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Benzodiazepine receptor ligands. 8: Synthesis and pharmacological evaluation of new pyrazolo[5,1-*c*] [1,2,4]benzotriazine 5-oxide 3- and 8-disubstituted: High affinity ligands endowed with inverse-agonist pharmacological efficacy

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Abstract—The synthesis and the binding study of new 3-arylesters and 3-heteroarylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 8-substituted are reported. The nature of these substituents (in terms of lipophilic and electronic features) seems to influence the binding affinity. High-affinity ligands were studied in mice in vivo for their pharmacological effects, considering six potential benzodiazepine actions: anxiolytic-like effects, muscle relaxant effects, motor coordination, anticonvulsant action, spontaneous motor activity, and ethanol-potentiating action. Compounds **4d** and **6d** showed an inverse-agonist profile. 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

The ability of benzodiazepines to strengthen the inhibitory action of GABA in the brain offers a clinical therapy of primary importance in treating neurological disorders such as anxiety, sleep disturbance, muscle spasms and epilepsy. However, some side effects limit the clinical usefulness of BZs, such as memory impairment, cognition and motor disturbances, potentiation of the effects of alcohol, tolerance, and physical dependence. Classical benzodiazepines (Bz) act as positive allosteric modulators, binding a specific site on the $GABA_A$ receptor complex composed of α-, β-, and γ-subunits, arranged in a pentameric structure.[1,2] Sensitivity to Bz is conferred by the γ-subunit (γ_2 genetic variant) and adjacent α-subunit (α_1, α_2, α_3, and α_5 genetic variants). The heterogeneity of $GABA_A$/BzR complex subtypes associated with regionally distinct distribution in the central nervous system has been indicated as the major factor responsible for the therapeutic actions displayed by BZs.[3–6]

Based on a vivo point mutation strategy recent studies[7] have demonstrated that sedation, anterograde amnesia, and part of seizure protection are mediated through α_1-subtypes, anxiolysis through α_2-subtypes and muscle relaxant effects through α_2-, α_3-, and α_5-subtypes.

The search for subtype specific ligands is a promising approach in order to clarify the pharmacological role

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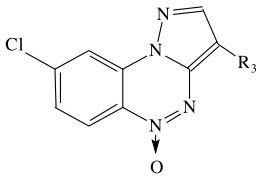
of subtype receptors and to obtain new drugs with higher therapeutic selectivity and a reduced side-effect profile. In light of this, our studies on pyrazolo [5,1-*c*][1,2,4]benzotriazines have been directed toward the search for new selective nonbenzodiazepine ligands at the $GABA_A$/BzR complex with preferential affinity or preferential efficacy.

Recently, we reported two new series of 8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 3-arylester (A series) and 3-heteroaryl derivatives (B series), respectively, which showed high affinity for BZR (K_i = 36.3–1.00 nM range) (Chart 1).[8–11]

For the sake of clarity, reference compounds listed in Chart 1 follow the same numerical succession of newly synthesized derivatives.

On the basis of structure–activity relationship (SAR) data from numerous derivatives, it was proposed that the 8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine system, the same in the two series (A and B), binds the $GABA_A$/BzR complex through N1 and N4 atoms by means of a hydrogen bond involving H_2 and H_1 donor receptor sites for this class of ligands.[12] The presence at the position 3 of a π-electron rich ring, providing a π–π interaction with receptor protein, improves the affinity of these ligands in both series.[9,10] The pharmacophoric descriptors are in accordance with those of the model formulated by other researchers.[13,14]

The lead compounds of two series, 3-(2-thienylmethoxycarbonyl)- and 3-(3-thienyl)-derivative, **6** and **11**, respectively, were found to have a selective in vivo activity. In particular, the ester **6** shows anxiolytic-like activity similar to diazepam without any sedative or amnesic properties, or interference from alcohol. The 3-heteroaryl derivative **11** displays selective anticonvulsant properties. Nevertheless, compounds **6** and **11**, tested for binding at $\alpha_{x(1-3)}\beta_2\gamma_2$, $\alpha_5\beta_3\gamma_2$ $GABA_A$/BzR complex subtypes, do not display pronounced preferential subtype selectivity.[9] Based on pharmacological results, it was proposed that **6** and **11** are functionally selective ligands with a mixed agonist/antagonist profile for various receptor subtypes.



A series

R_3 =$COOCH_2$-2-MeO-Phenyl **4**[8]

R_3 =$COOCH_2$-2-thienyl **6**[9]

B Series

R_3 = 3-thienyl **11**[10]

R_3 = 2-thienyl **12**[10]

R_3 = 3-furyl **13**[11]

R_3 = 2-furyl **14**[11]

Chart 1. .

In the present study, with the aim to investigate if chemical modification of the 8-substituent of the pyrazolo-[5,1-*c*][1,2,4]benzotriazine 5-oxide system could yield new ligands, with the same high affinity but improved efficacy and receptor subtype selectivity as compared to lead compounds **6** and **11**, we replaced the chlorine atom of molecules of two series, respectively, with substituents of different lipophilic (π) and electronic (σ) features and bulkiness (Es),[15] leaving the favorable substituent at the position 3 unchanged. Therefore, we synthesized new derivatives of both series, bearing at position 8 bromine or iodine atoms (fluorine atom has resulted in being detrimental for binding[16]), a methyl-, an ethoxy- a methoxy-, and a methylthio group. Some 3-arylester and 3-heteroaryl deoxy derivatives were also synthesized to confirm the different role of a 5-oxide group in the binding of ligands of two series, as previously evidenced.[9,10]

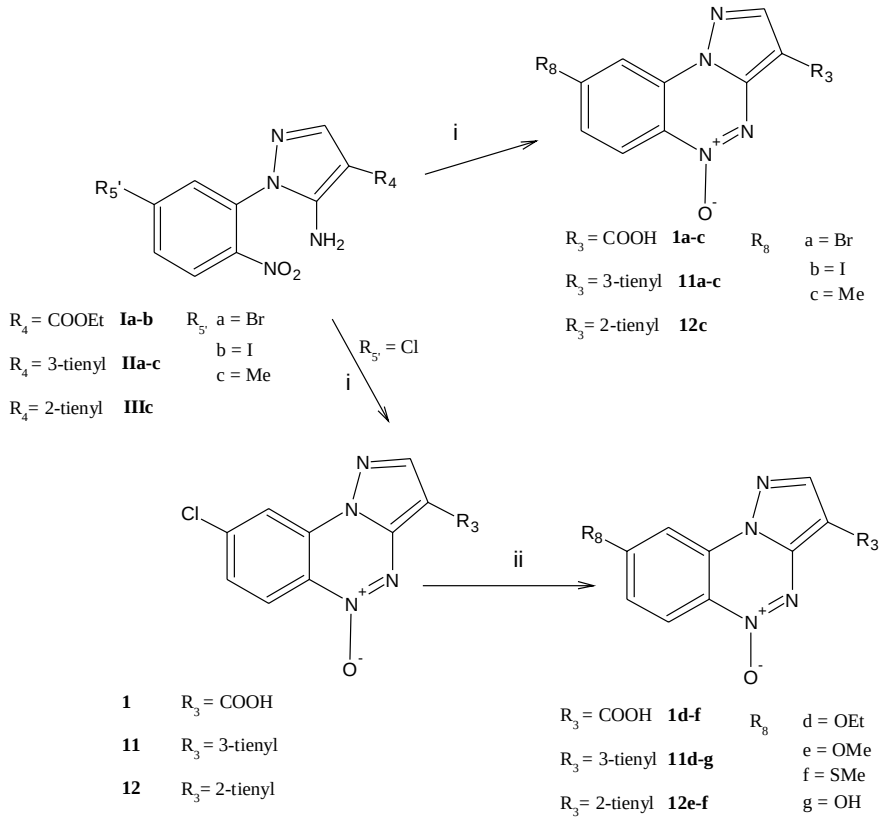
2. Chemistry

The crucial synthetic step to obtain the pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide system is the intramolecular cyclization between the nitro and amino group under basic conditions of the suitable 1-(2-nitrophenyl)-5-aminopyrazole, following a procedure reported in our previous papers.[17] The synthetic pathways which yielded desired final compounds are illustrated in Schemes 1 and 2; chemical and physical data of new compounds are shown in Tables 7 and 8 in Section 6.

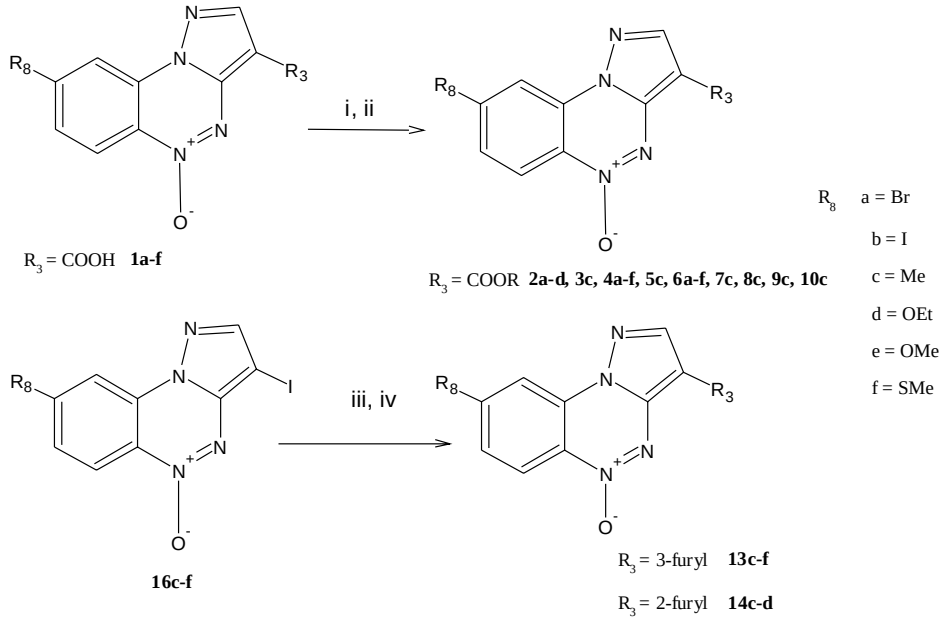
In this work, the appropriate 3-carboxy-8-substituted pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxides (**1a**,**b**,**c**,[17] **d**,[17] **e**, and **f**[18] see Table 7 in Section 6) were the starting material for the synthesis of 3-ester derivatives. Acids **1a** and **b**, (Scheme 1) 8-bromo- and 8-iodio-substituted, were achieved from the corresponding ethyl 1-(2-nitro-5-bromophenyl)-, and the ethyl 1-(2-nitro-5-iodiophenyl)-5-aminopyrazol-4-carboxylate **Ia** and **b** (see Table 8 in Section 6), which were, in turn, obtained from the suitable 5-bromo-2-nitro-,[19] or 5-iodio-2-nitrophenylhydrazine[20] and ethyl 2-cyano-3-ethoxypropeneate in ethanol.[17] Acid **1e**, 8-methoxy- was obtained from 3-carboxy-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide exploiting the aromatic nucleophilic substitution of chlorine atom, as reported to obtain **1d**[17] and **1f**.[18]

