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ScienceDirect
Bioorganic & Medicinal Chemistry 16 (2008) 4471–4489
Bioorganic & Medicinal Chemistry

Novel 3-aroylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxides 8-substituted, ligands at $GABA_A$/benzodiazepine receptor complex: Synthesis, pharmacological and molecular modeling studies

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Received 19 October 2007; revised 13 February 2008; accepted 19 February 2008
Available online 5 March 2008

Abstract—The synthesis and binding studies of a series of 3-acylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxides 8-substituted are reported. High-affinity ligands at benzodiazepine site on $GABA_A$ receptor complex ($GABA_A$/BzR complex) were obtained when the 3-aroyl substituent is represented by a five-member heteroaroyl ring (furoyl-, thenoyl-, and pyrroyl-). Moreover the type of heteroaroyl ring at position 3 influences the feature of the substituent at position 8 to obtain high-affinity ligands: a 'hydrogen-bond acceptor ring' at position 3 is synergic with an electron donor substituent at position 8, while a 'hydrogen-bond donor ring' is synergic with a withdrawing substituent. Compounds **8a**, **9b**, and **11** were deeply studied in vivo for their pharmacological effects considering six potential benzodiazepine actions: motor coordination, anticonvulsant action, spontaneous motor activity and explorative activity, anxiolytic-like effects, mouse learning and memory modulation, and ethanol-potentiating action. To rationalize and qualitatively interpret the $GABA_A$/Bz binding affinities of compounds **8a** and **11**, a dynamic molecular modeling study has been performed, with the aim of assessing the preferred geometry of protein–ligand complex.

1. Introduction

Gamma-aminobutyric acid (gaba), the major inhibitory neurotransmitter in mammalian brain, targets the ionophore $GABA_A$ and $GABA_C$ receptors and the metabotropic $GABA_B$ receptors. Of these, it is the $GABA_A$ receptor family which has been the most widely studied since this family is the site of action of a number of clinically important drugs, including benzodiazepines (BZs), barbiturates, and anesthetics. $GABA_A$ receptor is a hetero-oligomeric complex composed of five transmembrane spanning subunits assembled to form a pentamer: there are 16 different subunits (α1–6, β1–3, γ1–3, δ, ε, π, θ) or 19 if the so-called $GABA_C$ subunits are included (ρ1–3). The majority of $GABA_A$ receptor subtypes contain α-, β-, and γ-subunits arranged in a 2:2:1 stoichiometry and concatenated as γβαβα, counterclock-wise from synaptic cleft.[1] The Bzs binding occurs at the interface of α and γ subunits and the major BZ-sensitive $GABA_A$ receptors in the brain are α1β2γ2, α2β3γ2, α3β3γ2, α5β3γ2. These show distinct regional distribution in the brain which provokes the question of whether $GABA_A$ receptor subtypes are linked to distinct pharmacological functions.[2–4] Currently, the genet-

Keywords: $GABA_A$/benzodiazepine receptor ligands; Tricyclic heteroaromatic system; 3-Acyl derivatives pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxides.
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doi:10.1016/j.bmc.2008.02.058

ic approach (the use of transgenic mice with point mutation on α-subunit) and the development of ligands endowed with selective efficacy have permitted the obtainment of data suggesting that a certain subtype receptor might be responsible for mediating specific pharmacological effects of BZs.[2,5–8]

All data are consistent with an important role of α1-containing $GABA_A$ receptors in mediating sedative effects and part of anticonvulsant activity and amnesic action; α2- and/or α3-containing $GABA_A$ receptors in mediating the anxiolytic-like action of benzodiazepines; α5-containing $GABA_A$ receptors are important for the cognitive effects and/or in the neurobehavioral action of alcohol.[6,9–15]

The mechanism by which allosteric modulators, as benzodiazepines, influence the $GABA_A$ receptor currents is unclear. Recently, the conformational rearrangements triggered by benzodiazepines binding in or near the GABA binding site (considering that GABA binding pocket is physically linked to the Bzs binding site) have been reported.[16–18] It seems that $GABA_A$/BzR positive modulators can induce change at a specific region formed by 11 amino acids on GABA binding site (αMet113-αLeu132 or Loop E) and this could elicit, at the end, different functional effects.[16,17] Other model [18] provided evidence that the transduction of signal, after benzodiazepine binding at the α/γ interface, happens via γ subunit from the N-terminal region of transmembrane domain M1 through M2 and the proximal portion of the M2–M3 loop. The benzodiazepine binding 'produces', at the end, a decreased energy requirement for channel opening.

To obtain drugs which are more selective than BZs, the development of $GABA_A$ receptor subtype selective ligands or functionally selective ligands is the current strategy of medicinal chemistry researchers. With this aim in mind, we have identified in a previously synthesized compound, the 3-(3-thienyl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide,[19] the lead for our new research. This compound is a selective agonist endowed with anticonvulsant activity, acting trough the $GABA_A$/BZs receptor complex, since its effect were antagonized by flumazenil. Starting from this compound a chemical modification at position 3 was made obtaining new 3-acylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 8-derivatives. These compounds can be considered metabolically more stable than ester derivatives previously studied[20–22] and strictly related to Indiplon[23,24] and Ocinaplon,[25] two heteroarylmethanones endowed with selective agonist profile (respectively, sedative and anxiolytic activity) through the $GABA_A$/benzodiazepine receptor complex.[9,10,26–28] These compounds belong to pyrazolo[1,5-*a*]pyrimidines chemical family and the 3-acylpyrazolopyrimidine core is easily recovered in our pyrazolobenzotriazine system (see Chart 1). This modi-

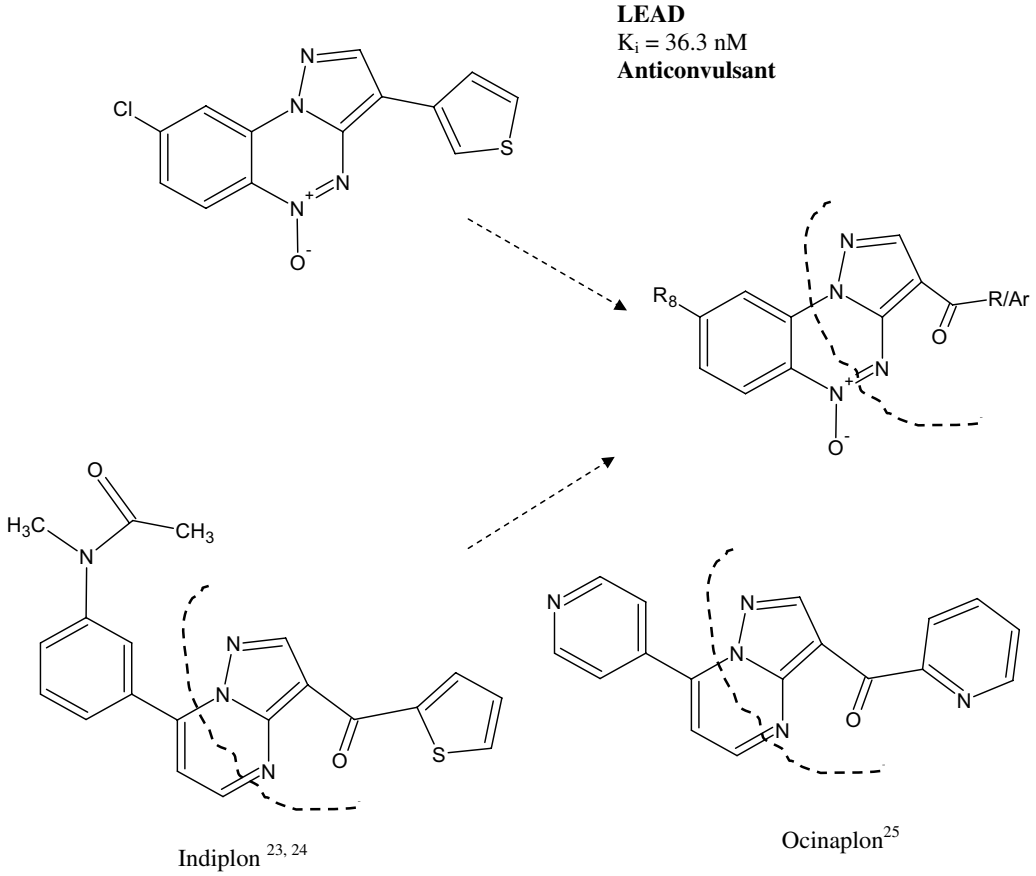
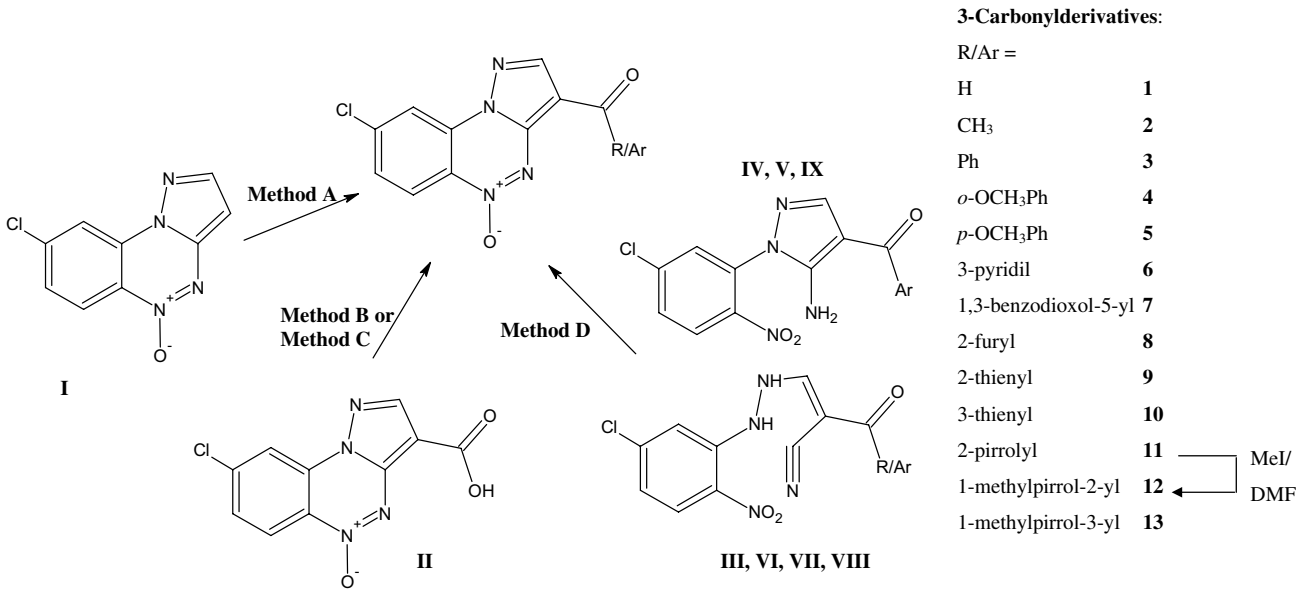


Chart 1.



Scheme 1. Reagents: **Method A**: **I**[30] $DMF/POCl_3$ for compound **1**. **Method B**: **II**[30] (i) thionyl chloride; (ii) ArH, $SnCl_2$ for compounds **5**, **8**–**9**, **11**–**13**; **12** was also obtained by the alkylation of **11**. **Method C**: **II**[30] (i) thionyl chloride; (ii) phenylboronic acid, Cs_2CO_3 for compound **3**. **Method D**: (i) NaOH 10% solution, 50–60 °C for compounds **2**, **4**, **6**–**10** (respectively, from starting material: **III**, **IV**, **V**, **VI**, **VII**, **VIII** and **IX**). For compounds **8** and **9** method B and D were indifferently used (yields shown on Supplementary data, Table 1).

fication at position 3 permits us to further explore the type of interaction that exists between the substituent at position 3 and the receptor protein. Moreover to define the role of 5-oxide group,[22,29] also in the new 3-aroyl derivatives series, we have provided for the synthesis of some 5-deoxy derivatives.

1.1. Chemistry

Two synthetic approaches to obtain the desired 3-acyl-8-chloropyrazolo[5,1-*c*] [1,2,4]benzotriazine 5-oxides **1**–**13** were developed consisting in: modification of position 3 (e.g., –H, –COOH substitution), by functionalization of this position with the keto group at a later stage of the tricyclic system synthesis (**method A, B, C** in Scheme 1); intramolecular cyclization under basic conditions of the 5-aminopyrazole or hydrazinyl derivative, bearing at 4-position the suitable keto group (**method D** in Scheme 1).

