3-Chloro-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (1a). From **1** and NCS. Yellow crystals. TLC eluent: dichloromethane/methanol 10:0.5 v/v; IR ν^{-1} 1586; ^{1}H NMR ($CDCl_3$) δ 8.48 (d, 1H, H-6); 8.38 (d, 1H, H-9); 8.03 (s, 1H, H-2); 7.60 (dd, 1H, H-7). Anal C, H, N.

6.1.3.2. 3-Chloro-8-bromopyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (2a). From **2** and NCS. Yellow crystals. TLC eluent: isopropyl ether/cyclohexane 8:3 v/v; IR ν^{-1}

1589; ^{1}H NMR ($CDCl_3$) δ 8.54 (d, 1H, H-9); 8.40 (d, 1H, H-6); 8.06 (s, 1H, H-2); 7.76 (dd, 1H, H-7). Anal C, H, N.

6.1.3.3. 3-Chloro-8-iodopyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (3a). From **3** and NCS. Yellow crystals. TLC eluent: isopropyl ether/cyclohexane 8:3 v/v; IR ν^{-1} 1586; ^{1}H NMR ($CDCl_3$) δ 8.76 (d, 1H, H-9); 8.22 (d, 1H, H-6); 8.06 (s, 1H, H-2); 7.98 (dd, 1H, H-7). Anal C, H, N.

6.1.3.4. 3-Chloro-8-methylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (4a). From **4** and NCS. Yellow crystals. TLC eluent: isopropyl ether/cyclohexane 8:3 v/v; IR ν^{-1} 1586; ^{1}H NMR ($CDCl_3$) δ 8.40 (d, 1H, H-6); 8.18 (d, 1H, H-9); 8.06 (s, 1H, H-2); 7.42 (dd, 1H, H-7); 2.64 (s, 3H, CH_3). Anal C, H, N.

6.1.3.5. 3-Chloro-8-ethoxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (5a). From **5** and NCS. Yellow crystals. TLC eluent: isopropyl ether/cyclohexane 8:3 v/v; IR ν^{-1} 1586; ^{1}H NMR ($CDCl_3$) δ 8.44 (d, 1H, H-6); 8.02 (s, 1H, H-2); 7.64 (d, 1H, H-9); 7.14 (dd, 1H, H-7); 4.25 (q, 2H, CH_2); 1.58 (t, 3H, CH_3). Anal C, H, N.

6.1.3.6. 3-Bromo-8-bromopyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (2b). From **2** and bromine. Orange crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1588; ^{1}H NMR ($CDCl_3$) δ 8.56 (d, 1H, H-9); 8.42 (d, 1H, H-6); 8.10 (s, 1H, H-2); 7.76 (dd, 1H, H-7). Anal C, H, N.

6.1.3.7. 3-Bromo-8-iodopyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (3b). From **3** and bromine. Orange crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1585; ^{1}H NMR ($CDCl_3$) δ 8.78 (d, 1H, H-9); 8.22 (d, 1H, H-6); 8.08 (s, 1H, H-2); 7.96 (dd, 1H, H-7). Anal C, H, N.

6.1.3.8. 3-Iodo-8-bromopyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (2c). From **2** and ICl. Orange crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1587; ^{1}H NMR ($CDCl_3$) δ 8.56 (d, 1H, H-9); 8.40 (d, 1H, H-6); 8.12 (s, 1H, H-2); 7.76 (dd, 1H, H-7). Anal C, H, N.

6.1.3.9. 3-Iodo-8-iodopyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (3c). From **3** and ICl. Dark yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1585; ^{1}H NMR ($CDCl_3$) δ 8.78 (d, 1H, H-9); 8.22 (d, 1H, H-6); 8.11 (s, 1H, H-2); 7.80 (dd, 1H, H-7). Anal C, H, N.

6.1.3.10. 3-Iodo-8-ethoxypyrazolo[5,1-*c*][1,2,4]benzotriazine (5cR). From **5R** and ICl. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; ^{1}H NMR ($CDCl_3$) δ 8.60 (d, 1H, H-6); 8.30 (s, 1H, H-2); 7.75 (d, 1H, H-9); 7.35 (dd, 1H, H-7); 4.35 (q, 2H, CH_2); 1.58 (t, 3H, CH_3). Anal C, H, N.

6.1.3.11. 3-Iodo-8-ethoxypyrazolo[5,1-*c*][1,2,4]benzotriazine 4-oxide (5′c). From **5′** and ICl. TLC eluent: diisopropylether/cyclohexane 8:3 v/v; ^{1}H NMR (DMSO-d_6) δ 8.40 (s, 1H, H-2); 7.86 (d, 1H, H-6); 7.60 (d, 1H, H-9); 7.28 (dd, 1H, H-7); 4.28 (q, 2H, CH_2); 1.40 (t, 3H, CH_3). Anal C, H, N.