The desired ester derivatives (see Table 7 in Section 6 and Scheme 2) **2a**–**b**, **3c**, and **10c** were prepared by treating the suitable 3-carboxylic acid (**1a**–**c**) with thionyl chloride, and the intermediate 3-carbonyl chloride was dissolved in 2-methyl-3-butene stabilized chloroform and added to the suitable alcohol. All other esters **4a**–**f**, **5c**, **6a**–**f**, **7c**, **8c**, and **9c** were obtained from acids **1a**–**f** by treatment with triethyl amine/ethyl chlorocarbonate in tetrahydrofurane (THF) to achieve the not isolated, mixed anhydride, which was reacted with suitable alcohol.

To evaluate the influence on biological activity of the 8-substituent (bromine, iodine, methyl-, ethoxy-,



Scheme 1. Reagents: (i) NaOH 10% solution; (ii) NaOH/EtOH (**d** compounds) or NaOH/MeOH (**e** compounds) or NaSMe/EtOH (**f** compounds) or NaOH 40% (**g** compound). **1c**,[17] **1d**[17] and **1f**.[18]



Scheme 2. Reagents: (i) $SOCl_2$; (ii) ROH for compounds **2a** and **b**, **3c**, and **10c**. (i) NEt_3; (ii) ClCOOEt/THF, ROH for compounds **3c**, **4a**–**f**, **5c**, **6a**–**f**, **7c**, **8c**, and **9c**. For compound **2c**, see Ref. 18 and for **2d**, see. Ref. 17 In compounds **2**, R_3 = COOEt; **3**, R_3 = $COOCH_2Ph$; **4**, R_3 = $COOCH_2$-2-OMePh; **5**, R_3 = $COOCH_2$-2-NO_2Ph; **6**, R_3 = $COOCH_2$-2-tienyl; **7**, R_3 = $COOCH_2$-3-tienyl; **8**, R_3 = $COOCH_2$-2-furyl; **9**, R_3 = $COOCH_2$-3-furyl; **10**, R_3 = COO-2-OMePh. (iii) Toluene, Tetrakis ($Pd(PPh_3)_4$); (iv) 2-furyl- or 3-furylboronic acid, Na_2CO_3 2 M.

methoxy-, and methylthio-group) in the 3-heteroaryl series, compounds **11a**–**g**, **12c**, **12e**–**f**, **13c**–**f**, and **14c**–**d** (Table 7 in Section 6 and Schemes 1 and 2) were synthesized. Compounds **11a**–**c**, 3-(thien-3-yl)- and **12c,** 3-(thien-2-yl)- were obtained reacting, as the first reaction step, the 5-bromo-2-nitro-,[19] 5-iodio-2-nitro-,[20] and

5-methyl-2-nitrophenylhydrazine[18] with 2-(thien-3-yl)- and 2-(thien-2-yl)-3-oxapropanenitrile[21,22] following a reported method.[10] The obtained 4-(thien-3-yl)-5-aminopyrazoles **IIa–c**, and 4-(thien-2-yl)-5-aminopyrazole **IIIc** (see Table 8 in Section 6) were then cyclized to pyrazolobenzotriazine 5-oxide system in alkaline medium.[10] Compounds **11d–f**, 8-ethoxy-, 8-methoxy-, and 8-methylthio derivative, were obtained in ethanol/sodium hydroxide, methanol/sodium hydroxide, and ethanol/sodium thiomethoxide, exploiting the aromatic nucleophilic substitution of the 3-(3-thienyl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxides.[10] The 8-hydroxy derivative **11g**, isolated as a by-product in the **11d** synthesis, was also characterized and used for the SAR. Compounds **12e–f** were obtained from 3-(2-thienyl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxides[10] and methanol/sodium hydroxide or ethanol/sodium thiomethoxide, respectively, in the same manner as compounds **11e–f** (Scheme 1).

The synthetic path to obtain the 8-methyl- and 8-ethoxy derivatives bearing at the position 3 a furyl ring, **13c–f** and **14c–d** (Scheme 2) exploited the palladium(0)catalyzed cross-coupling reaction between 3-iodio-8-methyl—**16c**, 3-iodio-8-ethoxy—**16d,** 3-iodio-8-methoxy—**16e**, and 3-iodio-8-methylthio pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide **16f** (see below), and 3-furyl- and 2-furylboronic acid.[11] For this purpose, 3-iodo derivatives **16c–f** were achieved from corresponding 8-substituted pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide, 8-methyl-[18] 8-ethoxy-[17] 8-methoxy-, **15e**, and 8-methylthiopyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide[18] by treatment with iodine monochlorine (ICl) in chloroform following the classical halogenation reaction. Compound **15e**, 8-methoxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide, was obtained by decarboxylation from the corresponding 3-carboxylic derivative **1e**.

The synthesis of the 5-deoxide derivatives **2bR**, **3cR**, **4aR**, and **11cR** (see Table 7) useful for structure–activity relationships (SAR) was performed by treatment of corresponding 5-oxide compounds **2b**, **3c**, **4a**, and **11c** with triethylphosphite (TEP).[18]

3. Biological results

The BZR binding affinity of new 3-ester- and 3-heteroaryl derivatives was evaluated by their ability to displace [^{3}H]flumazenil ([^{3}H]Ro15-1788) from its specific binding in bovine brain membrane. Binding data for 3-ester-(**2a–b**, **3c**, **4a–f**, **5c**, **6a–f**, **7c**, **8c**, **9c**, and **10c**), 3-heteroaryl derivatives (**11a–g**, **12c**, **e–f**, **13c–f**, and **14c–d**) and corresponding 5-deoxide compounds (**2bR**, **3cR**, **4aR**, and **11cR**), and for reference compounds (see Chart 1), useful for the SAR discussion, are reported in Tables 1 and 2, respectively.

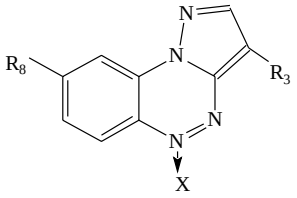
It appears from the results reported in Table 1 that the introduction at the position 8 of the 3-ester derivatives of a bromine (**2a**, **4a**, and **6a**) or iodine (**2b**, **4b**, and **6b**) yielded, in general, compounds with binding affinity lower than those of corresponding 8-chloroderivatives. Only the 8-bromo derivative **6a** displayed an affinity value twofold higher than that of reference compound **6**[9] (**6a**: K_i = 3.6 nM vs **6**: K_i = 6.8 nM). The order of substituents on binding affinity is Cl > Br > I. Therefore, the substitution at the position 8 of the chlorine atom of reference compounds (Chart 1, compounds **4**[8] and **6**,[9]) with bromine (**2a**, **4a**, and **6a**) or iodine (**2b**, **4b**, and **6b**), more lipophilic atoms than chlorine (Br π = 1.19, I π = 1.43 vs Cl π = 0.71),[15] slightly reduced the BZR recognition of ligands (see Table 1). Taking into account the similar electronic parameter of three halogen atoms (Br σ = 0.23, I σ = 0.28, Cl σ = 0.23)[15] it seems reasonable to hypothesize that the lipophilic and/or steric hindrance features (Br E_s = −1.16, I E_s = −1.40, and Cl E_s = −0.97)[15] may play an important role in anchoring ligands to receptor. A more lipophilic atom could alter the right lipophilic/hydrophilic balance of the whole ligand–receptor protein system; a bulkier halogen would not permit the perfect interaction of the molecule with receptor.

When the position 8 was substituted with a methyl- (**3c**, **4c**, **6c**, **7c**, **8c**, and **9c**), an ethoxy group (**4d**, **6d**), a methoxy group (**4e**, **6e**) or a methylthio group (**4f**, **6f**), high affinity ligands were in general obtained (K_i range 0.31–9.5 nM). The exception of compounds **5c** 3-(2-nitrobenzyloxycarbonyl)- and **10c** 3-(2-methoxyphenoxycarbonyl)-derivatives confirms that when at the position 3 there is an electron-poor ring such as 2-nitrobenzyloxy-, or too brief a spacer such as 2-methoxyphenoxy, complete loss of receptor recognition is obtained.[8] In particular, 8-ethoxy derivatives (**4d**, **6d**) and 8-methyl derivatives (**6c**, **7c**, **8c**, and **9c**) show the best affinity values, 3-8-fold higher with respect to corresponding 8-chlorosubstituted reference compounds (see Table 1). In general, the order with which different groups influence the binding affinity is EtO > Me > MeS > MeO. A lipophilic value (π) slightly lower than or comparable to that of chlorine (EtO π = 0.38, Me π = 0.60 vs Cl π = 0.71)[15] supported by electronic features (σ) opposite of chlorine (EtO σ = −0.24, Me σ = −0.17 vs Cl σ = 0.23)[15] increases binding affinity, as in the case of compounds **4d**, **6c**, and **d**, or maintains a good BzR affinity (~2-fold lower than that of 8-chloro derivative) as in the case of **4c**. When the lipophilic (π) and electronic (σ) values are opposite of chlorine (MeO π = −0.02 vs Cl π = 0.71; MeO σ = −0.27 vs Cl σ = 0.23)[15], the affinity value decreases as in the case of compounds **4e** and **6e**. The substitution of chlorine with a thiomethyl group (MeS π = 0.60 vs Cl π = 0.71; MeS σ = 0.00 vs Cl σ = 0.23)[15] as in compounds **4f** and **6f** seems negligible.