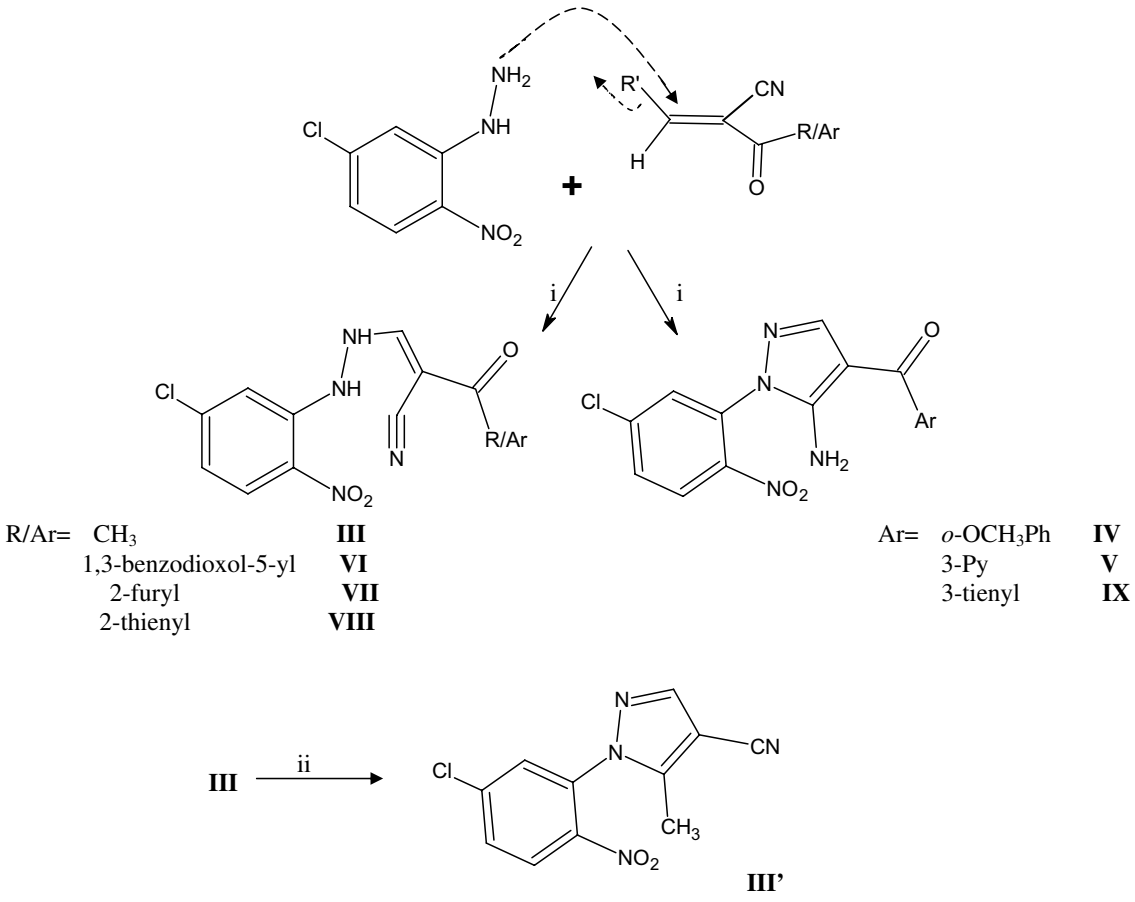
In the first approach (**method A**), the starting material 8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide[30] **I** was reacted according to the Vilsmayer conditions ($DMF/POCl_3$) and the desired 3-formyl derivative **1**, together with the corresponding 5-deoxide **1R**, was obtained after chromatographic separation (10/1 ratio).

When the starting material was the 3-carboxy-8-chloropyrazolo[5,1-*c*][1,2,4] benzotriazine 5-oxide[30] **II**, the treatment with thionyl chloride gave the corresponding 3-acylchloride as intermediate which, exploiting the Friedel–Craft reaction, was directly converted into final products, **5**, **8**–**9**, **11**–**13** (**method B**). Alternatively when the 3-acylchloride was reacted with phenyl boronic acid, (**method C**), in Suzuki coupling conditions compound **3** was obtained in good yield.

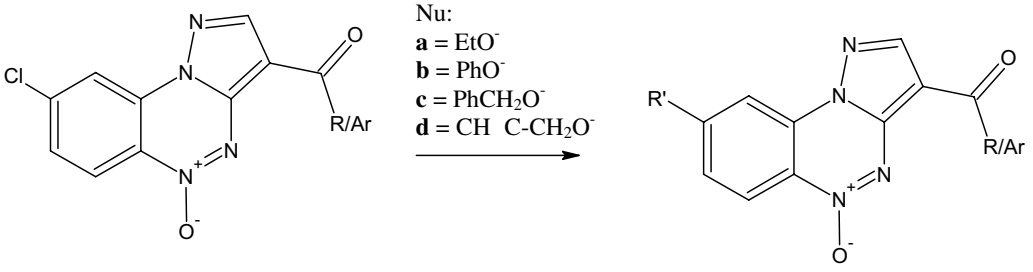
For the latter method (**method D** for compounds **2**, **4**, **6**–**10**), which gives the 3-acylpyrazolobenzotriazine system by ring closure in alkaline medium,[30] the synthesis of the starting compounds **III**–**IX** was developed as shown in Scheme 2. In particular the 5-chloro-2-nitrophenylhydrazine[31] was reacted with 2-ethoxymethylen-3-oxobutanenitrile[32] or with 2-(dimethylaminomethylen)-3-oxo-3-arylpropanenitrile[33–36] and the intermediate hydrazinyl derivatives **III**, **VI**, **VII**, **VIII** or the 5-aminopyrazoles **IV**, **V**, **IX** were obtained (Scheme 2) and used in the next cyclization in 10% sodium hydroxide solution.

A particular reactivity and regiospecificity depending on the type of acid catalyst used was observed when the 2-ethoxymethylen-3-oxobutanenitrile[32] was reacted with 5-chloro-2-nitrophenylhydrazine. In fact when the reaction was carried out in ethanol/acetic acid (Scheme 2) the ring closure proceeded involving, as the first step, the primary amino group of hydrazine giving the en-hydrazinyl derivative **III**.

In the attempt to obtain the 5-aminopyrazole-4-ethanone derivative, starting products (2-ethoxymethylen-3-oxobutanenitrile[32] and 5-chloro-2-nitrophenylhydrazine) were reacted using HCl concd as catalyst (Scheme 2). Also in this case the first intermediate was the en-hydrazinyl derivative **III** (not isolated and detected by TLC) which gave a regiospecific ring closure involving the $–COCH_3$ group instead of the –CN group and the final compound was the 1-(2-nitro-5-chlorophenyl)-5-methylpyrazole-4-carbonitrile, **III′**. It is important to note that this type of ring closure has occurred only for intermediate **III** in HCl concd conditions: the other hydrazinyl derivatives (**VI**, **VII**, and **VIII**) in the same condition never gave the correspond-



Scheme 2. Reagents and conditions: (i) ethanol/acetic acid, reflux; (ii) ethanol/HCl concd, reflux R = Me, R′ = OEt.[32] R = *o*-OCH_3Ph, 1,3-benzodioxol-5-yl, 3-Py, 2-furyl, 2-thienyl, 3-thienyl, R′ = NMe_2.[33–36]



Compd	R/Ar
2	Me
5	*p*-OMePh
6	3-pyridyl
8	2-furyl
9	2-thienyl
11	3-thienyl
12	2-pyrrolyl

Compd	R'
2a	EtO
2b	PhO
5a	EtO
6a	EtO
8a	EtO
8b	PhO
8c	$PhCH_2O$
8d	CH C-CH_2O
9a	EtO
9b	PhO
9c	$PhCH_2O$
11a	EtO
11b	PhO
12a	EtO
12b	PhO

Scheme 3. Reagents: 40% sodium hydroxide solution and ethanol for compounds **2a**, **5a**, **6a**, **8a**, **9a**, **11a**, and **12a**; 40% sodium hydroxide solution, tetrabutylammonium bromide NBu^+Br^-, ROH, CH_2Cl_2 for compounds **2b**, **8b**, **9b**, **11b**, **8c**, **9c**, and **8d**.

ing 4-carbonitrile derivatives, but evolve to 5-aminopyrazoles as verified by previously laboratory experiences (unpublished data).

To evaluate the influence of the 8-substituent on biological activity, the aromatic nucleophilic substitution of chlorine at position 8 was exploited, as depicted in Scheme 3. To obtain compounds **2a**, **5a**, **6a**, **8a**, **9a**, **11a**, and **12a**, starting materials **2**, **5**, **6**, **8**, **9**, **11**, and **12** were suspended in ethanol and 40% sodium hydroxide solution as previously reported.[29] Products **2b**, **8b**, **9b**, **11b**, **12b**, **8c**, **9c**, and **8d** were obtained by exploiting the phase transfer catalyzed (PTC)[37] chlorine displacement at position 8 of the benzotriazine system. In this procedure the suitable reagent (phenol, benzyl alcohol, or propargyl alcohol) is added to a two-phase system consisting of a strong aqueous sodium hydroxide solution (40%), a catalyst (tetrabutylammonium bromide, $Bu_4N^+Br^-$), and a methylene chloride solution of starting materials **2**, **8**, **9**, **11**, and **12** (see Scheme 3).

To better explore the role of the *N*-oxide group, compounds **9b** and **11** were N5-deoxygenated, using triethylphosphite (TEP) as previously reported,[38] yielding compounds **9bR** and **11R**. All chemical data of compounds described here are listed in Tables 1 and 2 available on 'Supplementary data'.

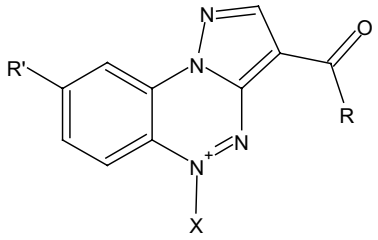
1.2. Binding studies

The $GABA_A$/BzR complex binding affinity of new 3-acylderivatives was evaluated by their ability to displace [^{3}H]flumazenil (Ro15-1788) from its specific binding in bovine brain membrane. Binding data for new, synthesized compounds (**1**–**13**, **2a**, **5a**, **6a**, **8a**, **9a**, **11a**, **12a**, **2b**, **8b**, **9b**, **11b**, **12b**, **8c**, **9c**, **8d**, **9bR** and **11R**) and for reference compounds are reported in Table 1.

The following observations arise from the binding studies' results. First of all, the type of substituent on the carbonyl group at position 3 resulted very important. In the series of 8-chloroderivatives compounds **1** and **2** (respectively, 3-formyl- and 3-acetyl derivatives) have no or low-affinity value (**1**, $I\% = 64\%$; **2**, $K_i = 551$ nM) as compounds bearing at position 3 a six-membered aroyl/heteroaroyl group (**3**, **4**, **5**, **6** or the larger **7**) which have a K_i in the range of 4200–1240 nM.

When the substituent of the carbonyl group is a five-membered heteroaroyl group (**8**, **9**, **10** and **11**) the affinity strongly increases up to a K_i value of 4 nM in the case of compound **11**. Probably heteroaroyl derivatives **8** (3-(2-furoyl)-, $K_i = 72$ nM) and **9**, (3-(2-thenoyl)-, **9**, $K_i = 62$ nM), interact with receptor protein in a different way with respect to 3-(pyrrol-2-carbonyl)derivative **11** ($K_i = 4.0$ nM). In compounds **8** and **9** the lone pairs of oxygen and, to a lesser degree, those of sulfur could be involved in a hydrogen-bond with the hydrogen-bond donor site (H_1) on the receptor protein. The presence of the 3-thenoyl at position 3 causes a reduction of the affinity value

Table 1. BZR ligand affinity of new 3-acylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide



Compound	R	R′	X	*I*%[a] or K_i[b] (nM)
1	H	Cl	O	64
2	Me	Cl	O	551
2a	Me	OEt	O	902
2b	Me	OPh	O	53
3	Ph	Cl	O	3494
4	*o*-OMe-Ph	Cl	O	4200
5	*p*-OMe-Ph	Cl	O	1240
5a	*p*-OMe-Ph	OEt	O	919
6	3-Py	Cl	O	2400
6a	3-Py	OEt	O	2000
7	1,3-Benzodioxol-5-yl	Cl	O	2900
8	2-Furyl	Cl	O	72
8a	2-Furyl	OEt	O	28
8b	2-Furyl	OPh	O	7
8c	2-Furyl	OCH_2Ph	O	1.4
8d	2-Furyl	$OCH_2C{=}CH$	O	3.5
9	2-Thienyl	Cl	O	62
9a	2-Thienyl	OEt	O	45
9b	2-Thienyl	OPh	O	8.9
9c	2-Thienyl	OCH_2Ph	O	2.7
10	3-Thienyl	Cl	O	121
11	2-Pyrrolyl	Cl	O	4
11a	2-Pyrrolyl	OEt	O	1836
11b	2-Pyrrolyl	OPh	O	276
12	1-Methylpyrrol-2-yl	Cl	O	5202
12a	1-Methylpyrrol-2-yl	OEt	O	819
12b	1-Methylpyrrol-2-yl	OPh	O	1300
13	1-Methylpyrrol-3-yl	Cl	O	380
9bR	2-Thienyl	OPh	—	14.7
11R	2-Pyrrolyl	Cl	—	1941
Daz[c]				10
Flu[c]				0.9

[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. 21.

of compound **10** with respect to the corresponding isomer 3-(2-thenoyl)derivative **9** (**10**, $K_i = 121$ nM, **9**, $K_i = 62$ nM): this fact could be explained by the different orientation of sulfur lone pairs that render the interaction weaker.