6.1.3.12. 3-Iodo-8-hydroxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (6). Compound **1c** (0.5 mmol) was dissolved in a minimal amount of diethylene glycol dimethyl ether and added with 10 mL of 40% sodium hydroxide solution. The suspension was kept at refluxing temperature for 4 h. and then, after cooling and dilution with water, was treated with hydrochloric acid 12 M. The precipitate was filtered and recrystallized by ethanol 80%. Dark yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 3400; ^{1}H NMR (DMSO-d_6) δ 8.30 (m, 2H, H-2 and H-6); 7.48 (d, 1H, H-9); 7.10 (dd, 1H, H-7). Anal C, H, N.

6.1.4. General procedure for the synthesis of 9–14, 17. A mixture of compound **1c** (100 mg, 0.288 mmol), 10 mL of dichloromethane, 5 mL of 40% sodium hydroxide solution, 0.1 mole of tetrabutylammonium bromide, and the suitable alcohol in large excess (5 mL) was vigorously stirred at 30–50 °C for 4–12 h. The organic layer was then separated and the aqueous layer extracted twice with 10 mL of dichloromethane. The combined organic extracts were evaporated and the residue was recovered with isopropyl ether and recrystallized by suitable solvent.

6.1.4.1. 3-Iodo-8-isopropyloxypyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-oxide (9). From **1c** and 2-propanol. Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; ^{1}H NMR ($CDCl_3$) δ 8.45 (d, 1H, H-6); 8.10 (s, 1H, H-2); 7.65 (d, 1H, H-9); 7.10 (dd, 1H, H-7); 4.85 (m, 1H, CH); 1.48 (d, 6H, CH_3). Anal C, H, N.

6.1.4.2. 3-Iodo-8-*n*-propyloxypyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-oxide (10). From **1c** and *n*-propanol. Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; ^{1}H NMR ($CDCl_3$) δ 8.47 (d, 1H, H-6); 8.10 (s, 1H, H-2); 7.67 (d, 1H, H-9); 7.17 (dd, 1H, H-7); 4.20 (t, 2H, CH_2O); 1.95 (m, 2H, CH_2); 1.10 (t, 3H, CH_3). Anal C, H, N.

6.1.4.3. 3-Iodo-8-*n*-butyloxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (11). From **1c** and *n*-butanol. Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; ^{1}H NMR ($CDCl_3$) δ 8.46 (d, 1H, H-6); 8.10 (s, 1H, H-2); 7.66 (d, 1H, H-9); 7.15 (dd, 1H, H-7); 4.20 (t, 2H, CH_2O); 1.90 (m, 2H, CH_2); 1.57 (m, 2H, CH_2); 1.14 (t, 3H, CH_3). Anal C, H, N.

6.1.4.4. 3-Iodo-8-*n*-pentyloxypyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-oxide (12). From **1c** and *n*-pentanol. Yellow crystals. TLC eluent: toluene/ethyl acetate 8:2 v/v; ^{1}H NMR ($CDCl_3$) δ 8.46 (d, 1H, H-6); 8.10 (s, 1H, H-2); 7.66 (d, 1H, H-9); 7.16 (dd, 1H, H-7); 4.22 (t, 2H, CH_2O); 1.92 (q, 2H, CH_2); 1.48 (m, 4H, –CH_2–CH_2–); 0.98 (t, 3H, CH_3). Anal C, H, N.

6.1.4.5. 3-Iodo-8-methylcyclopropyloxypyrazolo[5,1-*c*]-[1,2,4]benzotriazine 5-oxide (13). From **1c** and methylcy-

clopropanol. Yellow crystals. TLC eluent: chloroform/methanol 10:1 v/v; ^{1}H NMR ($CDCl_3$) δ 8.46 (d, 1H, H-6); 8.10 (s, 1H, H-2); 7.64 (d, 1H, H-9); 7.17 (dd, 1H, H-7); 4.10 (t, 2H, CH_2O); 1.38 (m, 1H, CH); 0.76 (m, 2H, CH_2); 0.45 (m, 2H, CH_2). Anal C, H, N.

6.1.4.6. 3-Iodo-8-(2-methoxyethoxy)pyrazolo[5,1-*c*]-[1,2,4]benzotriazine 5-oxide (14). From **1c** and 2-methoxyethanol. Yellow crystals. TLC eluent: toluene/ethyl acetate 8:3 v/v; ^{1}H NMR ($CDCl_3$) δ 8.48 (d, 1H, H-6); 8.10 (s, 1H, H-2); 7.70 (d, 1H, H-9); 7.22 (dd, 1H, H-7); 4.38 (m, 2H, CH_2O); 3.87 (m, 2H, CH_2); 3.50 (s, 3H, OCH_3). Anal C, H, N.