As far as the 3-heteroaryl series (3-/2-tienyl, 3-/2-furyl) is concerned (Table 2), in general good affinity values are shown (K_i range 10–37 nM). Also in this series the substitution of chlorine atom of position 8 with methyl-, ethoxy-, and methoxy-electron-donating groups becomes favorable for binding (see compounds **11c**, **11e**, **13c–d**, and **14c–d**).

The lower affinity binding of compound **11b**, 8-iodine derivative, than 8-chloro derivative could be due to the

Table 1. BZR ligand affinity of pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 3-ester derivatives



R8: **a= Br**
b= I
c=Me
d=OEt
e=OMe
f=SMe

Compound	R_3	R_8	X	I%[a]	K_i[b] (nM)	8-Cl[c]
2a	COOEt	Br	O	95	65.2 ± 5.3	35
2b	COOEt	I	O	90	93.0 ± 8	
2bR	COOEt	I	—	95	121 ± 10	
3c	$COOCH_2Ph$	Me	O	100	15 ± 2	11.6
3cR	$COOCH_2Ph$	Me	—	93	26.5 ± 3	
4a	$COOCH_2$-2-MeOPh	Br	O	100	5 ± 0.3	**4** 1.0
4aR	$COOCH_2$-2-MeOPh	Br	—	99	7.3 ± 0.5	
4b	$COOCH_2$-2-MeOPh	I	O	100	10 ± 1	
4c	$COOCH_2$-2-MeOPh	Me	O	98	2.3 ± 0.2	
4d	$COOCH_2$-2-MeOPh	OEt	O	99	0.31 ± 0.03	
4e	$COOCH_2$-2-MeOPh	OMe	O	99	4.6 ± 0.4	
4f	$COOCH_2$-2-MeOPh	SMe	O	100	3.8 ± 0.4	
5c	$COOCH_2$-2-NO_2Ph	Me	O	61	—	
6a	$COOCH_2$-2-tienyl	Br	O	99	3.6 ± 0.3	**6** 6.8
6b	$COOCH_2$-2-tienyl	I	O	100	11 ± 2	
6c	$COOCH_2$-2-tienyl	Me	O	99	1.41 ± 0.2	
6d	$COOCH_2$-2-tienyl	OEt	O	100	0.85 ± 0.05	
6e	$COOCH_2$-2-tienyl	OMe	O	100	9.5 ± 1.0	
6f	$COOCH_2$-2-tienyl	SMe	O	100	4.0 ± 0.7	
7c	$COOCH_2$-3-tienyl	Me	O	100	3.97 ± 0.4	13.3
8c	$COOCH_2$-2-furyl	Me	O	99	4.57 ± 0.5	24.4
9c	$COOCH_2$-3-furyl	Me	O	100	4.39 ± 0.6	11.4
10c	COO-2-MeOPh	Me	O	59	—	
Daz[d]					10 ± 1.1	
Flu[d]					0.9 ± 0.5	

[a] Percent of inhibition of specific [^{3}H]Ro15-1788 binding at 10 μM concentration are means ± SEM of five determinations.
[b] K_i values are means ± SEM of five determinations.
[c] The first column represents the number of reference compound 8-chloro derivatives; the second column represents K_i values (nM) of corresponding 3-aryl- heteroarylester-8-chloro derivatives: see Refs. 8–11,16,18.
[d] See Ref. 8.

different dimension of iodine with respect to chlorine (I $E_s = -1.40$, Cl $E_s = -0.97$).[15]

The significant loss of affinity of **11g** (K_i = 690 nM), bearing a hydroxyl group at the 8-position, confirms the unsuitable substitution with a hydrophilic group at that position.[18]

With regard to the importance of the 5-oxide, that reinforces the hydrogen bond between N_4/H_2 receptor donor site,[18] it appears that in the 3-arylester series the auxiliary role of the 5-oxide group to reinforce binding is negligible because the π–π interaction works together in a more significant manner at anchorage.[9] In fact, the affinity values of 5-oxide and 5-deoxide derivatives are comparable: **3cR** K_i = 26.5 nM vs **3c** K_i = 15.2 nM, **4aR** K_i = 7.3 nM vs **4a** K_i = 5 nM.

In the 3-heteroaryl series, instead, the decrease of affinity of **11cR**, *N*-deoxy derivative, confirms the need for a 5-oxide group to recognize the receptor protein.[9,10]

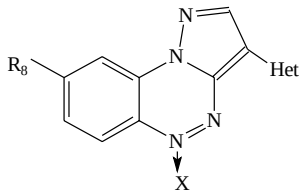
3.1. Binding of selected compounds at $\alpha_{x(1,2,5)}\beta_2\gamma_2$GABA$_A$/BzR complex subtype

Compound **4d** and **6d**, which showed a selective anxiogenic activity (see Section 4), were tested, in comparison with diazepam (full agonist) and zolpidem (α_1-selective agonist), for their ability to displace [^{3}H]Ro15-1788 from recombinant rat $\alpha_{x(1,2,5)}\beta_2\gamma_2$ GABA$_A$/BzR complex subtypes, which are stably expressed in human embryonic kidney cells (HEK293). As can be observed in Table 3, compound **4d** displayed preferential subtype selectivity α_1/α_2 (K_i = 0.81 nM vs 70 nM) and no recognition to α_5 subtype receptor. Compound **6d** recognized all studied GABA$_A$/BzR complex subtypes and displayed preferential subtype selectivity α_1/α_2, α_5 (K_i = 2.3 nM vs 10 nM and 57 nM, respectively).

4. Pharmacological results

Some of the newly synthesized molecules, **4d**, **6a**, **c**, **d**, **e**, **7c** (A series) **11a**, **c**, and **d** (B series), were studied in mice in vivo for their pharmacological effects. Six

Table 2. BZR ligand affinity of pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 3-heteroaryl derivatives



R8: **a= Br** **b= I** **c=Me** **d= OEt** **e= OMe** **f= SMe**

Compound	Het	R_8	X	I%[a]	K_i[b] (nM)	8-Cl[c]
11a	3-Tienyl	Br	O	99	40 ± 3	**11** 36.3
11b	3-Tienyl	I	O	93	88 ± 7	
11c	3-Tienyl	Me	O	95	16 ± 1.7	
11cR	3-Tienyl	Me	—	90	318 ± 20	
11d	3-Tienyl	OEt	O	93	35.9 ± 0.27	
11e	3-Tienyl	OMe	O	98	19.0 ± 2	
11f	3-Tienyl	SMe	O	96	37 ± 7	
11g	3-Tienyl	OH	O	75	690 ± 53	
12c	2-Tienyl	Me	O	95	22 ± 2	**12** 10.3
12e	2-Tienyl	OMe	O	98	17 ±	
12f	2-Tienyl	SMe	O	94	56 ±	
13c	3-Furyl	Me	O	100	13.2 ± 2	**13** 12
13d	3-Furyl	OEt	O	97	12.6 ± 2	
13e	3-Furyl	OMe	O	97	27 ±	
13f	3-Furyl	SMe	O	96	39 ±	
14c	2-Furyl	Me	O	100	10.7 ± 2	**14** 20
14d	2-Furyl	OEt	O	99	10.2	
Daz[d]					10 ± 1.1	
Flu[d]					0.9 ± 0.5	

[a] Percent of inhibition of specific [^{3}H]Ro15-1788 binding at 10 μM concentration are means ± SEM of five determinations.
[b] K_i values are means ± SEM of five determinations.
[c] The first column represents the number of reference compound 8-chloro derivatives; the second column represents K_i values (nM) of corresponding 3-heteroaryl-8-chloro derivatives: see Refs. 11,12.
[d] See Ref. 9.

potential benzodiazepine actions were considered: eventual anxiolytic-like effects were screened using light/dark choice test, muscle relaxant effects with grip-strength meter test, and motor coordination with the rota-rod test. The anticonvulsant action was evaluated using the new drugs against pentylenetetrazole-induced convulsions and the hole-board test was adopted to discover eventual alterations in mouse spontaneous motor activity caused by drugs which previously had a bend on anxiogenic-like properties. Finally, the drugs were tested also for their ethanol-potentiating action. Diazepam was used as the positive reference molecule (see Tables 4–6 and Fig. 1).

In all the tests, only molecules **4d**, **6d**, **e**, and **11d** (8-ethoxy- and 8-methoxy derivatives of A and B series) demonstrated to have selective anxiogenic-like effects. Compounds **6a** and **11a** (8-bromo derivatives of A and B series), and compounds **6c**, **7c**, and **11c** (8-methyl derivative ligands whose in vivo data are not reported) endowed with high affinity binding value (K_i range 1.41–40 nM) showed no or negligible pharmacological action. These in vivo results, which seem to be in contrast with binding data, may be attributed to poor oral bioavailability and/or to rapid metabolization.