In the case of compound **11**, which bears a 3-(pyrrol-2-ylcarbonyl) moiety, the much higher potency ($K_i = 4.0$ nM) could likely be due to a hydrogen-bond interaction between the pyrrole-NH and the receptor protein. The hypothesis that a hydrogen-bond acceptor site (A_2) may exist in the proximity of, or located at the S_1 site of BzR is yet suggested by Primofiore et al.[39] On the other hand, the importance of the pyrrole NH is

Table 2. Effects of the new compounds in comparison with diazepam on motor coordination and convulsion

Treatment[a]	mg/kg po	Motor coordination rota-rod test (16 rpm) falls in 30″			Anticonvulsant activity		
		n	Pretest	30′	*n*	Shock latency (s)	Convulsion latency (s)
CMC 1%[b]	0.1 ml	22	4.2 ± 0.25	1.3 ± 0.3	38(7)[c]	273.3 ± 11.6	522.25 ± 60.35
Diazepam	1 (ip)	6	4.8 ± 0.4	4.5 ± 0.4[*]	48	No shocks[***]	No convulsions[***]
8a	3	10	4.2 ± 0.4	0.6 ± 0.3	10(1)	nd	328.5 ± 38.0[*]
	10	10	4.2 ± 0.4	0.8 ± 0.3	10(1)	nd	488.2 ± 26.0
	30	10	3.5 ± 0.3	0.6 ± 0.2			
9b	3	10	3.8 ± 0.5	0.8 ± 1.1	13(8)	224.5 ± 19.5	460.3 ± 68.9
	10	10	3.8 ± 0.3	1.6 ± 0.8	12(7)	279.7 ± 34.4	542.6 ± 69.3
	30	10	3.6 ± 0.5	2.0 ± 0.8	16(5)	614.1 ± 66.8[***]	766.6 ± 92.6[*]
11	3	17	4.1 ± 0.4	0.6 ± 0.2	14(2)	297.5 ± 17.2	498.8 ± 64.2
	10	17	4.0 ± 0.3	1.2 ± 0.2	14(2)	264.7 ± 17.5	647.8 ± 144.8
	30	17	4.6 ± 0.4	0.8 ± 0.3	14(3)	329.6 ± 31.5	535.2 ± 90.5

[a] Treatment with new compounds was performed 30 min and diazepam (ip) 20 min before the test. In the rota-rod test the effect of treatments was performed 15, 30, 45 and 60 min after the drug administration. The complete data are reported in Supplementary data (Table 4).
[b] Carboxymethylcellulose 1%.
[c] Number of dead mice.
[*] $P < 0.05$.
[***] $P < 0.001$ versus control mice.

evidenced by the lack of affinity of the 3-(1-methylpyrrol-2-ylcarbonyl) derivative, **12**, K_i = 5202 nM. The shift of the *N*-methylpyrrole group from position 2-yl to position 3-yl gave compound **13** with a better K_i value than compound **12** (**13**: K_i = 380 nM vs **12**: K_i = 5202 nM).

When the 3-carbonylpyrazolobenzotriazine scaffold was substituted at position 8 with groups such as -OEt, (compounds **2a**, **5a**, **6a**, **8a**, and **9a**) that previously showed a general positive influence on affinity value,[29,40] or –OPh (compounds **2b**, **8b**, and **9b**), it was observed that the order of substituent on binding was OPh > OEt»Cl. The influence of 8-substitution is particularly evident if at position 3 is present a five-membered ring (furoyl- or thenoyl-): an increase of affinity, with respect to **8** and **9**, was shown (**8a**, K_i = 28 nM and **8b**, K_i = 7 nM vs **8**, K_i = 72 nM; **9a**, K_i = 45 nM and **9b**, K_i = 8.9 nM vs **9**, K_i = 62 nM). The importance of the methylene spacer, as in the case of the 8-benzyloxy group, is evidenced in compounds **8c** and **9c** which show an affinity value from 4- to 7-fold higher compared to the corresponding 8-phenoxy derivatives **8b** and **9b** (**8c**, K_i = 1.4 nM and **9c** K_i = 2.7 nM vs **8b**, K_i = 7 nM and **9b** K_i = 8.9 nM). When the 3-(fur-2-ylcarbonyl)pyrazolobenzotriazine scaffold was substituted at position 8 with a propargyloxy group (**8d**) the binding-affinity value is in the same range of K_i (3.5 nM) of **8c**. This could probably be explained by the large size of the lipophilic pocket in which the 8-substituent fits, by an involvement of the ligand–receptor protein π-systems and by a probable hydrogen bond that involves the ethereal oxygen (see Section 1.3).

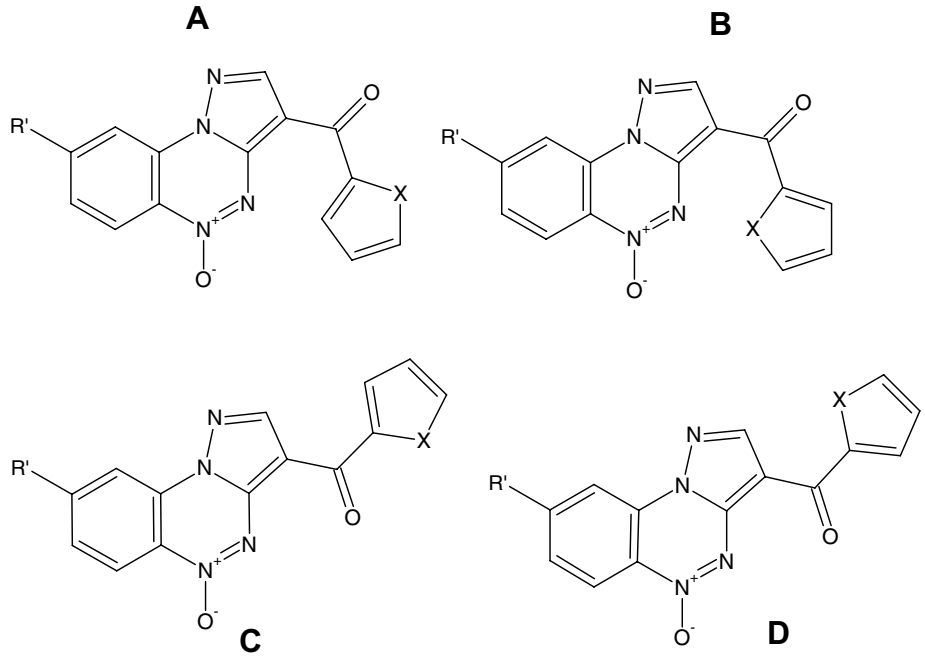
Surprisingly, when the 3-(1H-pyrrol-2-ylcarbonyl)pyrazolobenzotriazine scaffold is substituted at position 8 with a –OEt (**11a**), or a –OPh group (**11b**), the affinity values were drastically reduced with respect to the 8-chloro derivative **11** (**11a**, K_i = 1836 nM and **11b**, K_i = 276 vs **11**, K_i = 4 nM). In this case we could assume that the NH hydrogen bond with the receptor protein 'shifts' the whole molecule and this does not permit the fitting of the 8-substituents, which are more awkward than chloride (see Section 1.3).

The different role of N_5-oxide in these new derivatives is evidenced in compounds **9bR** and **11R**. The lack of N_5-oxide group drastically reduces the affinity value only in the case of compound **11** (**11R**, K_i = 1941 nM vs **11**, K_i = 4 nM). It appears that, in this case, the potential three-centered hydrogen bond in which the 5-oxide group is involved,[19,38] is necessary to better anchor the molecule to the receptor protein. In the case of compound **9b** the lack of the N_5-oxide group maintains the affinity value in the same value range (**9bR** K_i = 14.7 nM vs **9b** K_i = 8.9 nM), therefore the *N*-oxide group results to be secondary to anchor the molecule on the receptor protein.

1.3. Molecular modeling studies

In the present study we used the comparative (homology) model developed by Ernst et al.[41,42] to try to qualitatively interpret the $GABA_A$/Bz binding affinities of compounds **8a** (K_i = 28 nM) and **11** (K_i = 4 nM), 3-(fur-2-ylcarbonyl)- and 3-(1H-pyrrol-2-yl carbonyl) derivatives, respectively. It could be hypothesized that the different features of the heteroaroyl moiety present in the two compounds, H-bond acceptor for 2-furoyl and H-bond donor for 2-pyrroyl, are responsible for the different way of interaction with receptor protein.

A dynamic molecular modeling study has been performed with the aim of assessing the preferred geometry of protein–ligand complex, starting from the likely con-



X = O R' = OEt	**8a**
X = NH R' = Cl	**11**

Figure 1. Four possible conformers (**A**–**D**) for each compound.

formations of the ligands. For each compound (**8a** and **11**) four conformers A, B, C and D (Fig. 1) were selected and 'inserted' in the binding site in four different modes to obtain for each compound sixteen possible conformations (Fig. 2; for example, conformer A has been inserted in mode A1, A2, A3, and A4 and so on for each conformer).

Ligands **8a** and **11** were docked in the α1 (from Ile17 to Gly223) and γ2 (from Ile30 to Gly234) peptide chains that were cut out from pentameric construct downloaded from Margot Ernst's web page.[43] The extracellular domain of the α1, β2, and γ2 subunits is truncated N-terminally, respectively, at Ile17, Val16, and Ile30 and C-terminally at Gly223, Gly219, and Gly234. The binding site has been evidenced considering the most likely residues that mediate the binding at $GABA_A$/BzR complex,[44–50] and to construct the complex, a protein portion of 20 Å radius has been used.

A molecular dynamic (MD) simulation of the complex was carried out (see Section 3) and for each simulation those conformers of ligand–protein complex that showed an effective hydrogen-bond interaction were collected. With the term 'effective hydrogen-bond interaction' we mean those hydrogen bonds whose distance between acceptor and donor atoms is below 3.5 Å.

For compound **8a** (Fig. 3) those conformers of ligand–protein complex that simultaneously exhibited strong hydrogen-bond interactions involving the ethereal oxygen atom of the ethoxy group and the oxygen atom of the furane ring and the receptor protein were selected. These selected conformers showed the lowest conformational energy, that is 40 kcal/mol less with respect to the mean value of all conformers collected during the MD simulation. In particular, the oxygen atom of the furane ring could engage a hydrogen bond either with hydroxyl residue of γThr142 or the same residue on γThr81, while the ethereal oxygen atom could engage the hydrogen bond either with γArg144 (NH guanidine) or, alternatively, with αHis101 (NH imidazole ring). The carbonyl oxygen atom forms a strong hydrogen bond with γArg132 (NH guanidine) and in part with the hydroxyl group of γThr142. The N4-atom of the benzotriazine system and the oxygen of the *N*-oxide group alternatively interact with γArg132 (NH guanidine) and with the hydroxyl group of αSer205; the same oxygen of the *N*-oxide group in some conformations forms a hydrogen bond with the hydroxyl group of αTyr209 (Fig. 3).