6.1.4.7. 3-Iodo-8-(2-propynyloxy)pyrazolo[5,1-*c*] [1,2,4]-benzotriazine 5-oxide (17). From **1c** and propargyl alcohol. Yellow crystals. TLC eluent: toluene/ethyl acetate 8:3 v/v; ^{1}H NMR ($CDCl_3$) δ 8.50 (d, 1H, H-6); 8.10 (s, 1H, H-2); 7.84 (d, 1H, H-9); 7.22 (dd, 1H, H-7); 4.98 (s, 2H, CH_2O); 2.70 (s, 1H, CH). Anal C, H, N.

6.1.4.8. 3-Iodo-8-(2-*N*,*N*-dimethylethoxy)pyrazolo-[5,1-*c*][1,2,4]benzotriazine 5-oxide (15). From **1c** (100 mg, 0.288 mmol), *N*,*N*-dimethylethanolamine (5 mL) and 10% sodium hydroxide solution (1.5 mL). The reaction mixture was maintained at 50 °C and monitored by TLC. After starting material disappeared, the solvent was evaporated and the residue treated with water and extracted twice with chloroform. The evaporation yielded an orange residue which was recrystallized by ethanol 80%. TLC eluent: $CHCl_3$/MeOH 10:1 v/v; ^{1}H NMR ($CDCl_3$) δ 8.48 (d, 1H, H-6); 8.10 (s, 1H, H-2); 7.70 (d, 1H, H-9); 7.20 (dd, 1H, H-7); 4.40 (m, 2H, CH_2O); 2.90 (m, 2H, CH_2N); 2.50 (m, 6H, $(CH_3)_2$). Anal C, H, N.

6.1.4.9. 3-Iodo-8-(2-chloroethoxy)pyrazolo[5,1-*c*]-[1,2,4]benzotriazine 5-oxide (16). From **6** and 1,2-chlorobromoethane. In this case starting material was added to 40% sodium hydroxide solution and the reaction mixture was stirred at room temperature for 15 min. After this time a solution of 1,2-chlorobromoethane (1.5 mL) in dichloromethane (5 mL) and 0.1 mol tetrabutylammonium bromide were added and the reaction was maintained at 50-60 °C. After the normal workup a residue was recovered. Yellow crystals. TLC eluent: chloroform/methanol 10:1 v/v; ^{1}H NMR ($CDCl_3$) δ 8.50 (d, 1H, H-6); 8.10 (s, 1H, H-2); 7.70 (d, 1H, H-9); 7.22 (dd, 1H, H-7); 4.50 (t, 2H, CH_2O); 3.92 (t, 2H, CH_2Cl). Anal C, H, N.

6.2. Radioligand binding assay

6.2.1. Binding studies. [^{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.[42] 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 et al. [^{3}H]Ro 15-1788 binding studies were performed as previously reported. Clonal mammalian cell lines, expressing relatively high levels of $GABA_A$ receptor subtypes ($\alpha_1\beta_2\gamma_2$, $\alpha_1\beta_2\gamma_2$, $\alpha_3\beta_2\gamma_2$, $\alpha_5\beta_3\gamma_2$) 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]Ro 15-1788 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]Ro 15-1788 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 triplicate 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.

6.3. 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 12 h/12 h light/dark cycles (lights on at 7:00). The cages were brought into the experimental room the day before the experiment, for acclimatization purposes. All experiments were performed between 10:00 and 15:00.

6.3.1. 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 numbers of falls from the rod in 30 s, 25 min after drug administration were counted.

6.3.2. Grip-strength meter test. The grip strength meter measures forelimb grip-strength in rodents. The apparatus is formed by a Perspex basis on which is located a grasping trapeze. Mouse instinctively grabs the trapeze, when raised by trail, trying to stop this involuntary backward movement until the pulling force overcomes animal's grip strength. After the animal loses its grip,

the peak preamplifier automatically stores the peak pull force and shows it on a liquid crystal display (data not shown).

6.3.3. Hole board test. The hole board test was used to evaluate the effects of drugs on a mouse's explorative capacity and curiosity. Mice were placed individually on the board and left free to explore both panel and holes for 5 min, 30 min after drug administration.

6.3.4. 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.

6.3.5. 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.

6.3.6. Ethanol-induced sleeping time test. Ethanol (4 g/kg ip) was injected 30 min after drug administration. The duration of a loss of the righting reflex was measured as the sleep time. If the mice slept more than 210 min, the end-point was recorded as 210 min.

Drugs. Diazepam (Valium 10—Roche), Flumazenil (Roche), Pentylenetetrazole (PTZ) (Sigma), and Zolpidem (Tocris) were the drugs 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 and injected sc. All benzodiazepine receptor ligands were administered by the po route, except for flumazenil which was administered ip. Drug concentrations were prepared in such a way that the necessary dose could be administered in a 10 ml/kg volume of carboxymethylcellulose (CMC) 1% by the po, ip or sc routes.

Statistical analysis. Results are given as means ± 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 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 & Murray. *P* values of less than 0.05 were considered significant.

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