4.1. Effects on motor coordination

First of all, we studied the effects of the substances on mouse performance in the rota-rod test. As expected, diazepam (0.3, 1, and 3 mg/kg po) 30 min after the treatment dose-dependently increased the number of falls from rotating rod, reaching statistical significance at the dose of 3 mg/kg in comparison with the vehicle-treated group of mice (Table 4). None of the newly synthesized substances induced any effect on mice endurance on the rota rod, as reported in Table 4. Only compounds **6e** and **11a** at the highest dose used (30 mg/kg po) induced some excitation in the mice, demonstrating to have some motor disturbance.

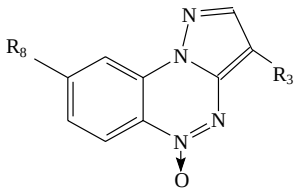
4.2. Muscle relaxant effects

We then wanted to study in more detail eventual myorelaxant effects. In the grip-strength meter test, diazepam at the dosage of 3 mg/kg po induced a statistically significant muscle relaxant effect (Table 5). Significant effect was observed also with compound **11a** at both doses (10 and 30 mg/kg po). Compounds **4d**, **6a**, **6d, 6e**, and **11d** showed no pharmacological effect.

4.3. Effects on spontaneous motor activity

A very common effect of all the old benzodiazepine ligands is some degree of sedation. The hole-board test was more sensitive, with respect to the two previous tests (rota-rod and grip-strength meter test), in checking this kind of behavior: diazepam already at the lower dosage

Table 3. Affinity value at recombinant $\alpha_{x(1,2,5)}\beta_2\gamma_2$ $GABA_A$/BzR complex subtypes



Compound	R_3	R_8	K_i (nM) or inhibition (I) %[a] (μM)		
			α_1	α_2	α_5
4d	$COOCH_2$-2-MeOPh	OEt	0.81	70	I% 25%
6d	$COOCH_2$-2-tienyl	OEt	2.3	10	57
6[b]	$COOCH_2$-2-tienyl	Cl	3.00	26	8.6
Daz[c]			14.1 ± 2.1	7.80 ± 1.1	9.80 ± 1.1
Zolpidem[d]			26.7	156	>10000

[a] K_i values represent means ± SEM derived from three independent experiments, conducted in triplicate; I% Percent of inhibition of specific [^{3}H]Ro15-1788 binding at 10 μM concentration are means ± SEM of five determinations.
[b] See Ref. 9.
[c] See Ref. 10.
[d] See Ref. 13.

Table 4. Motor coordination, anticonvulsant, and anxiolytic-like effects of new compounds in comparison with those of diazepam

Treatment[a]	mg/kg po	Motor coordination rota-rod test		Anticonvulsant activity		Anxiety activity light–dark box	
		n	No. of falls in 30 s	Against PTZ-induced attacks (%)	*n*	No. transfer in 5′	Time (s) in light in 5′
CMC 1%[b]	0.1 ml	20	0.30 ± 0.12	5	20	15.6 ± 1.9	66.1 ± 6.5
Diazepam	0.3	10	0.5 ± 0.27	70***			
	1	15	0.6 ± 0.21	100***	9	23.4 ± 4.5**	79.7 ± 9.7
	3	6	1.2 ± 0.4*	100***	17	22.7 ± 2.3*	147.5 ± 14.2**
Flumazenil	100				16	17.0 ± 2.7	66.5 ± 6.7
4d	10	10	0.1 ± 0.1	0			
	30	9	0.4 ± 0.24	0	5	10.2 ± 2.2	69.2 ± 10.9
6a	10	10	0.6 ± 0.22	20	11	10.8 ± 2.2	69.2 ± 14.1
	30	10	0.3 ± 0.15	20	10	8.1 ± 2.6	81.4 ± 14.9
6d	10	11	0.13 ± 0.09	6.6	9	5.7 ± 2.2**	39.6 ± 9.7*
	30	10	0.35 ± 0.11	17.6	17	6.9 ± 1.6**	42.7 ± 8.4
6d+Flu	10				19	14.4 ± 1.9	66.8 ± 10.7
	100						
6d+daz	10	12	0.58 ± 0.19	41.6			
	0.3						
6e	10	10	0.2 ± 0.13	20	8	6.9 ± 2.5*	37.7 ± 9.7*
	30	10	0.5 ± 0.22[c]	0	8	8.9 ± 1.9*	63.0 ± 11.6
11a	10	12	0.25 ± 0.13	16	13	7.15 ± 2.26	45.7 ± 8.8
	30	10	0.6 ± 0.16[c]	0	10	9.7 ± 2.2	69.7 ± 11.78
11a+daz	10	10	0.5 ± 0.17	40			
	0.3						
11a+daz	10				10	7.2 ± 2.3	128.7 ± 36.2
	1						
11d	3	10	0.7 ± 0.2	0			
	10	10	0.6 ± 0.26	10			
	30	10	0.3 ± 0.15	0	5	6.6 ± 1.3*	46.2 ± 8.2

[a] Treatment with new compounds and diazepam (po) was performed 30 min and flumazenil (ip) 40 min before the test.
[b] Carboxymethylcellulose 1%.
[c] Excitation.
* $P < 0.05$ versus cont rol mice.
** $P < 0.01$ versus control mice.
*** $P < 0.001$ versus control mice.

of 1 mg/kg po diminished mouse movements on the board significantly and at the dose of 3 mg/kg also curiosity toward the holes.

Additional experiments were carried out for compounds **4d** and **6d** to evaluate their effects on mice spontaneous motor activity. Neither compound **4d** nor **6d** was found

Table 5. Myorelaxant activity of compounds **4d**, **6a**, **6d**, **6e, 11a**, and**11d** in the grip-strength meter test

Treatment[a]	mg/kg po	Myorelaxant activity grip-strength meter	
		n	Test 35′ (g)
CMC 1%	0.1 ml	16	64.5 ± 1
Diazepam	0.3	5	66.0 ± 3.1
	1	11	61.9 ± 1.74
	3	13	44.2 ± 4.4***
4d	10	10	64.6 ± 1.9
	30	10	63.2 ± 2.5
6a	10	11	60.5 ± 2.3
	30	10	62.9 ± 2.3
6d	10	10	64.2 ± 1.5
	30	10	63.9 ± 1.4
6e	10	10	63.1 ± 2.3
	30	10	60.4 ± 1.7
11a	10	13	57.6 ± 3.0*
	30	10	56.0 ± 3.4*
11d	10	8	63.3 ± 1.5
	30	8	59.2 ± 1.4

[a] The test was performed 30 and 20 min after administration of compounds and diazepam (po), respectively.
* $P < 0.05$ versus control mice.
*** $P < 0.001$ versus control mice.

Table 6. Effect of compounds **4d** and **6d** on animal spontaneous motility, in the comparison of diazepam, in the mouse hole-board test

Treatment[a]	mg/kg po	Sedative activity Hole board		
		n	Hole	Plane
CMC 1%	0.1 ml	21	49.8 ± 3.1	135.9 ± 7.6
Diazepam	1	10	39.7 ± 4.2	96.9 ± 10.3*
	3	11	23.4 ± 6.3**	59.7 ± 8.6***
4d	10	8	55.5 ± 2.9	155.8 ± 11.6
	30	8	58.8 ± 6.2	159.2 ± 13.1
4d+daz	10	8	51.8 ± 5.0	106.1 ± 9.1
	1			
6d	3	8	58.3 ± 4.3	154.0 ± 9.2
	10	13	58.3 ± 4.4	135.5 ± 10.4
	30	9	56.8 ± 4.7	154.0 ± 10.9
6d+daz	10	10	52.8 ± 6.6	125.4 ± 9.3^
	0.3			

[a] The test was performed 30 and 20 min after administration of compounds and diazepam (po), respectively.
* $P < 0.05$ versus control mice.
** $P < 0.01$ versus control mice.
*** $P < 0.001$ versus control mice.
^ $P < 0.05$ versus diazepam 1 mg/kg treated mice (T_s test).

to be active (Table 6). For this reason, we decided to evaluate the antagonistic activity of these two compounds as they have very high affinity values for $GABA_A$ receptor α_1 subtype (0.81 and 2.3 nM, respectively). In fact, **4d** (10 mg/kg po) diminished, although not significantly, the sedative effect of diazepam (1 mg/kg po) in this test, while the effect of compound **6d** reached statistic significance (Table 6); the opinion that sedation is caused by activation of the $GABA_A$ receptor α_1 subtype was thus confirmed.[6,23]

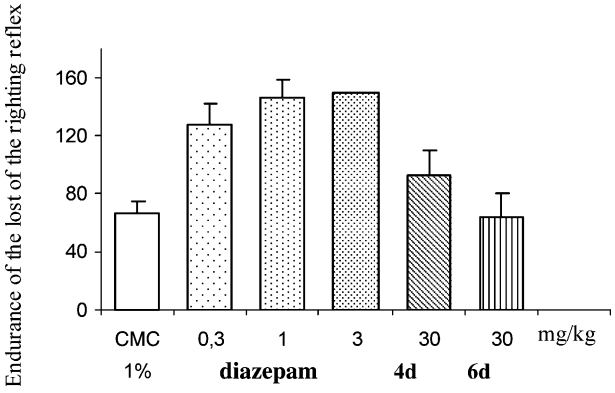


Figure 1. Effect of compounds **4d** and **6d** (30 mg/kg) on ethanol-induced sleeping time, in comparison with that of diazepam (po). Each column represent the mean ± SEM of 10 mice. Substances were administered 30 min before ethanol (4 g/kg ip). $P < 0.001$ (Student's *t* test).