Analogously, for compound **11** (Fig. 4) those conformers of ligand–protein complex were selected that showed the stronger hydrogen bond between pyrrole-NH and protein and the lowest conformational energy (−4860 kcal/mol) with respect to the mean value 60 kcal/mol less of all conformers collected during MD simulation. The pyrrole-NH group of compound **11** interacts, by a strong hydrogen bond, with two different amino acids of the receptor protein; depending on the amino acid involved, molecule **11** can adopt two alterna-

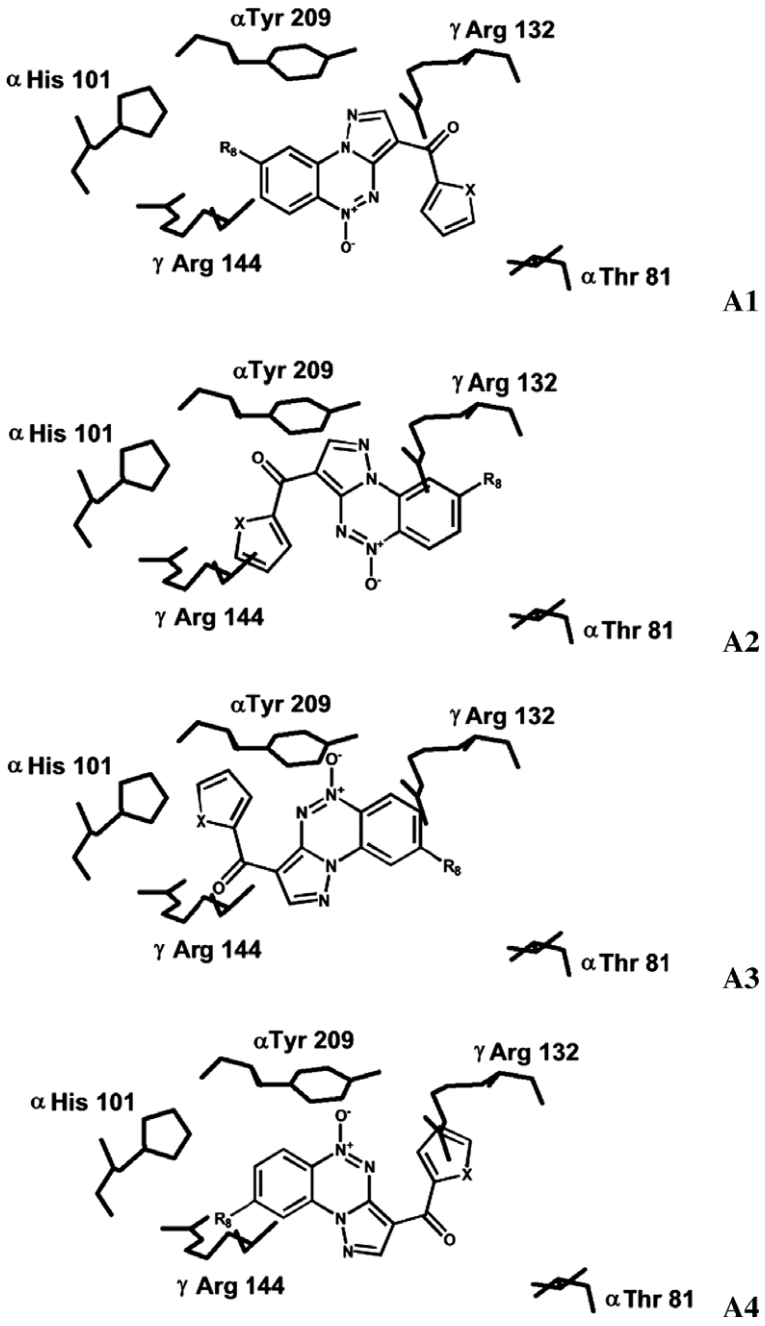


Figure 2. Modes of binding (**1**–**4**) of each conformer (below example for conformer **A**).

tive modes of anchoring in the receptor pocket, arbitrarily called 'binding mode type 1' and 'binding mode type 2' (Fig. 4a and b).

In 'binding mode type 1' (Fig. 4a), the pyrrole-NH interacts with the amidic oxygen (side chain) of αGln203, and the oxygen of *N*-oxide group alternatively interacts, by strong hydrogen bond, with the hydroxyl group of αTyr209 and of αSer205; the N4-atom of the benzotriazine system interacts with αTyr209 (hydroxy group). The carbonyl oxygen atom binds alternatively with γArg144 (NH guanidine) or αTyr159 (hydroxyl group).

In 'binding mode type 2' (Fig. 4b) the pyrrole-NH interacts with the carbonyl oxygen (backbone CO) of αTyr159, and the oxygen of the *N*-oxide group and the N4-atom of benzotriazine system alternatively interact, by strong hydrogen bond, with αSer205. The carbonyl oxygen atom binds alternatively with γArg132 (NH guanidine) or αTyr159 (hydroxyl group).

The binding modes of compounds **8a** and **11** permit us to hypothesize why compound **11a** (8-ethoxy-3-(1H-pyrrol-2-ylcarbonyl)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide) has a low affinity binding value (K_i = 1836 nM). Since the driving force of interaction of compounds that bear the 3-carbonylpyrrole moiety with receptor protein is the strong hydrogen bond between the pyrrole-NH and the corresponding amino acids (in both 'binding modes type 1 and 2', vide supra), compound **11a** would

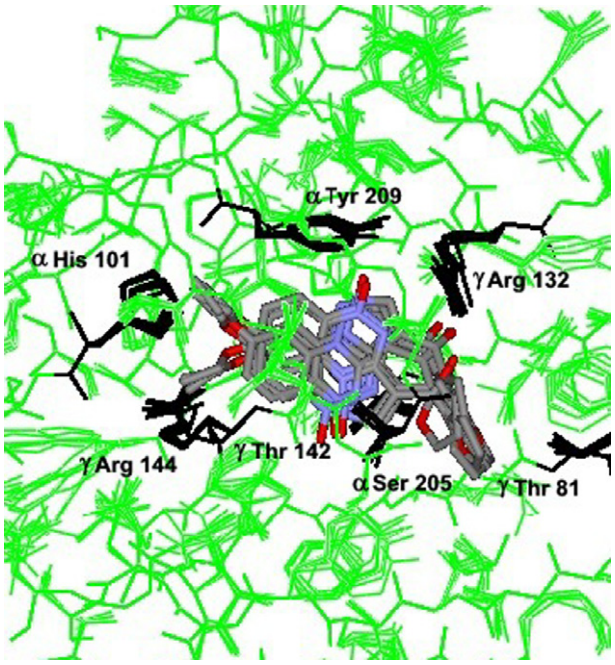


Figure 3. Docking of compound **8a**, 3-(fur-2-ylcarbonyl)-8-ethoxypyrazolo[5,1-*c*][1,2,4] benzotriazine 5-oxide: conformers with the lowest conformational energy in the receptor protein.

anchor in the same manner. Unexpectedly compound **11a** cannot bind the receptor protein in '1 or 2 type modes' due to steric hindrance of the 8-ethoxy group. In fact in 'binding mode type 1' the substituent at position 8 presents a strong repulsive interaction with γThr81, while in 'binding mode type 2' the amino acids γArg144 and αHis101 cannot act as hydrogen bond donors (as hypothesized for 8-ethoxy group of compound **8a**) because, probably, the 8-substituted benzotriazine aromatic moiety gives steric hindrance (Fig. 5a and b).

1.4. In vivo pharmacology

Compounds **8a** and **11** inserted on molecular modeling studies were chosen also for in vivo testing. Compound **9b**, 3-thenoyl derivative, was studied to evaluate how the insertion of keto group influences the in vivo effect, with respect to 3-thienyl derivative (lead, Chart 1) that showed selective anticonvulsant activity.[19] Six potential benzodiazepine actions were considered: motor coordination was screened with the rota-rod test and the anticonvulsant action was evaluated using the new drugs against pentilenetetrazole-induced convulsions (Table 2); spontaneous motility and explorative activity with hole-board test (Table 3); potential anxiolytic-like effects were screened using light/dark choice test (Fig. 6); mouse learning and memory modulation were evaluated by passive-avoidance test (Fig. 7); and finally, the drugs were tested also for their ethanol-potentiating action (Fig. 8). Diazepam was used as the positive reference molecule.

1.5. Effect on motor coordination

The effect of compounds **8a**, **9b**, and **11** was evaluated in comparison with diazepam on mouse rota-rod test, in order to point out motor incoordination activity (In Table 2 are reported only the pretest and the falls after 30 min from treatment; The Complete data are reported in Supplementary data (Table 4)). The reference compound (diazepam 1 mg/kg ip) increased the number of falls from the rotating rod, reaching statistical significance (Table 2). None of the newly synthesized substances induced any effect on the number of mouse falls from the rota-rod evaluated after 15, 30, 45, and 60 min (see Supplementary data); moreover it is evidenced that mice learned to stay on the rota-rod since the number of falls was lower in time-dependent manner.

1.6. Effect against chemically induced convulsions

Anticonvulsant activity was studied in mice using pentilentetrazole (PTZ) as chemical convulsant agent: the latency time (s) to shocks and convulsions was evaluated. Diazepam (1 mg/kg ip) completely protected against PTZ-induced shocks and convulsions (Table 2), while **8a** only reduced (3 mg/kg po) the convulsion

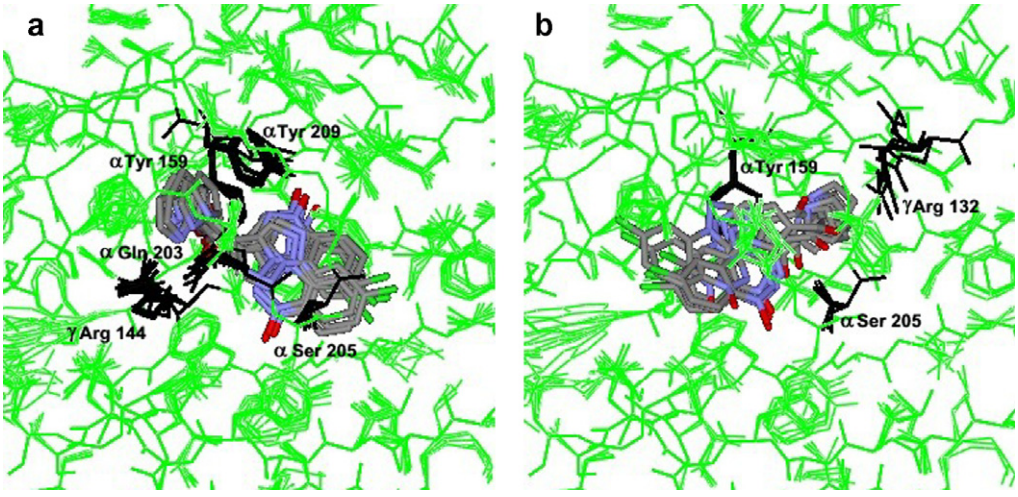


Figure 4. Docking of compound **11**, 3-(1H-pyrrol-2-ylcarbonyl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide. Two alternative modes of anchoring in the receptor protein: '*binding mode type 1*' in (a) and '*binding mode type 2*' in (b).

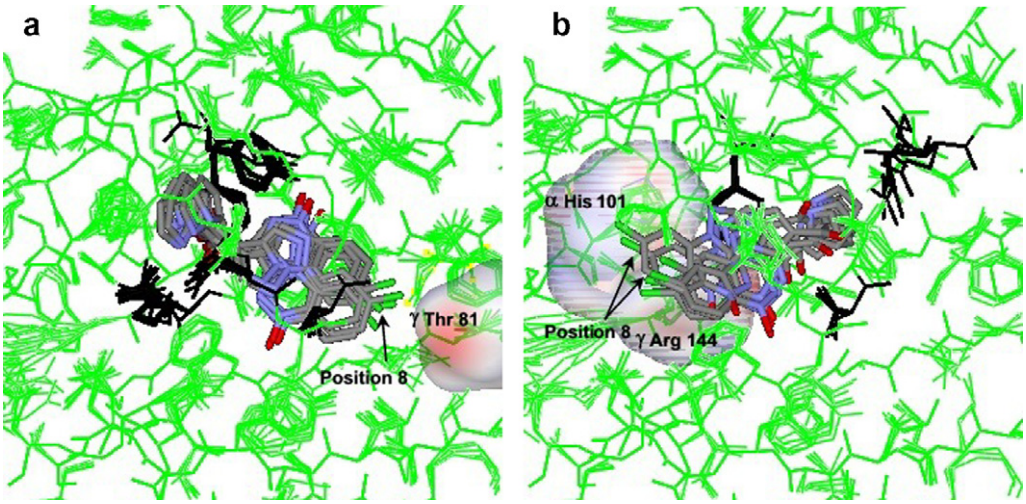


Figure 5. In panel (a) and (b) are evidenced amino acids not involved in binding of compound **11**.

Table 3. Effect of compounds **8a**, **9b**, and **11** on spontaneous motility and explorative activity in comparison of diazepam evaluated in the mouse hole-board test

Treatment[a]	mg/kg po	Hole board		
		n	Hole	Plane
CMC 1%	0.1 ml	31	33.53 ± 2.3	66.36 ± 1.6
Diazepam	3 (ip)	11	13.4 ± 6.3**	49.7 ± 6.6**
8a	3	10	19.4 ± 0.5**	68.0 ± 3.0
	10	10	30.7 ± 1.3	65.1 ± 3.5
	30	10	36.9 ± 5.0	59.0 ± 7.9
9b	3	15	23.1 ± 2.9	62.8 ± 8.0
	10	15	32.4 ± 5.6	73.0 ± 7.9
	30	15	21.2 ± 3.6*	52.2 ± 11.5
11	3	17	29.2 ± 3.0	67.1 ± 2.8
	10	17	39.1 ± 2.2	68.5 ± 3.1
	30	17	33.5 ± 2.2	56.3 ± 2.0**

[a] The test was performed 30 and 20 min after the administration of compounds (po) and the diazepam (ip), respectively.
* $P < 0.05$.
** $P < 0.01$ versus control mice.

latency. Compound **11** was devoid of any effect on PTZ-shock and convulsion latency and compound **9b** increases the latency of both parameters in a significant manner.

1.7. Effects on spontaneous motility and curiosity

A very common effect of the old benzodiazepine ligands is represented by sedation. The hole-board test is sensitive in checking this kind of behavior as demonstrated by the significant reduction of spontaneous motility and curiosity exerted by diazepam at the dose of 3 mg/kg ip.

Experiments carried out for compounds **8a**, **9b**, and **11** were finalized to evaluate their effects on mice spontaneous motility and explorative activity. Compounds **8a** and **9b** were able to diminish curiosity at the dose of 3 mg/kg po and 30 mg/kg po, respectively. By contrast compound **11** at the dose of 3 and 10 mg/kg po did not show any alteration of both spontaneous and explorative activities while at the dose of 30 mg/kg po it did diminish the panel movements (Table 3).

1.8. Effect on anxiety

Effects on mouse anxiety of newly synthesized molecules in comparison with diazepam were studied using a light/dark box apparatus. In our experiments, compound **9b** showed a good anxiolytic-like effect at doses of 10 and 30 mg/kg po with an efficacy comparable to that shown by diazepam (Fig. 6). Also compound **11**, reveled an anxiolytic activity at the dose of 10 mg/kg but not at the dose of 3 and 30 mg/kg po. The anxiolytic-like effects of **9b** and **11** were completely antagonized by flumazenil (at a dose of 100 mg/kg ip, dose at which flumazenil was able to antagonize the anxiolytic effect of diazepam) suggesting the agonist profile at $GABA_A$/BzR complex of the newly synthesized ligands (Fig. 6).

1.9. Effects on learning and memory

In order to clarify the effect that the newly synthesized compounds have on learning and memory processes, mice performance on passive-avoidance test was investigated. In this assay, the parameters taken into consideration are represented by retention and training latencies expressed in sec. While the training latencies did not differ among the various groups, some retention latencies are significantly different from the others. Particularly, as can be observed in Figure 7, compounds **9b** (10 and 30 mg/kg po) and **11** (3 and 10 mg/kg po) improved, in a statistically significant manner, the mouse memory processes. In the same experimental test the full-agonist diazepam (1 mg/kg ip) showed an amnesic activity.

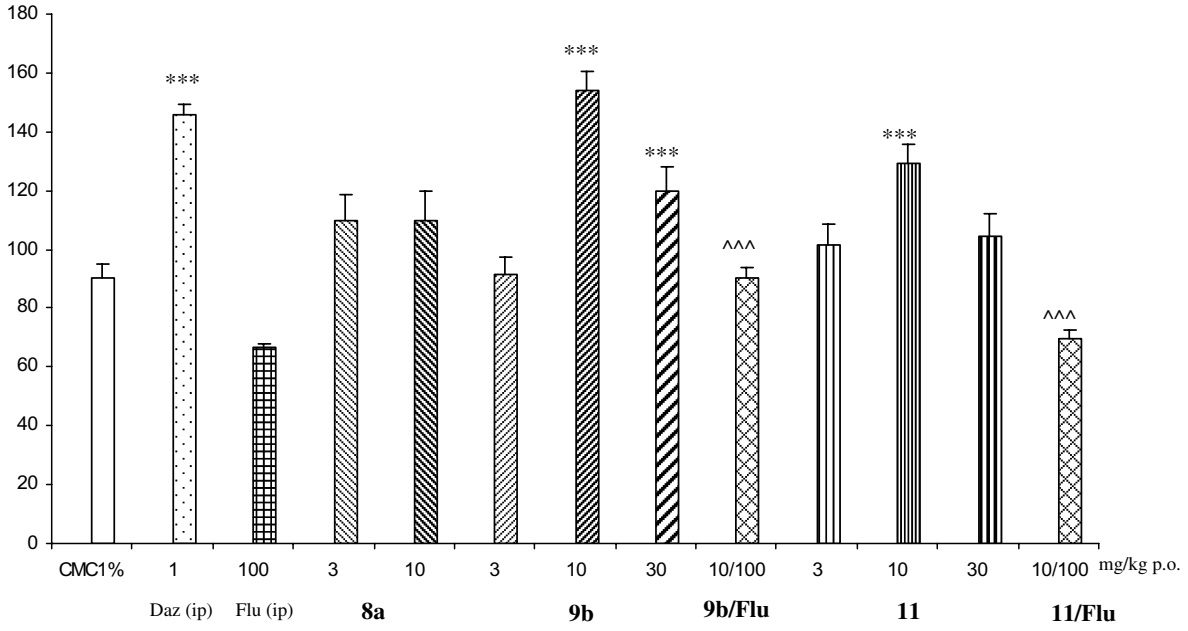


Figure 6. Effect of compounds **8a**, **9b** and **11** in comparison with diazepam (Daz) on mouse *light-dark box test*. Columns represent the time, expressed in s, spent in illuminated compartment. Treatment with compounds (po) and flumazenil (Flu) was performed 30 min and with diazepam 20 min before the test. Each column represents means ± SEM of 14–21 mice. $^{**}P < 0.01$, $^{***}P < 0.001$ versus controls. $^{\wedge\wedge\wedge}P < 0.001$ versus **9b**-treated or **11**-treated mice.

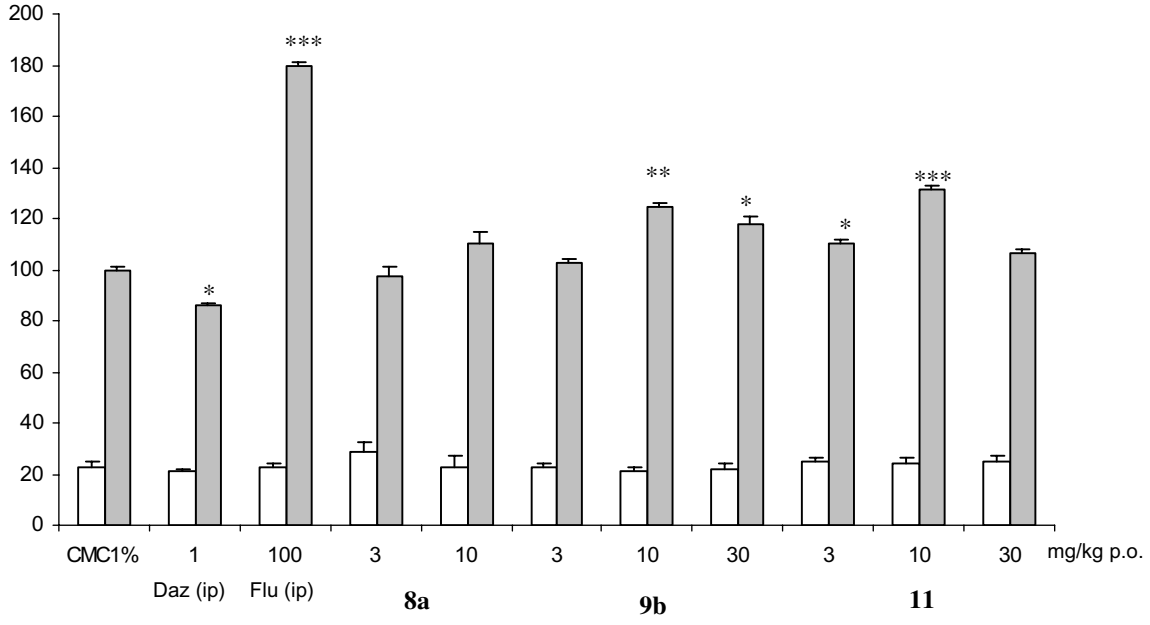


Figure 7. Effect of compounds **8a**, **9b**, and **11** in comparison with diazepam (Daz) and flumazenil (Flu) on *mouse passive-avoidance test*. White histograms represent the training latency and grey histograms represent the retention latency. Mice were treated 30 min before the training test. The retention test was performed 24 h later. Each column represents means ± SEM of 15–18 mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus controls.

1.10. Effect on ethanol-induced sleeping time

In the test of ethanol-induced sleeping time compounds **9b** and **11** at the dose of 3, 10 and 30 mg/kg po increased the duration of loss of righting reflex (Fig. 8). On the contrary **8a** did not modify this behavioral parameter. As expected, the reference drug diazepam (daz, ip) enhanced sedation in a statistically significant manner. Compounds **9b** and **11** up to a dose of 300 mg/kg po did not exhibit any modification of behavioral parameters. Mice treated with **9b** and **11** at the dose of 300 mg/kg po appeared on observation to be wholly comparable to that of the control, to the extent that researchers, who were unaware of the treatment received by the animals, were unable to distinguish between the various groups.

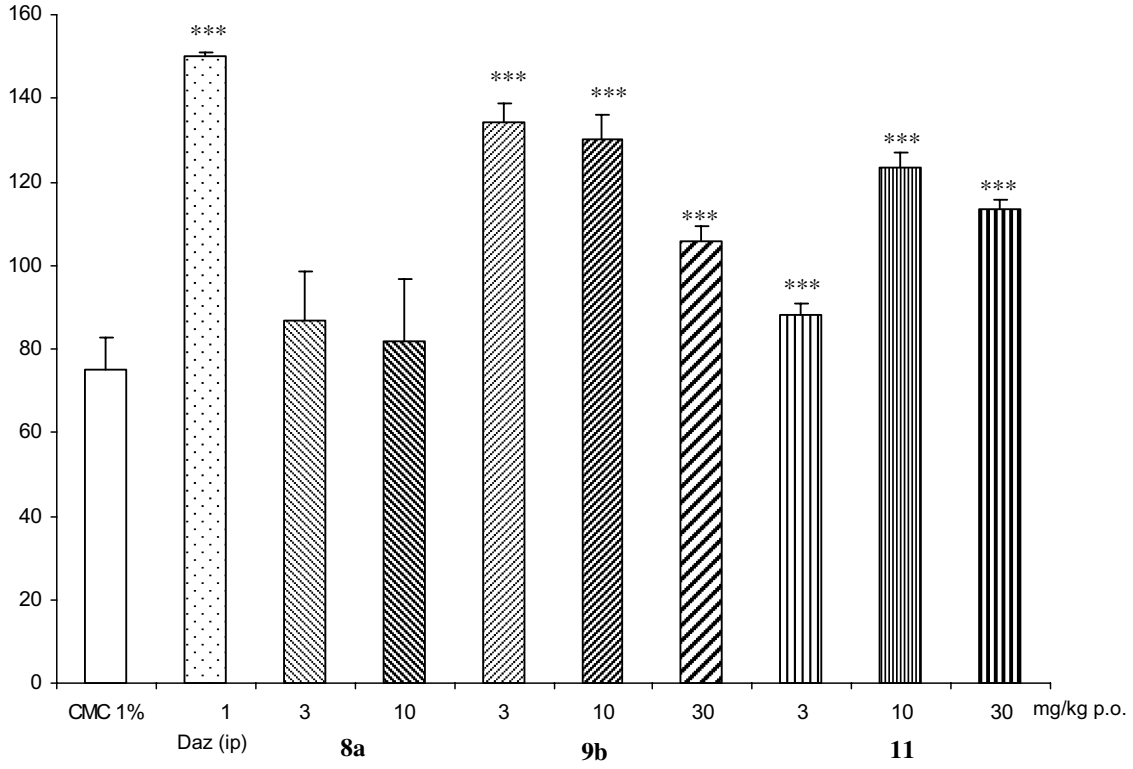


Figure 8. Effect of compounds **8a**, **9b**, and **11** in comparison with diazepam (Daz) on *mouse ethanol-induced sleeping time*. Each column represents means ± SEM of 12–14 mice. Substances were administered 30 min after ethanol (4 g/kg ip). $^{***}P < 0.001$ versus controls.