4.4. Effects on mouse anxiety

With the reference molecule, diazepam, at the dose of 3 mg/kg po, we observed a strong increase not only in time spent in the lit compartment of the light/dark apparatus, but the number of mouse transfers were augmented already at the dose of 1 mg/kg po, too. This molecule, at the same doses, as demonstrated before (Tables 5 and 6), caused reduction of motor coordination, myorelaxation, and sedation, effects also reported by many other authors.[4,24] Subsequently, the increased parameters in the light/dark choice test should be considered reliable indicators of anxiolytic-like behavior as suggested by other authors.[25,26] In the opposite manner, all the newly synthesized compounds did not affect the mouse spontaneous motor activity (Table 6), but in the light/dark choice test the compounds **6d** and **e** decreased the time spent in the lit compartment and the number of transfers from one compartment into the other, in a statistically significant way (Table 4), while the compounds **4d** and **11d** (30 mg/kg po) behaved in the same manner but not statistically significantly, and the compounds **6a** and **11a** became inactive. So, not only the decreased time, but also the diminished number of transfers seems to indicate an anxiogenic-like action of our molecules.[26,27] The anxiogenic-like effect of **6d** was completely antagonized by the nonselective benzodiazepine antagonist, flumazenil (Table 4), confirming the inverse agonist action of **6d** on the $GABA_A$/BzR complex. The activation of α_2 subtype receptors has been proposed as being responsible for the anxiolytic-like effect of benzodiazepine ligands.[28,29] Since compound **6d** in the hole-board test and in PTZ-induced convulsion test (see below) resulted to antagonize diazepam effects and then block α_1 subtype receptor (for which it has a very high affinity), anxiogenic-like behavior seems to depend on inverse antagonism for α_2 subtype receptors, for which the affinity is also high (K_i = 10 nM).

4.5. Study against chemically induced convulsions

A further approach to study the newly synthesized molecules was to investigate their potency against

pentylenetetrazole (PTZ)-induced convulsions. At the dose used (90 mg/kg sc), PTZ caused convulsions in 95% of mice. Diazepam already at the dosage of 0.3 mg/kg po significantly protected 70% of mice from clonic generalized convulsions (Table 4), while none, among the newly synthesized molecules, differed statistically from carboxymethylcellulose vehicle in causing protection from convulsions. Compound **11a** (30 mg/kg po) caused some excitation and no protection from convulsions; it has been used as a potential antagonist toward diazepam-induced protection and it actually partially antagonized diazepam-induced protection, but not in a statistically significant way (Table 4). Compound **6d** also behaved in the same manner.

4.6. Effect on ethanol-induced sleeping time

The potency with which diazepam is able to potentiate ethanol-induced sleeping time is surprising (Fig. 1). Already at the lowest dose, diazepam (0.3 mg/kg po), together with ethanol (4 g/kg ip), caused a statistically significant additive action: six mice out of eight lost the righting reflex for the entire 2.5-h observation time, while the control mice slept only for 66.0 ± 9.5 min. Conversely, the effect of **6d** at the highest anxiogenic-like dose was comparable to that of the control group and the other compound, **4d**, demonstrated no significant loss of the righting reflex, as well (Fig. 1). Ethanol-reinforcing actions are often connected to the activation of the α_5 subunit of the $GABA_A$ receptor.[30,31] As far as the α_5 subunit is concerned, **6d** binds to it with an intermediate ($K_i = 57$ nM) and **4d** with no affinity. Also in this case, if the additive effects are assumed to be due to activation of this subunit, **6d** might behave on it like antagonists.

5. Conclusion

The study here reported on new BZR ligands, A and B series, 3-heteroarylester- and 3-heteroarylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 8-substituted, has focused the role of the substituent at the position 8, which appears to be in synergy with the substituent at position 3 but per se to have a crucial role in binding and in in vivo activity. The lipophilic and steric features of the optimum 8-substituent, to achieve high affinity ligands, have been determined improving the pharmacophore/receptor model proposed for this class of ligands.[12]

The replacement of the 8-chlorine of lead compounds **6** and **11** (see Chart 1) is favorable when the substituent is endowed with a π value (lipophilic feature) in the range 0.38–1.19 (substituents as EtO-, Me-, MeS-, and Br-) and the steric hindrance lower than iodine ($E_s = -1.40$).[15] Interestingly, while these lipophilic and steric hindrance limits seem to be the driving force to achieve high binding ligands, the electronic parameter (σ) of 8-substituent seems to influence the subtype receptor selectivity and pharmacological efficacy. In fact replacing the chlorine, electron-withdrawing atom ($\sigma = 0.23$)[15] with an electron-donor group, as EtO- which has a σ value opposite ($\sigma = -0.24$),[15] the high affinity ligand **6d** with preferential α_1/α_2 subtype selectivity and no recognition at α_5 subtype receptor was obtained.

The same 8-substitution in the A and B series compounds yielded the ethoxy derivatives **4d**, **6d**, and **11d**, all showing inverse agonist profile in vivo as anxiogenic effect, opposite the lead compounds (**4**, **6**, and **11** in Chart 1).[9,10] Recently, it has been described [32,33] that new molecules with a semirigid heterocyclic structure bearing various ethereal groups are endowed with inverse-agonist efficacy.

Taken together, the data above reported suggest that an 8-substituent with suitable lipophilic and steric features supports the hydrophobic interaction of the 3-ligand substituent with receptor protein in the lipophilic pocket (L_1/L_2) influencing the affinity binding; on the other hand, dependent on its electronic parameter, the same 8-substituent is also capable per se of interacting with 'critical residues'[34,35] on the same pocket of various subtype receptors, eliciting different pharmacological effects.

These preliminary findings require further investigation either through biological and electrophysiological studies or through the synthesis of new derivatives to confirm this binding hypothesis. In particular in this paper, a new ester **6d** endowed with inverse-agonist efficacy, opposite to reference compound **6** which showed an anxiolytic profile without side effects, is identified. This compound **6d** causes no effects on mice spontaneous motor activity (i.e., no sedative action), no protection from PTZ-induced convulsions, and no ethanol-reinforcing actions. On the other hand, **6d** antagonizes the sedative effect of diazepam and diazepam-induced convulsion, suggesting an antagonist effect on α_1 subtype receptor. The anxiogenic effect could be due to inverse agonism to the α_2 subtype receptor. It could become a new lead, and suitable chemical modification could possibly shift its inverse agonist effect onto other subtype receptors, that is, α_5 subtype receptor, thus yielding new ligands useful in therapy as cognition enhancers in Alzheimer's disease and related dementias.[36,37]

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 Varian Gemini at 200 MHz). Chemical shifts were expressed in δ ppm, using DMSO-d_6 or $CDCl_3$ as solvent. The coupling constant values ($J_{H6\text{-}H7,H7\text{-}H6}$; $J_{H7\text{-}H9,H9\text{-}H7}$;) were in agreement with the

assigned structure. The chemical and physical data of new compounds are shown in Tables 7 and 8; Microanalyses were performed with a Perkin-Elmer 260 analyzer for C, H, N, and the results were within ±0.4% of the theoretical value (Table 9).

6.1.1. General procedure for the synthesis of Ia and b. The suitable 5-bromo-2-nitro-[19] or 5-iodio-2-nitrophenylhydrazine[20] was reacted with ethyl 2-cyano-3-ethoxypropeneate following a reported method.[18]

6.1.1.1. Ethyl 1-(2-nitro-5-bromophenyl)-5-aminopyrazol-4-carboxylate Ia. From 5-bromo-2-nitrophenylhydrazine.[19] Orange crystals. TLC eluent: chloroform/ethanol 10:1 v/v; IR ν^{-1} 3438, 3324, 1683; ^{1}H NMR (DMSO-d_6) δ 8.06 (d, 1H, H-3′); 7.98 (m, 2H, H-4′ and H-6′); 7.68 (s, 1H, H-3); 6.62 (br s, exch. 2H, NH_2); 4.20 (q, 2H, CH_2); 1.32 (t, 3H, CH_3). Anal. C, H, N.

6.1.1.2. Ethyl 1-(2-nitro-5-iodiophenyl)-5-aminopyrazol-4-carboxylate Ib. From 5-iodio-2-nitrophenylhydrazine.[20] Orange crystals. TLC eluent: chloroform/ethanol 10:1 v/v; IR ν^{-1} 3387, 3324, 1682; ^{1}H NMR (DMSO-d_6) δ 8.20 (m, 2H, H-4′ and H-6′); 7.85 (d, 1H, H-3′); 7.68 (s, 1H, H-3); 6.62 (br s, exch. 2H, NH_2); 4.20 (q, 2H, CH_2); 1.32 (t, 3H, CH_3). Anal. C, H, N.

6.1.2. General procedure for the synthesis of IIa–c, and IIIc. The suitable 5-bromo-2-nitro-[19] or 5-iodio-2-nitro-[20] 5-methyl-2-nitrophenylhydrazine[18] was reacted with 2-(thien-3-yl)- and 2-(thien-2-yl)-3-oxapropanenitrile[10] following a reported methods[10] Derivatives **IIa** and **b** were obtained after evaporation of solvent and recrystallized. Compounds **IIc** and **IIIc**, 1-(2-nitro-5-methylphenyl)-4-(thien-3-yl)-5-aminopyrazole and 1-(2-nitro-5-methylphenyl)-4-(thien-2-yl)-5-aminopyrazole, respectively, were not characterized. In this case only the evaporation of reaction solvent (ethanol) was made: the red-brown oils obtained were used for synthesis of **11c** and **12c**, respectively.

6.1.2.1. 1-(2-Nitro-5-bromophenyl)-4-(thien-3-yl)-5-aminopyrazole IIa. Brown crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 3461, 3180; ^{1}H NMR (DMSO-d_6) δ 8.02 (d, 1H, H-3′); 7.96 (d, 1H, H-6′); 7.88 (dd, 1H, H-4′); 7.76 (s, 1H, H-3); 7.56 (dd, 1H, H-4″ 4-thienyl); 7.52 (m, 1H, H-2″ 4-thienyl); 7.38 (dd, 1H, H-5″ 4-thienyl); 5.54 (br s, exch. 2H, NH_2). Anal. C, H, N.