2. Conclusions

The synthesis of these compounds has permitted us to further analyze the SAR for pyrazolo[5,1-*c*][1,2,4]benzotriazine ligands. Interesting confirmations that arise from biological results concern the lipophilic pocket in which the 3-substituent fits: the limited size and particular requisites are shown even in the presence of additive carbonyl group with respect 3-heteroaryl derivative.[19] In fact, 3-aroyl derivatives with six-membered rings or bulkier substituents (compounds **3**, **4**, **5**, **5a**, **6**, **6a**, **7**) show a very low-affinity value (K_i range 919–4200 nM). On the other hand the small methyl group does not determine important increases on binding-affinity (compound **2**). The presence, instead, of electron-rich five-membered heteroaryl rings (as furoyl-, thenoyl-, pyrroyl-), retains significant affinity to BzR. In general the presence at position 8 of electron donating group as –OEt, –OPh, –OCH_2Ph, and in position 3 of a furoyl- or thenoyl ring (compounds **8a**–**c** and **9a**–**c**) increase the affinity binding. By molecular modeling studies the increased binding of 8-alkoxy- and 8-aryloxyderivatives would be due to an additional hydrogen bond that the ethereal oxygen could engage with two amino acids (γArg144 or αHis101) on the receptor protein.

Molecular modeling study strengthens our hypothesis of ligand–receptor protein interaction,[19] assuming as topological model the pharmacophore/model proposed by Cook and co-workers[51–54]: the pyrazolobenzotriazine system binds the $GABA_A$/BZ complex through N1 and/or N4 atom by means of a hydrogen bond involving H-donor sites on receptor protein. Substituents at positions 3 and 8 interact with lipophilic pockets of receptor proteins (L_1/L_2) and their influence on binding seem to depend on electronic or chemical features. Moreover, the interdependence between positions 3 and 8 was shown, since the type of heteroaryl ring at position 3 enables molecule to exert a further hydrogen bond with receptor protein by ethereal oxygen at position 8. In fact the presence of a ring able to be 'hydrogen-bond acceptor' toward receptor protein (furane oxygen in compound **8a**) 'permits' the molecule to engage another hydrogen bond, through the ethereal oxygen of the 8-substituent. On the contrary, the presence at position 3 of heteroaroyl ring able to be 'hydrogen-bond donor' (as 1H-pyrrol-2-yl, compound **11**) shifts completely the molecule in the receptor protein and two alternative modes of anchorage can be suggested (Fig. 4a and b). The pyrrole NH plays a crucial role in the receptor binding pocket because, first of all, it is involved in a strong hydrogen bond in both anchorage modes and its alkylation determines a loss of affinity; second the cooperative binding of pyrrole NH and *N*-oxide group is evidenced since the *N*-deoxide ligand (**11R**) has low affinity; this is in contrast with compound **9bR** (3-thenoyl derivative) which maintains the affinity value of the corresponding 5-oxide derivative, **9b**. On the other hand an 8-alkyloxy substituent on 3-pyrrol-2-ylcarbonyl derivative, **11a**, is not tolerated probably because this substituent can lie near to sterically forbidden sites and the ethereal oxygen cannot form the hydrogen bond, as instead happens for compounds **8a**.

From a pharmacological point of view, we might consider **9b** and **11** selective anxiolytic-efficacy ligands, with a similar efficacy (for compound **9b**) to that of diazepam even if at a greater doses (10 mg/kg), but without its undesirable side effect. The selective effect of **9b** and **11** could be ascribed to an agonist-efficacy at α2-subtype; moreover the activation of these subtypes could induce an increase of loss righting reflex endurance,[55] as evidenced for the same compounds (Fig. 8). On the other hand ligands **9b** and **11** not only show no impairment on learning and memory mouse performance in statistically significant manner, but they are able to increase the retention latency demonstrating their ability to ameliorate mnemonic processes. These data can lead to the interpretation that compounds **9b** and **11** could have an antagonist profile at α1-containing receptor subtypes-like flumazenil; in fact to these subtypes has been attributed the amnesic effect of benzodiazepines.[56] These pharmacological aspects permit to distinguish the examined compounds from the old BDZ-ligands that were endowed with amnesic properties.

In summary, the introduction at position 3 of pyrazolobenzotriazine system of a heteroaroyl group, as in Ocinaplon and Indiplon, lets us to individuate a new series of $GABA_A$/BZ receptor ligands, endowed with high affinity. With respect to lead (anticonvulsant activity, see Chart 1), the tested compounds show selective anxiolytic activity devoid of side effects. It is further confirmed that the positions 3 and 8 of pyrazolobenzotriazine system are 'key point' to modify to obtain selective-efficacy ligands.

3. Experimental

3.1. Chemistry

Melting points were determined with a Gallenkamp apparatus and were 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 reported in Supplementary data. Microanalyses were performed with a Perkin-Elmer 260 analyzer for C, H, N, and the results were within ±0.4% of the theoretical value.

3.2. General procedure for the synthesis of III–IX

A suspension of 2-nitro-5-chlorophenylhydrazine[30] (2.0 mmol) and 2-ethoxymethylene-3-oxobutanenitrile[32] or [(2-dimethylamino)methylene]-3-oxo-3-arylpropanenitrile[33–36] (2.0 mmol) in ethanol (50 mL) and acetic acid was refluxed until the starting material disappeared (6 h). The work up of final solution gave the (5-aminopyrazol-4-yl)arylmethanones **IV**, **V**, **IX** and the enhydrazinyl derivatives **III**, **VI**, **VII** and **VIII**. Compound **III′**, the 5-methylpyrazole-4-carbonitrile derivative, is obtained by the treatment of the 2-nitro-5-chlorophenylhydrazine[30] and 2-ethoxymethylene-3-oxo butane nitrile with ethanol/HCl.[32]

3.2.1. 2-Acetyl-3-(2-(5-chloro-2-nitrophenyl)hydrazinyl) acrylonitrile (III). Orange crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1} 3369, 2203; ^{1}H NMR ($CDCl_3$) δ 11.6 (d, 1H, NH exch.); 9.80 (s, 1H, NH); 8.20 (d, 1H, H-3′); 7.57 (d, 1H, CH); 7.11 (d, 1H, H-6′); 7.00 (dd, 1H, H-4′); 2.50 (s, 3H, CH_3). Anal. C, H, N.

3.2.2. 1-(2-nitro-5-chlorophenyl)-5-methylpyrazole-4-carbonitrile (III′). Red-brown crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1} 2232; ^{1}H NMR ($CDCl_3$) δ 8.13(d, 1H, H-3′); 7.90 (s, 1H, H-3); 7.73 (dd, 1H, H-4′); 7.54 (s, 1H, H-6′); 2.45 (s, 3H, CH_3). Anal. C, H, N.

3.2.3. [5-Amino-1-(5-chloro-2-nitrophenyl)-1H-pyrazol-4-yl](2-methoxyphenyl)methanone (IV). Dark-yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1} 3401, 3324, 1629; ^{1}H NMR ($CDCl_3$) δ 8.04 (d, 1H, H-3′); 7.68 (d, 1H, H-6′); 7.63 (dd, 1H, H-4′); 7.52 (s, 1H, H-3); 7.45 (m, 2H, H-4″ and H-6″ 2-MeOPh); 7.05 (m, 2H, H-3″ and H-5″ 2-MeOPh); 6.00 (br s, 2H, NH_2 exch.); 3.90 (s, 3H, OCH_3). Anal. C, H, N.

3.2.4. [5-Amino-1-(5-chloro-2-nitrophenyl)-1H-pyrazol-4-yl](pyridin-3-yl)methanone (V). Dark-yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1} 3401, 3324, 1624; ^{1}H NMR ($CDCl_3$) δ 8.70 (dd, 1H, H-4 Py); 8.50 (d, 1H, H-2 Py); 8.10 (s, 1H, H-3); 8.08 (d, 1H, H-6 Py); 8.00 (d, 1H, H-3′); 7.65 (dd, 1H, H-4′); 7.45 (d, 1H, H-6′); 6.20 (br s, 2H, NH_2 exch.). Anal. C, H, N.

3.2.5. 2-(1,3-Benzodioxol-5-yl)-3-(2-(5-chloro-2-nitrophenyl)hydrazinyl)acrylonitrile (VI). Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1} 3369, 2203; ^{1}H NMR ($CDCl_3$) δ 12.00 (d, 1H, NH exch.); 10.00 (s, 1H, NH exch.); 8.20 (d, 1H, H-3′); 7.80 (d, 1H, CH exch.); 7.70 (dd, 1H, H-6″); 7.48 (d, 1H, H-2″); 7.20 (d, 1H, H-5′); 7.00 (dd, 1H, H-4′); 6.90 (d, 1H, H-5″); 6.01 (s, 2H, CH_2O). Anal. C, H, N.

3.2.6. 2-(2-Furylcarbonyl)-3-(2-(5-chloro-2-nitrophenyl) hydrazinyl)acrylonitrile (VII). Dark-yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1} 3324, 2215, 1610; ^{1}H NMR ($CDCl_3$) δ 11.90 (d, 1H, NH exch.); 9.85 (s, 1H, NH exch.); 8.22 (d, 1H, H-3′); 8.05 (d, 1H, CH exch.); 7.70 (m, 2H, H-5 and H-3 furane); 7.18 (d, 1H, H-6′); 7.00 (dd, 1H, H-4′); 6.62 (dd, 1H, H-4 furane). Anal. C, H, N.

3.2.7. 2-(2-Thienylcarbonyl)-3-(2-(5-chloro-2-nitrophenyl) hydrazinyl)acrylonitrile (VIII). Dark-yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/

v; IR ν cm^{-1} 3345, 2213, 1609; ^1H NMR ($CDCl_3$) δ 11.90 (d, 1H, NH exch.); 9.85 (s, 1H, NH exch.); 8.22 (d, 1H, H-3′); 8.05 (d, 1H, CH exch.); 7.70 (m, 2H, H-5 and H-3 thien.); 7.18 (d, 1H, H-6′); 7.00 (dd, 1H, H-4′); 6.62 (dd, 1H, H-4 thien.). Anal. C, H, N.

3.2.8. [5-Amino-1-(5-chloro-2-nitrophenyl)-1H-pyrazol-4-yl](thien-3-yl)methanone (IX). Ivory crystals. TLC eluent: toluene/ethyl acetate 8:3 v/v; IR ν cm^{-1} 3345, 2213, 1609; ^1H NMR ($CDCl_3$) δ 8.06 (d, 1H, H-3′); 8.02 (d, 1H, H-6′); 7.97 (s, 1H, H-2); 7.67 (m, 2H, H-4′ and H-2′ thien.); 7.58 (m, 1H, H-3′ thien.); 7.41 (m, 1H, H-4″ thien.). Anal. C, H, N.

3.2.9. 3-Formyl-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine5-oxide (1). To 6 mL of DMF maintained at 0°C was added $POCl_3$ (0.8 mL, 8.6 mmol) and the solution was stirred for 30 min; the starting material, 8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide[30] (4.0 mmol) was slowly added. The reaction was maintained at 60 °C for 48 h and monitored by TLC. Ice was added into the brown solution and the formed precipitate was filtered. The reaction mixture was purified by chromatography column using diisopropylether/cyclohexane (8:3) as eluent and the eluted bands were characterized: the 5-deoxide derivative 3-formyl-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine (faster eluted band), **1R**, and 3-formyl-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide **1**.

Compound **1**. Yellow crystals. TLC eluent: *i*-propylether/cyclohexane 8:3 v/v; IR ν cm^{-1} 1677, 1565; ^1H NMR ($CDCl_3$) δ 10.28 (s, 1H, CHO.); 8.50 (m, 3H, H-2, H-6 and H-9); 7.70 (dd, 1H, H-7). Anal. C, H, N.