6.1.2.2. 1-(2-Nitro-5-iodiophenyl)-4-(thien-3-yl)-5-aminopyrazole IIb. Red crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 3461, 3340; ^{1}H NMR ($CDCl_3$) δ 8.08 (d, 1H, H-6′); 7.96 (dd, 1H, H-4′); 7.76 (d, 1H, H-3′); 7.68 (s, 1H, H-3); 7.43 (dd, 1H, H-4″ 4-thienyl); 7.22 (m, 2H, H-2″, H-5″ 4-thienyl); 3.80 (br s, exch. 2H, NH_2). Anal. C, H, N.

6.1.3. General procedure for the synthesis of 1a and b. A suspension of**Ia** and **b** (0.5 mmol) in 25 mL of 10% sodium hydroxide was kept at room temperature for 24 h. After treatment with hydrochloric acid 6 M, the desired acids, which were filtered and purified by recrystallization, were obtained.

6.1.3.1. 3-Carboxy- 8-bromopyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 1a. Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1660, 1593; ^{1}H NMR (DMSO-d_6) δ 12.5 (br s, exch. 1H, OH); 8.60 (s, 1H, H-2); 8.54 (d, 1H, H-9); 8.35 (d, 1H, H-6); 7.95 (dd, 1H, H-7). Anal. C, H, N.

6.1.3.2. 3-Carboxy-8-iodiopyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 1b. Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 3481, 1713, 1588; ^{1}H NMR (DMSO-d_6) 12.8 (br s, exch. 1H, OH); 8.65 (d, 1H, H-9); 8.55 (s, 1H, H-2); 8.12 (m, 2H, H-6, H-7). Anal. C, H, N.

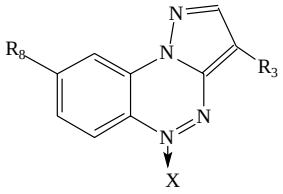
6.1.4. 3-Carboxy-8-methoxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 1e. A suspension of 3-carboxy-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide[17] (0.5 mmol) was reacted with methanol/10% sodium hydroxide 20 mL for 4 h. After treatment with hydrochloric acid 6 M, the desired acids, which were filtered and purified by recrystallization, were obtained. Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 3400, 1560; ^{1}H NMR (DMSO-d_6) 8.57 (s, 1H, H-2); 8.39 (d, 1H, H-6); 7.72 (d, 1H, H-9); 7.35 (dd, 1H, H-7); 4.1 (s, 3H, CH_3O). Anal. C, H, N.

6.1.5. General procedure for the synthesis of ester derivatives 2a–b, 3c, 4a–f, 5c, 6a–f, 7c, 8c, 9c, and 10c. The starting acids **1a**–**c** (3-carboxy-8-methylpyrazolo-[5,1-*c*][1,2,4]benzotriazine 5-oxide),[18] **1d** (3-carboxy-8-ethoxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide),[17] **1e**, and **f**[18] were transformed into ester derivatives by means of two methods, A and B.

Method A: the ethyl esters **2a** and **b**, the benzyl ester **3c**, and the phenoxyester **10c** were obtained from acids **1a**–**c**[18] (0.32 mmol) by means of the corresponding 3-carbonyl chlorides which were suspended in 2-methyl-3-butene stabilized chloroform (5 mL) and then the suitable alcohol was added. In the case of ester **10c**, addition of some pellets of sodium hydroxide was needed.

Method B: all other esters (**4a**–**f**, **5c**, **6a**–**f**, **7c**, **8c**, and **9c**) were obtained treating the corresponding acids (100 mg) (**1a**–**f**) in tetrahydrofurane (THF) with triethyl amine (1:3.5) in ice bath for 30′. To the suspension was added ethylchlorocarbonate (1:2) and maintained under stirring for 1 h, from 0 °C to room temperature, to permit the anhydride to form. The suitable alcohol was added (1:2.5) and the mixture was heated at 60 °C for 8–18 h and monitored by TLC. The final suspension was diluted with water and extracted with chloroform which was in turn washed with sodium hydrogen carbonate solution and, after the normal workup, the residue was treated with isopropyl ether or ethyl ether, filtered, and recrystallized by a suitable solvent.

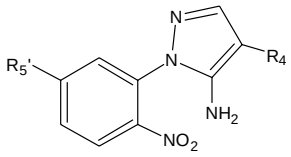
Table 7. Chemical data for pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxides



Compound	R_3	R_8	X	Yield (%)	Mp °C (recryst. solvent)
1a	COOH	Br	O	80	296° (methoxyethanol)
1b	COOH	I	O	75	295–6° (methoxyethanol)
1c[a]	COOH	Me			
1d[b]	COOH	OEt			
1e	COOH	Ome	O	55	288–90° (methoxyethanol)
1f[a]	COOH	SMe	O		
2a	COOEt	Br	O	92	197–8° (ethanol 80%)
2b	COOEt	I	O	90	232–3° (ethanol 80%)
2bR	COOEt	I	—	62	195–6° (ethanol)
2c[a]	COOEt	Me			
2d[b]	COOEt	OEt			
3c	$COOCH_2Ph$	Me	O	85	170–2° (2-propanol)
3cR	$COOCH_2Ph$	Me	—	64	185–6° (2-propanol)
4a	$COOCH_2$-2-MeOPh	Br	O	40	200–1° (ethanol)
4b	$COOCH_2$-2-MeOPh	I	O	40	189–90° (ethanol)
4c	$COOCH_2$-2-MeOPh	Me	O	45	193–5° (ethanol)
4d	$COOCH_2$-2-MeOPh	OEt	O	29	224–5° (ethanol)
4e	$COOCH_2$-2-MeOPh	Ome	O	36	200–2° (2-propanol)
4f	$COOCH_2$-2-MeOPh	SMe	O	45	225–6° (2-propanol)
4aR	$COOCH_2$-2-MeOPh	Br	—	65	179–80° (ethanol)
5c	$COOCH_2$-2-NO_2Ph	Me	O	70	217–8° (methoxyethanol)
6a	$COOCH_2$-2-tienyl	Br	O	95	187–8° (CHX–EtOAc)[c]
6b	$COOCH_2$-2-tienyl	I	O	75	214–5° (ethanol)
6c	$COOCH_2$-2-tienyl	Me	O	35	174–5° (2-propanol)
6d	$COOCH_2$-2-tienyl	OEt	O	70	184–5° (ethanol)
6e	$COOCH_2$-2-tienyl	Ome	O	36	198–9° (2-propanol)
6f	$COOCH_2$-2-tienyl	SMe	O	37	184–5° (methoxyethanol)
7c	$COOCH_2$-3-tienyl	Me	O	52	152–3° (ethanol)
8c	$COOCH_2$-2-furyl	Me	O	38	163–4° (ethanol 80%)
9c	$COOCH_2$-3-furyl	Me	O	35	165–6° (ethanol 80%)
10c	COO-2-MeOPh	Me	O	56	223–4° (ethanol)
11a	3-Tienyl	Br	O	80	224–5° (methoxyethanol)
11b	3-Tienyl	I	O	70	233–4° (methoxyethanol)
11c	3-Tienyl	Me	O	75	224–5° (methoxyethanol)
11cR	3-Tienyl	Me	-	68	195–6° (methoxyethanol)
11d	3-Tienyl	OEt	O	55	211–2° (methoxyethanol)
11e	3-Tienyl	Ome	O	61	226–7° (methoxyethanol)
11f	3-Tienyl	SMe	O	45	227–8° (methoxyethanol)
11g	3-Tienyl	OH	O	30	>300° (methoxyethanol)
12c	2-Tienyl	Me	O	48	207–8° (methoxyethanol)
12e	2-Tienyl	Ome	O	70	218–9° (methoxyethanol)
12f	2-Tienyl	SMe	O	45	195–7° (2-propanol)
13c	3-Furyl	Me	O	74	192–3° (methoxyethanol)
13d	3-Furyl	OEt	O	30	189–9°(diisopropylether)[d]
13e	3-Furyl	Ome	O	60	213–4° (methoxyethanol)
13f	3-Furyl	SMe	O	59	194–5° (methoxyethanol)
14c	2-Furyl	Me	O	82	202–3° (methoxyethanol)
14d	2-Furyl	OEt	O	30	207–8°[d] (diisopropylether)
15e	H	Ome	O	60	218–9° (methoxyethanol)
16c	I	Me	O	62	228–9° (2-propanol)
16d	I	OEt	O	85	244–5° (methoxyethanol)
16e	I	Ome	O	94	234–5° (ethanol)
16f	I	SMe	O	58	229–30° (ethanol)

[a] See Ref. 18.
[b] See Ref. 17.
[c] Cyclohexane/ethyl acetate.
[d] Separated by chromatography column is reported the eluent solvent.

Table 8. Chemical data for new 5-aminopyrazoles



R5′: **a= Br** **b= I** **c= Me**

Compound	R_4	$R_{5'}$	MF (MW)	Yield (%)	Mp °C (recryst. solvent)
Ia	COOEt	Br	$C_{12}H_{11}O_4N_4Br$ (355.06)	78	139–40° (ethanol 80%)
Ib	COOEt	I	$C_{12}H_{11}O_4N_4I$ (402.15)	60	169–70° (ethanol 80%)
Ic[a]	COOEt	Me			
I[b]	COOEt	Cl			
IIa	3-Tienyl	Br	$C_{13}H_9O_2N_4SBr$ (365.11)	50	133–4° (ethanol 80%)
IIb	3-Tienyl	I	$C_{13}H_9O_2N_4SI$ (412.11)	45	162–3° (ethanol)
IIc	3-Tienyl	Me	$C_{14}H_{12}O_2N_4S$ (300.15)		Not separated
II[c]	3-Tienyl	Cl			
IIIc	2-Tienyl	Me	$C_{14}H_{12}O_2N_4S$ (300.15)		Not separated
III[c]	2-Tienyl	Cl			

[a] See Ref. 18.
[b] See Ref. 17.
[c] See Ref. 10.