Compound **1R**. Yellow crystals. TLC eluent: *i*-propylether/cyclohexane 8:3 v/v; IR ν cm^{-1} 1677; ^1H NMR ($CDCl_3$) δ 10.80 (s, 1H, CHO); 8.72 (m, 2H, H-2 and H-6); 8.58 (d, 1H, H-9); 7.85 (dd, 1H, H-7). Anal. C, H, N.

3.3. General procedure for the synthesis of 2, 4, 6–10

The suitable intermediates **III**–**IX** (0.5 mmol) were suspended in sodium hydroxide solution 10% and stirred at 50 °C for 36 hours. When the staring material disappeared the reaction was stopped and treated with water/ice: the precipitate was filtered and recrystallized by a suitable solvent.

3.3.1. 3-Acetyl-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (2). From **III**. Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1} 1660; ^1H NMR ($CDCl_3$) δ 8.60 (s, 1H, H-2); 8.52 (d, 1H, H-6); 8.47 (d, 1H, H-9); 7.67 (dd, 1H, H-7); 2.8 (s, 3H, CH_3). Anal. C, H, N.

3.3.2. 3-(2-Methoxybenzoyl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (4). From **IV**. Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1} 1630; ^1H NMR ($CDCl_3$) δ 8.48 (d, 1H, H-6); 8.46 (d, 1H, H-9); 8.45 (s, 1H, H-2); 7.64 (dd, 1H, H-7); 7.53 (m, 2H, H-4′ and H-6′); 7.10 (t, 1H, H-5′); 7.04 (d, 1H, H-3′); 3.75 (s, 3H, OCH_3). Anal. C, H, N.

3.3.3. 3-(Pyridin-3-ylcarbonyl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (6). From **V**. Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1} 1626; ^1H NMR ($CDCl_3$) δ 9.12 (d, 1H, H-2′ Py); 8.88 (dd, 1H, H-4′ Py); 8.60 (s, 1H, H-2); 8.50 (m, 2H, H-6 and H-9); 8.25 (dd, 1H, H-6′ Py); 7.70 (dd, 1H, H-7); 7.55 (dd, 1H, H-5′ Py). Anal. C, H, N.

3.3.4. 3-(1,3-Benzodioxol-5-ylcarbonyl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (7). From **VI**. Brown crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1} 1660; ^1H NMR ($CDCl_3$) δ 8.50 (m, 3H, H-6, H-2 and H-9); 7.68 (dd, 1H, H-7); 7.50 (dd, 1H, H-6′); 7.48 (d, 1H, H-2′); 6.90 (d, 1H, H-5′); 6.10 (s, 2H, OCH_2O). Anal. C, H, N.

3.3.5. 3-(Fur-2-ylcarbonyl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (8). From **VII**. Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1} 1650; ^1H NMR ($CDCl_3$) δ 8.85 (s, 1H, H-2); 8.55 (d, 1H, H-6); 8.50 (d, 1H, H-9); 7.75 (d, 1H, H-5′ fur); 7.68 (dd, 1H, H-7); 7.60 (d, 1H, H-3′ fur); 6.68 (dd, 1H, H-4′ fur). Anal. C, H, N.

3.3.6. 3-(Thien-2-ylcarbonyl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (9). From **VIII**. Brown crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1} 1630; ^1H NMR ($CDCl_3$) δ 8.62 (s, 1H, H-2); 8.52 (d, 1H, H-6); 8.49 (d, 1H, H-9); 8.04 (d, 1H, H-3′ thien.); 7.77 (d, 1H, H-5′ thien.); 7.69 (dd, 1H, H-7); 7.23 (t, 1H, H-4′ thien.). Anal. C, H, N.

3.3.7. 3-(Thien-3-ylcarbonyl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (10). From **IX**. Yellow crystals. TLC eluent: petroleum benzine/ethyl acetate 31 v/v; IR ν cm^{-1} 1620, 1552; ($CDCl_3$) δ 8.62 (s, 1H, H-2); 8.53 (d, 1H, H-6); 8.50 (d, 1H, H-9); 8.29 (m, 1H, H-2′ thien.); 7.73 (dd, 1H, H-5′ thien.); 7.68 (dd, 1H, H-7); 7.42 (t, 1H, H-4′ thien.). Anal. C, H, N.

3.4. General procedure for the synthesis of 3, 5, 11–13

The starting material, 3-carboxy-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide[30] (0.4 mmol) was reacted with 2.0 mL of thionyl chloride and maintained at 60 ° for 30′; the final solution was evaporated in vacuo and the corresponding 3-carbonylchloride intermediate was utilized as raw material in Suzuki coupling for compound **3** or in Friedel–Crafts reaction to obtain compounds **5**, **11**–**13**. Compounds **8** and **9** were also alternatively obtained by this synthetic procedure using furane or tiophene as aryl compounds (yields shown on Supplementary data).

3.4.1. 3-Benzoyl-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (3). The raw intermediate 3-carbonyl chloride (0.4 mmol) was suspended in 8 mL of absolute toluene. Phenylboronic acid (0.2 mmol), cesium carbonate (0.5 mmol) and an excess of triphenylphosphine palladium(0) (Tetrakis) (10 mg) were added; the mixture was maintained at refluxing temperature under nitrogen

for 2 days. The final suspension was diluted with ethyl acetate and washed with water and a saturated solution of sodium bicarbonate. The ethyl acetate solution was then dried over sodium sulfate and evaporated under pressure. The residue was recrystallized by a suitable solvent.

Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1} 1677; ^1H NMR ($CDCl_3$) δ 8.68 (s, 1H, H-2); 8.50 (m, 2H, H-6 and H-9); 7.68 (dd, 1H, H-7); 7.42 (m, 2H, H-2′ and H-6′); 7.25 (m, 3H, H-3′, H-4′ and H-5′). Anal. C, H, N.

3.5. General procedure for Friedel–Craft reaction to obtain compounds 5, 11–13

The raw intermediate 3-carbonyl chloride (0.4 mmol) was suspended in a solution of 15 mL of methylene chloride and 0.8 mmol of anhydrous tin tetrachloride; the red solution was maintained at reflux, then 1.2 mmol of suitable aryl compounds were added. The reaction was monitored by TLC and when the starting material disappeared, the reaction was quenched by treatment with HCl 1:1, diluted with methylene chloride and the organic layer was separated. The methylene solution was washed with a saturated solution of sodium bicarbonate, dried over sodium sulfate and evaporated. The residue was purified by recrystallization.

3.5.1. 3-(4-Methoxybenzoyl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (5). From 3-carbonyl chloride derivative and anisole; dark-yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1} 1630; ^1H NMR ($CDCl_3$) δ 8.50 (m, 3H, H-2, H-6 and H-9); 7.94 (d, 2H, H-2′ and H-6′); 7.65 (dd, 1H, H-7); 7.01 (d, 2H, H-3′ and H-5′); 3.9 (s, 3H, OCH_3). Anal. C, H, N.

3.5.2. 3-(1H-pyrrol-2-ylcarbonyl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (11). From 3-carbonyl chloride derivative and pyrrole; yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1} 3263, 1613; ^1H NMR ($CDCl_3$) δ 9.92 (br s, 1H, NH exch.); 8.86 (s, 1H, H-2); 8.52 (d, 1H, H-6); 8.48 (d, 1H, H-9); 7.65 (dd, 1H, H-7); 7.33 (m, 1H, H-5′ pyrr.); 7.18 (m, 1H, H-3′ pyrr.); 6.40 (m, 1H, H-4′ pyrr.). Anal. C, H, N.

3.5.3. 3-(1-Methyl-1H-pyrrol-2-ylcarbonyl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (12)

3.5.4. 3-(1-Methyl-1H-pyrrol-3-ylcarbonyl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (13). From the reaction of 3-carbonyl chloride derivative and *N*-methylpyrrole the two isomers were detected: purification by chromatography column gave as faster band eluted compound **12** and as second band the isomer (1-methyl-1H-pyrrol-3-yl) derivative **13**.

Compound **12**. Yellow crystals. TLC eluent: toluene/ethyl acetate 8:2 v/v; IR ν cm^{-1} 1613; ^1H NMR ($CDCl_3$) δ 8.50 (m, 2H, H-2, H-6); 8.48 (d, 1H, H-9); 7.64 (dd, 1H, H-7); 6.96 (m, 2H, H-3′ and H-5′ pyrr.); 6.32 (m, 1H, H-4′ pyrr.); 4.10 (s, 3H, CH_3-N). Anal. C, H, N.

Compound **12** was also obtained by the alkylation of compound **11**: to a solution of DMF (10 mL) and **11** (0.13 mmol) were added anhydrous potassium carbonate (0.13 mmol) and methyl iodide (0.26 mmol). The reaction was maintained at 35 °C and monitored by TLC and when starting material disappeared water/ice was added. The precipitate, compound **12**, was filtered and recrystallized by ethanol. Yield 15%.

Compound **13**. Yellow crystals. TLC eluent: toluene/ethyl acetate 8:2 v/v; IR ν cm^{-1} 1613; ^1H NMR ($CDCl_3$) δ 8.60 (s, 1H, H-2); 8.52 (d, 1H, H-6); 8.48 (d, 1H, H-9); 7.64 (dd, 1H, H-7); 7.52 (m, 1H, H-2′ pyrr.); 6.82 (m, 1H, H-5′ pyrr.); 6.67 (m, 1H, H-4′ pyrr.); 3.80 (s, 3H, CH_3–N). Anal. C, H, N.

3.6. General procedure for the synthesis of compounds 2a, 5a–6a, 8a–9a, 11a–12a

Starting compounds **2**, **5**–**6**, **8**–**9**, **11**, and **12** (0.1 mmol) were suspended in ethanol (10 mL) and 10% sodium hydroxide solution (5 mL) and were maintained at room temperature for one day. The final suspension was filtered or extracted with ethyl acetate. The precipitate was recrystallized from a suitable solvent or the solution was evaporated in vacuum and the residue purified.

3.6.1. 3-Acetyl-8-ethoxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (2a). Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1} 1663; ^1H NMR ($CDCl_3$) δ 8.58 (s, 1H, H-2); 8.47 (d, 1H, H-6); 7.72 (d, 1H, H-9); 7.23 (dd, 1H, H-7); 4.30 (q, 2H, CH_2); 2.8 (s, 3H, $COCH_3$); 1.50 (t, 3H, CH_3). Anal. C, H, N.

3.6.2. 3-(4-Methoxybenzoyl)-8-ethoxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (5a). Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1} 1629 ^1H NMR ($CDCl_3$) δ 8.52 (s, 1H, H-2); 8.47 (d, 1H, H-6); 7.96 (d, 2H, H-2′ and H-6′); 7.75 (d, 1H, H-9); 7.22 (dd, 1H, H-7); 7.01 (d, 2H, H-3′ and H-5′); 4.35 (q, 2H, CH_2); 3.9 (s, 3H, OCH_3); 1.60 (t, 3H, CH_3). Anal. C, H, N.

3.6.3. 3-(Pyridin-3-ylcarbonyl)-8-ethoxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (6a). Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1} 1630; ^1H NMR ($CDCl_3$) δ 9.15 (d, 1H, H-2′ Py); 8.85 (dd, 1H, H-4′ Py); 8.58 (s, 1H, H-2); 8.48 (d, 1H, H-6); 8.20 (dd, 1H, H-6′ Py); 7.80 (d, 1H, H-9); 7.50 (dd, 1H, H-5′ Py); 7.19 (dd, 1H, H-7); 4.30 (q, 2H, CH_2); 1.50 (t, 3H, CH_3). Anal. C, H, N.

3.6.4. 3-(Fur-2-ylcarbonyl)-8-ethoxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (8a). Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1} 1650 ^1H NMR ($CDCl_3$) δ 8.81 (s, 1H, H-2); 8.48 (d, 1H, H-6); 7.78 (d, 1H, H-9); 7.72 (d, 1H, H-5′ fur); 7.63 (d, 1H, H-3′ fur); 7.22 (dd, 1H, H-7); 6.66 (dd,

1H, H-4′ fur); 4.30 (q, 2H, CH_2); 1.60 (t, 3H, CH_3). Anal. C, H, N.

3.6.5. 3-(Thien-2-ylcarbonyl)-8-ethoxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (9a). Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1} 1630; ^1H NMR ($CDCl_3$) δ 8.64 (s, 1H, H-2); 8.49 (d, 1H, H-6); 8.12 (m, 1H, H-3′ thien.); 7.76 (m, 2H, H-9 and H-5′ thien.); 7.24 (m, 2H, H-7 and H-4′ thien.); 4.35 (q, 2H, CH_2). Anal. C, H, N.