6.1.5.1. 3-Ethoxycarbonyl-8-bromopyrazolo[5,1-*c*]-[1,2,4]benzotriazine 5-oxide 2a. From acid **1a**, method A. Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1719, 1572; ^{1}H NMR (DMSO-d_6) δ 8.65 (s, 1H, H-2); 8.55 (d, 1H, H-9); 8.35 (d, 1H, H-6); 7.95 (dd, 1H, H-7); 4.25 (q, 2H, CH_2); 1.35 (t, 3H, CH_3). Anal. C, H, N.

6.1.5.2. 3-Ethoxycarbonyl-8-iodiopyrazolo[5,1-*c*]-[1,2,4]benzotriazine 5-oxide 2b. From acid **1b**, method A. Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1713, 1557; ^{1}H NMR ($CDCl_3$) δ 8.85 (d, 1H, H-9); 8.50 (s, 1H, H-2); 8.25 (d, 1H, H-6); 8.00 (dd, 1H, H-7); 4.40 (q, 2H, CH_2); 1.40 (t, 3H, CH_3). Anal. C, H, N.

6.1.5.3. 3-Benzyloxycarbonyl-8-methylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 3c. From acid **1c**,[18] method A. Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1720, 1570; ^{1}H NMR ($CDCl_3$) δ 8.55 (s, 1H, H-2); 8.45 (d, 1H, H-6); 8.25 (d, 1H, H-9); 7.45 (m, 6H, Ph and H-7); 5.45 (s, 2H, CH_2); 2.65 (s, 3H, CH_3). Anal. C, H, N.

6.1.5.4. 3-(2-Methoxybenzyloxycarbonyl)-8-bromopyrazolo[5,1-*c*][1,2, 4]benzotriazine 5-oxide 4a. From acid **1a**, method B. Yellow crystals. TLC eluent: isopropyl ether/cyclohexane 8:3 v/v; IR ν^{-1} 1711, 1566; ^{1}H NMR ($CDCl_3$) δ 8.62 (d, 1H, H-9); 8.48 (s, 1H, H-2); 8.40 (d, 1H, H-6); 7.80 (dd, 2H, H-7); 7.55 (d, 1H, H-3′ Ph); 7.32 (t, 1H, H-5′ Ph); 6.98 (t, 1H, H-4′ Ph); 6.92 (d, 1H, H-6′ Ph); 5.48 (s, 2H, CH_2); 3.87 (s, 3H, OCH_3). Anal. C, H, N.

6.1.5.5. 3-(2-Methoxybenzyloxycarbonyl)-8-iodiopyrazolo[5,1-*c*][1,2, 4]benzotriazine 5-oxide 4b. From acid **1b**, method B. Yellow crystals. TLC eluent: isopropyl ether/cyclohexane 8:3 v/v; IR ν^{-1} 1709, 1564; ^{1}H NMR ($CDCl_3$) δ 8.86 (d, 1H, H-9); 8.52 (s, 1H, H-2); 8.24 (d, 1H, H-6); 8.02 (dd, 2H, H-7); 7.56 (d, 1H, H-3′ Ph); 7.34 (t, 1H, H-5′ Ph); 7.00 (t, 1H, H-4′ Ph); 6.94 (d, 1H, H-6′ Ph); 5.48 (s, 2H, CH_2); 3.87 (s, 3H, OCH_3). Anal. C, H, N.

6.1.5.6. 3-(2-Methoxybenzyloxycarbonyl)-8-methylpyrazolo[5,1-*c*][1,2, 4]benzotriazine 5-oxide 4c. From acid **1c**[18] method B. Yellow crystals. TLC eluent: chloroform/methanol 10:0.1 v/v; IR ν^{-1} 1711, 1564; ^{1}H NMR ($CDCl_3$) δ 8.52 (s, 1H, H-2); 8.44 (d, 1H, H-6); 8.24 (d, 1H, H-9); 7.54 (m, 2H, H-7 and H-3′ Ph); 7.34 (t, 1H, H-5′ Ph); 7.00 (t, 1H, H-4′ Ph); 6.94 (d, 1H, H-6′ Ph); 5.49 (s, 2H, CH_2); 3.89 (s, 3H, OCH_3); 2.67 (s, 3H, CH_3). Anal. C, H, N.

6.1.5.7. 3-(2-Methoxybenzyloxycarbonyl)-8-ethoxypyrazolo[5,1-*c*][1,2, 4]benzotriazine 5-oxide 4d. From acid **1d**[17] method B. Yellow crystals. TLC eluent: isopropyl ether/cyclohexane 8:3 v/v; IR ν^{-1} 1711, 1564; ^{1}H NMR ($CDCl_3$) δ 8.52 (s, 1H, H-2); 8.45 (d, 1H, H-6); 7.65 (d, 1H, H-9); 7.60 (dd, 1H, H-7); 7.15 (m, 2H H-3′ and H-5′ Ph); 7.00 (t, 1H, H-4′ Ph); 6.94 (d, 1H, H-6′ Ph); 5.47 (s, 2H, CH_2); 4.30 (q, 2H, CH_2); 3.89 (s, 3H, OCH_3); 1.60 (t, 3H, CH_3). Anal. C, H, N.

6.1.5.8. 3-(2-Methoxybenzyloxycarbonyl)-8-methoxypyrazolo[5,1-*c*][1,2, 4]benzotriazine 5-oxide 4e. From acid **1e** method B. Yellow crystals. TLC eluent: isopropyl ether/cyclohexane 8:3 v/v; IR ν^{-1} 1711, 1564; ^{1}H NMR ($CDCl_3$) δ 8.55 (s, 1H, H-2); 8.48 (d, 1H, H-6); 7.74 (d, 1H, H-9); 7.60 (d, 1H, H-3′ Ph); 7.32 (t, 1H, H-5′ Ph); 7.22 (dd, 1H, H-7); 7.01 (t, 1H, H-4′ Ph); 6.93 (d, 1H, H-6′ Ph); 5.50 (s, 2H, CH_2); 4.10 (s, 3H, OCH_3); 3.59 (s, 3H, OCH_3). Anal. C, H, N.

6.1.5.9. 3-(2-Methoxybenzyloxycarbonyl)-8-methylthiopyrazolo[5,1-*c*][1,2, 4]benzotriazine 5-oxide 4f. From acid **1f**[18] method B. Yellow crystals. TLC eluent: isopropyl

Table 9. Elemental analyses

Compound	Formula	Anal. Calcd			Anal. Found		
		C	H	N	C	H	N
2a	$C_{12}H_9O_3N_4Br$	42.75	2.69	16.62	42.96	2.81	16.88
2b	$C_{12}H_9O_3N_4I$	37.52	2.36	14.59	37.88	2.72	14.79
2bR	$C_{12}H_9O_2N_4I$	39.15	2.46	15.22	38.90	2.15	14.99
3c	$C_{18}H_{14}O_3N_4$	64.66	4.22	16.76	64.89	4.58	16.78
3cR	$C_{18}H_{14}O_2N_4$	67.91	4.43	17.60	67.63	4.15	17.25
4a	$C_{18}H_{13}O_4N_4Br$	50.37	3.05	13.05	50.64	3.35	13.28
4b	$C_{18}H_{13}O_4N_4I$	45.40	2.75	11.76	45.72	2.95	12.03
4c	$C_{19}H_{16}O_4N_4$	62.63	4.43	15.38	62.31	4.26	15.18
4d	$C_{20}H_{18}O_5N_4$	58.11	4.22	14.65	57.84	3.97	14.35
4e	$C_{19}H_{16}O_5N_4$	60.00	4.24	14.73	59.84	3.93	14.57
4f	$C_{19}H_{16}O_4N_4S$	57.57	4.07	14.13	57.78	4.42	14.36
4aR	$C_{18}H_{13}O_3N_4Br$	52.32	3.17	13.56	52.69	3.37	13.85
5c	$C_{18}H_{13}O_5N_5$	56.99	3.45	18.46	56.78	3.13	18.20
6a	$C_{15}H_9O_3N_4SBr$	44.46	2.24	13.83	44.79	2.57	13.99
6b	$C_{15}H_9O_3N_4SI$	39.84	2.01	12.39	40.05	2.26	12.59
6c	$C_{16}H_{12}O_3N_4S$	56.46	3.55	16.46	56.82	3.76	16.74
6d	$C_{17}H_{14}O_4N_4S$	55.13	3.81	15.13	55.44	4.18	15.36
6e	$C_{16}H_{12}O_4N_4S$	53.93	3.39	15.72	53.64	3.62	15.99
6f	$C_{16}H_{12}O_3N_4S_2$	51.60	3.25	15.04	51.84	3.46	15.38
7c	$C_{16}H_{12}O_3N_4S$	56.46	3.55	16.46	56.78	3.74	16.80
8c	$C_{16}H_{12}O_4N_4$	59.26	3.73	17.28	59.58	4.03	17.54
9c	$C_{16}H_{12}O_4N_4$	59.26	3.73	17.28	59.35	3.85	17.35
10c	$C_{18}H_{14}O_4N_4$	61.71	4.03	15.99	61.99	4.35	16.28
11a	$C_{13}H_7ON_4SBr$	44.97	2.03	16.14	44.62	1.98	16.01
11b	$C_{13}H_7ON_4SI$	39.61	1.79	14.21	39.78	1.85	14.35
11c	$C_{14}H_{10}ON_4S$	59.56	3.57	19.84	59.85	3.90	19.99
11cR	$C_{14}H_{10}N_4S$	63.14	3.78	21.04	62.89	3.57	19.85
11d	$C_{15}H_{12}O_2N_4S$	57.68	3.87	17.94	57.98	4.03	18.02
11e	$C_{14}H_{10}O_2N_4S$	56.37	3.38	18.78	56.72	3.58	19.15
11f	$C_{14}H_{10}ON_4S_2$	53.49	3.21	17.82	53.74	3.51	18.00
11g	$C_{13}H_8O_2N_4S$	54.92	2.84	19.71	54.61	2.65	19.54
12c	$C_{14}H_{10}ON_4S$	56.56	3.57	19.84	56.19	3.20	19.74
12e	$C_{14}H_{10}O_2N_4S$	56.37	3.38	18.78	56.48	3.64	18.98
12f	$C_{14}H_{10}ON_4S_2$	53.49	3.21	17.82	53.74	3.51	18.04
13c	$C_{14}H_{10}O_2N_4$	63.15	3.79	21.04	63.54	3.99	21.38
13d	$C_{15}H_{12}O_3N_4$	60.81	4.08	18.91	61.04	4.35	19.06
13e	$C_{14}H_{10}O_3N_4$	59.57	3.57	19.85	59.84	3.67	20.10
13f	$C_{14}H_{10}O_2N_4S$	56.37	3.38	18.78	56.68	3.57	18.95
14c	$C_{14}H_{10}O_2N_4$	63.15	3.79	21.04	63.29	3.98	21.34
14d	$C_{15}H_{12}O_3N_4$	60.81	4.08	18.91	61.04	4.27	19.21
15e	$C_{10}H_8O_2N_4$	55.55	3.73	25.91	55.84	3.98	26.15
16c	$C_{10}H_7ON_4I$	63.83	2.16	17.18	63.54	1.89	16.99
16d	$C_{11}H_9O_2N_4I$	37.10	2.55	15.73	37.36	2.86	15.99
16e	$C_{10}H_7O_2N_4I$	35.11	2.06	16.38	35.36	2.38	16.65
16f	$C_{10}H_7ON_4IS$	33.53	1.97	15.64	35.85	2.13	15.84