3.6.6. 3-(1H-pyrrol-2-ylcarbonyl)-8-ethoxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (11a). Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1} 3263, 1613; ^1H NMR ($CDCl_3$) δ 10.20 (br s, 1H, NH exch.); 8.71 (s, 1H, H-2); 8.50 (d, 1H, H-6); 7.75 (d, 1H, H-9); 7.41 (m, 1H, H-5′ pyrr.); 7.22 (m, 2H, H-7 and H-3′ pyrr.); 6.41 (m, 1H, H-4′ pyrr.); 4.30 (q, 2H, CH_2); 1.50 (t, 3H, CH_3). Anal. C, H, N.

3.6.7. 3-(1-Methyl-1H-pyrrol-2-ylcarbonyl)-8-ethoxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (12a). Yellow crystals. TLC eluent: petroleum benzine/ethyl acetate 2:1 v/v; IR ν cm^{-1} 1613; ^1H NMR ($CDCl_3$) δ 8.50 (s, 1H, H-2); 8.47 (d, 1H, H-6); 7.75 (d, 1H, H-9); 7.20 (dd, 1H, H-7); 7.03 (m, 1H, H-3′ pyrr.); 6.95 (m, 1H, H-5′ pyrr.); 6.25 (m, 1H, H-4′ pyrr.); 4.35 (q, 2H, CH_2); 4.1 (s, 3H, NCH_3); 1.50 (t, 3H, CH_3). Anal. C, H, N.

3.7. General procedure for the synthesis of compounds 2b, 8b–9b, 11b–12b, 8c–9c

A mixture of compounds **2**, **5**–**6**, **8**–**9**, **11** ,and **12** (0.2 mmol), 10 mL of dichloromethane, 5 mL of 40% sodium hydroxide solution, 0.1 mole of tetrabutylammonium bromide, and a large excess (5 mL) of the suitable reagent (phenol or benzyl alcohol) 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 a suitable solvent.

3.7.1. 3-Acetyl-8-phenoxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (2b). From **2** and phenol. Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1} 1663, 1358; ^1H NMR ($CDCl_3$) δ 8.56 (s, 1H, H-6); 8.51 (d, 1H, H-2); 7.71 (d, 1H, H-9); 7.53 (t, 2H, H-3′ and H-5′); 7.38 (t, 1H, H-4′); 7.33 (dd, 1H, H-7); 7.20 (d, 2H, H-2′ and H-6′); 2.70 (s, 3H, $COCH_3$). Anal. C, H, N.

3.7.2. 3-(Fur-2-ylcarbonyl)-8-phenoxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (8b). From **8** and phenol. Yellow crystals. TLC eluent: toluene/ethyl acetate 8:3 v/v; IR ν cm^{-1} 1650, 1358; ^1H NMR ($CDCl_3$) δ 8.75 (s, 1H, H-2); 8.53 (d, 1H, H-6); 7.75 (d, 1H, H-9); 7.71 (d, 1H, H-5′ fur); 7.60 (d, 1H, H-3′ fur); 7.53 (t, 2H, H-3′ and H-5′); 7.38 (t, 1H, H-4′); 7.32 (dd, 1H, H-7); 7.10 (d, 2H, H-2′ and H-6′); 6.64 (dd, 1H, H-4′ fur). Anal. C, H, N.

3.7.3. 3-(Thien-2-ylcarbonyl)-8-phenoxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (9b). From **9** and phenol. Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1} 1630, 1358; ^1H NMR ($CDCl_3$) δ 8.55 (s, 1H, H-2); 8.53 (d, 1H, H-6); 8.05 (m, 1H, H-3′ thien.); 7.73 (m, 2H, H-9 and H-5′ thien.); 7.53 (t, 2H, H-3′ and H-5′); 7.40 (t, 1H, H-4′); 7.32 (dd, 1H, H-7); 7.20 (m, 3H, H-2′, H-6′ and H-4′ thien.). Anal. C, H, N.

3.7.4. 3-(1H-pyrrol-2-ylcarbonyl)-8-phenoxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (11b). From **11** and phenol. Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1} 3263, 1613; ^1H NMR ($CDCl_3$) δ 10.10 (br s, 1H, NH exch.); 8.62 (s, 1H, H-2); 8.52 (d, 1H, H-6); 7.73 (d, 1H, H-9); 7.52 (t, 2H, H-3′ and H-5′); 7.32 (m, 2H, H-4′ and H-5′ pyrr.); 7.20 (m, 4H, H-2′, H-6′ and H-3′ pyrr.); 6.41 (m, 1H, H-4′ pyrr.). Anal. C, H, N.

3.7.5. 3-(1-Methyl-1H-pyrrol-2-ylcarbonyl)-8-phenoxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (12b). From **12** and phenol. Yellow crystals. TLC eluent: toluene/ethyl acetate 8:3 v/v; IR ν cm^{-1} 1613; ^1H NMR ($CDCl_3$) δ 8.52 (d, 1H, H-6); 8.42 (s, 1H, H-2); 7.72 (d, 1H, H-9); 7.64 (dd, 1H, H-7); 7.52 (t, 2H, H-3′ and H-5′); 7.38 (t, 1H, H-4′); 7.30 (dd, 1H, H-7); 7.20 (d, 2H, H-2′, H-6′); 6.96 (dd, 1H, H-5′ pyrr.); 6.92 (m, 1H, H-3′ pyrr.); 6.21 (m 1H, H-3′ pyrr.); 4.10 (s, 3H, CH_3-N). Anal. C, H, N.

3.7.6. 3-(Fur-2-ylcarbonyl)-8-benzyloxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (8c). From **8** and benzyl alcohol. Yellow crystals. TLC eluent: toluene/ethyl acetate 8:3 v/v; IR ν cm^{-1} 1635; ^1H NMR ($CDCl_3$) δ 8.82 (s, 1H, H-2); 8.50 (d, 1H, H-6); 7.91 (d, 1H, H-9); 7.72 (d, 1H, H-5′ fur); 7.63 (d, 1H, H-3′ fur); 7.48 (m, 5H, Ph); 7.30 (dd, 1H, H-7); 6.67 (dd, 1H, H-4′ fur); 5.38 (s, 2H, OCH_2). Anal. C, H, N.

3.7.7. 3-(Thien-2-ylcarbonyl)-8-benzyloxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (9c). From **9** and benzyl alcohol. Yellow crystals. TLC eluent: toluene/ethyl acetate 8:3 v/v; IR ν cm^{-1} 1635; ^1H NMR ($CDCl_3$) δ 8.63 (s, 1H, H-2); 8.50 (d, 1H, H-6); 8.11 (m, 1H, H-3′ thien.); 7.90 (d, 1H, H-9); 7.75 (m, 1H, H-5′ thien.); 7.52 (m, 5H, Ph); 7.32 (dd, 1H, H-7); 7.24 (m, 1H, H-4′ thien.); 5.40 (s, 2H, OCH_2). Anal. C, H, N.

3.7.8. 3-(Fur-2-ylcarbonyl)-8-propargyloxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (8d). From **8** and benzyl alcohol. Yellow crystals. TLC eluent: toluene/ethyl acetate 8:3 v/v; IR ν cm^{-1} 1635; ^1H NMR ($CDCl_3$) δ 8.82 (s, 1H, H-2); 8.51 (d, 1H, H-6); 7.92 (d, 1H, H-9); 7.73 (d, 1H, H-5′ fur); 7.61 (d, 1H, H-3′ fur); 7.30 (dd, 1H, H-7); 6.67 (dd, 1H, H-4′ fur); 5.00 (s, 2H, OCH_2); 2.70 (s, 1H, CH). Anal. C, H, N.

3.8. General procedure for the synthesis of compounds 9bR and 11R

Starting materials, compounds **9b** and **11**, were treated with triethylphosphite (TEP) in toluene abs as previously reported.[38]

3.8.1. 3-(Thien-2-ylcarbonyl)-8-phenoxypyrazolo[5,1-*c*][1,2,4]benzotriazine (9bR). From **9b**. Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; ^{1}H NMR ($CDCl_3$) δ 8.70 (m, 2H, H-2 and H-6); 8.30 (dd, 1H, H-3′ thien.); 7.82 (d, 1H, H-9); 7.78 (dd, 1H, H-5′ thien.); 7.55 (m, 3H, H-3′ and H-5′ and H-7); 7.38 (m, 1H, H-4′); 7.23 (m, 3H, H-2′, H-6′ and H-4′ thien.). Anal. C, H, N.

3.8.2. 3-(1H-pyrrol-2-ylcarbonyl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine (11R). From **11**; Yellow crystals. TLC eluent: diisopropylether/ciclohexane 8:3 v/v; IR ν cm^{-1} 3263; ^{1}H NMR ($CDCl_3$) δ 11.2 (br s, 1H, NH exch.); 8.81 (s, 1H, H-2); 8.65 (d, 1H, H-6); 8.52 (d, 1H, H-9); 7.78 (dd, 1H, H-7); 7.41 (m, 1H, H-5′ pyrr.); 7.20 (m, 1H, H-3′ pyrr.); 6.41 (m, 1H, H-4′ pyrr.). Anal. C, H, N.

3.9. 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.[57] 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. 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 were 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. Non-specific 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.

3.10. 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.

3.10.1. 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 16 rpm. The treatment was performed before the test. The numbers of falls from the rod in 30 s were counted 15, 30, 45 and 60 min after drug administration.

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

3.10.3. 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 centee of the lighted area, facing away from the dark one, and allowing it to explore the novel environment for 5 min. The time spent in the illuminated side was measured. This test exploited the conflict between the animal's tendency to explore a new environment and its fear of bright light.

3.10.4. Pentylenetetrazole (PTZ)-induced seizure. PTZ (90 mg/kg sc) was injected 30 min after the administration of drugs. The number of clonic generalized convulsions was noted over a period of 30 min.

3.10.5. Passive-avoidance test. The test was performed according to the step-through method described by Jarvik and Kopp.[58] 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. The maximum entry latency allowed in the training and retention sessions was, respectively, 60 and 180 s.

3.10.6. 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. The end-point was recorded as 150 min.

3.10.7. Drugs. Diazepam (Valium 10–Roche), Flumazenil (Tocris Cooksonl Ltd, UK), Pentylenetetrazole (PTZ) (Sigma) were the drugs used. All drugs were dissolved in isotonic (NaCl 0.9%) saline solution and injected sc/ip. All the new compounds were administered by the po route and were suspended in 1% carboxymeth-

ylcellulose (CMC) sodium salt and sonicated immediately before use. Drug concentrations were prepared in such a way that the necessary dose could be administered in a 10 ml/kg volume of CMC 1% by the po, ip or sc routes.

3.10.8. Statistical analysis. All experimental results are given as means ± SEM. An analysis of variance, ANOVA, followed by Fisher's protected least significant difference procedure for post hoc comparison, was used to verify significance between two means of behavioral results. Data were analyzed with the StataView software for Macintosh (1992). *P* values of less than 0.05 were considered significant.

3.11. Molecular modeling

The model[41] of the $GABA_A$ receptor was downloaded from Ernst web page.[43] The structure of ligands was generated from the standard fragmentary library of the BUILDER module of the INSIGHT II program.[59] The complex ligand–protein was generated according to the position of essential amino acids for ligands binding according to literature data.[44–50]

Geometry optimizations were achieved with AMBER force field of DISCOVER module of the INSIGHTII program by applying the Conjugate Gradients algorithm with a convergence criterion of 0.01 kcal/mol.

3.12. Dynamics simulations

To assess the preferred conformation of the ligand and in the context of the selected conformer, a 100 ps molecular dynamics simulation of the complex was carried out. The calculations were performed in vacuum with the DISCOVER module of the INSIGHT II program[59] using AMBER force field. A multiple-step procedure was used. The complex was energetically minimized. The minimized system was used as initial structure for the subsequent molecular dynamics (MD) simulation. The system was heated gradually until 310 K. Coordinates were saved every 500 fs yielding 200 structures. The total time of complex molecular dynamics simulation was 1.6 ns; during the total dynamics simulation were collected 3200 conformations. The dynamics simulation was carried out keeping the backbone atoms constrained.

Supplementary data

Chemical data of intermediates **III**–**IX** (Table 1), of final compounds (Table 2), analytical data (Table 3) and motor coordination table (Table 4). Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.02.058.

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