ether/cyclohexane 8:3 v/v; IR ν^{-1} 1710, 1560; ^{1}H NMR ($CDCl_3$) δ 8.54 (s, 1H, H-2); 8.40 (d, 1H, H-6); 8.07 (d, 1H, H-9); 7.59 (d, 1H, H-3′ Ph); 7.48 (dd, 1H, H-7); 7.32 (t, 1H, H-5′ Ph); 7.01 (t, 1H, H-4′ Ph); 6.94 (d, 1H, H-6′ Ph); 5.50 (s, 2H, CH_2); 3.90 (s, 3H, OCH_3); 2.70 (s, 3H, SCH_3). Anal. C, H, N.

6.1.5.10. 3-(2-Nitrobenzyloxycarbonyl)-8-methylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 5c. From acid **1c**[18] method B. Yellow crystals. TLC eluent: chloroform/methanol 10:0.1 v/v; IR ν^{-1} 1715, 1570; ^{1}H NMR ($CDCl_3$) δ 8.59 (s, 1H, H-2); 8.47 (d, 1H, H-6); 8.28 (d, 1H, H-9); 8.19 (d, 1H, H-3′ Ph); 8.07 (d, 1H, H-6′ Ph); 7.75 (t, 1H, H-5′ Ph); 7.52 (m, 2H, H-7 and H-4′ Ph); 5.87 (s, 2H, CH_2); 2.69 (s, 3H, CH_3). Anal. C, H, N.

6.1.5.11. 3-(2-Thienylmethoxycarbonyl)-8-bromopyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 6a. From acid **1a** method B. Yellow crystals. TLC eluent: chloroform/methanol/acetic acid 10:0.5:0.1 v/v/v; IR ν^{-1} 1727, 1567; ^{1}H NMR (DMSO-d_6) δ 8.66 (s, 1H, H-2); 8.54 (d, 1H, H-9); 8.34 (d, 1H, H-6); 7.96 (dd, 1H, H-7); 7.58 (m, 1H, H-3′ 2-thienyl); 7.28 (m, 1H, H-5′ 2-thienyl); 7.04 (m, 1H, H-4′ 2-thienyl); 5.52 (s, 2H, CH_2). Anal. C, H, N.

6.1.5.12. 3-(2-Thienylmethoxycarbonyl)-8-iodiopyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 6b. From acid **1b** method B. Yellow crystals. TLC eluent: isopropyl ether/cyclohexane 8:3 v/v; IR ν^{-1} 1723, 1565; ^{1}H NMR ($CDCl_3$) δ 8.85 (d, 1H, H-9); 8.52 (s, 1H, H-2); 8.25 (d, 1H, H-6); 8.05 (dd, 1H, H-7); 7.35 (m, 1H,

H-3′ 2-thienyl); 7.28 (m, 1H, H-5′ 2-thienyl); 7.02 (m, 1H, H-4′ 2-thienyl); 5.55 (s, 2H, CH_2). Anal. C, H, N.

6.1.5.13. 3-(2-Thienylmethoxycarbonyl)-8-methylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 6c. From acid **1c**[18] method B. Yellow crystals. TLC eluent: chloroform/methanol 10:0.1 v/v; IR ν^{-1} 1700, 1565; ^{1}H NMR ($CDCl_3$) δ 8.50 (s, 1H, H-2); 8.44 (d, 1H, H-6); 8.23 (d, 1H, H-9); 7.52 (dd, 1H, H-7); 7.35 (m, 1H, H-3′ 2-thienyl); 7.24 (m, 1H, H-5′ 2-thienyl); 7.02 (m, 1H, H-4′ 2-thienyl); 5.58 (s, 2H, CH_2); 2.67 (s, 3H, CH_3). Anal. C, H, N.

6.1.5.14. 3-(2-Thienylmethoxycarbonyl)-8-ethoxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 6d. From acid **1d**[17] method B. Yellow crystals. TLC eluent: chloroform/methanol 10:0.1 v/v; IR ν^{-1} 1700, 1565; ^{1}H NMR ($CDCl_3$) δ 8.51 (s, 1H, H-2); 8.46 (d, 1H, H-6); 7.70 (d, 1H, H-9); 7.34 (m, 1H, H-3′ 2-thienyl); 7.22 (m, 2H, H-7 and H-5′ 2-thienyl); 7.02 (m, 1H, H-4′ 2-thienyl); 5.58 (s, 2H, CH_2); 4.30 (q, 2H, CH_2); 1.58 (t, 3H, CH_3). Anal. C, H, N.

6.1.5.15. 3-(2-Thienylmethoxycarbonyl)-8-methoxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 6e. From acid **1e** method B. Yellow crystals. TLC eluent: isopropyl ether/cyclohexane 8:3 v/v; IR ν^{-1} 1718, 1560; ^{1}H NMR ($CDCl_3$) δ 8.53 (s, 1H, H-2); 8.48 (d, 1H, H-6); 7.74 (d, 1H, H-9); 7.36 (dd, 1H, H-7); 7.25 (dd, 1H, H-5′ 2-thienyl); 7.22 (dd, 1H, H-3′ 2-thienyl); 7.02 (m, 1H, H-4′ 2-thienyl); 4.10 (s, 3H, OCH_3). Anal. C, H, N.

6.1.5.16. 3-(2-Thienylmethoxycarbonyl)-8-methylthiopyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 6f. From acid **1f**[18] method B. Yellow crystals. TLC eluent: isopropyl ether/cyclohexane 8:3 v/v; IR ν^{-1} 1709, 1570; ^{1}H NMR ($CDCl_3$) δ 8.53 (s, 1H, H-2); 8.40 (d, 1H, H-6); 8.05 (d, 1H, H-9); 7.49 (dd, 1H, H-7); 7.36 (dd, 1H, H-5′ 2-thienyl); 7.25 (dd, 1H, H-3′ 2-thienyl); 7.03 (m, 1H, H-4′ 2-thienyl); 5.60 (s, 2H, CH_2); 2.70 (s, 3H, SCH_3). Anal. C, H, N.

6.1.5.17. 3-(3-Thienylmethoxycarbonyl)-8-methylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 7c. From acid **1c**[18] method B. Yellow crystals. TLC eluent: chloroform/methanol 10:0.1 v/v; IR ν^{-1} 1740, 1570; ^{1}H NMR ($CDCl_3$) δ 8.52 (s, 1H, H-2); 8.44 (d, 1H, H-6); 8.23 (d, 1H, H-9); 7.50 (m, 2H, H-7 and H-2′ 3-thienyl); 7.34 (m, 1H, H-4′ 3-thienyl); 7.22 (m, 1H, H-5′ 3-thienyl); 5.44 (s, 2H, CH_2); 2.67 (s, 3H, CH_3). Anal. C, H, N.

6.1.5.18. 3-(2-Furylmethoxycarbonyl)-8-methylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 8c. From acid **1c**[18] method B. Yellow crystals. TLC eluent: chloroform/methanol 10:0.1 v/v; IR ν^{-1} 1710, 1570; ^{1}H NMR ($CDCl_3$) δ 8.51 (s, 1H, H-2); 8.44 (d, 1H, H-6); 8.24 (d, 1H, H-9); 7.52 (dd, 1H, H-7); 7.45 (d, 1H, H-3′ 2-furyl); 6.55 (d, 1H, H-5′ 2-furyl); 6.40 (m, 1H, H-4′ 2-furyl); 5.38 (s, 2H, CH_2); 2.67 (s, 3H, CH_3). Anal. C, H, N.

6.1.5.19. 3-(3-Furylmethoxycarbonyl)-8-methylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 9c. From acid **1c**[18] method B. Yellow crystals. TLC eluent: chloroform/methanol 10:0.1 v/v; IR ν^{-1} 1730, 1570; ^{1}H NMR ($CDCl_3$) δ 8.50 (s, 1H, H-2); 8.45 (d, 1H, H-6); 8.24 (d, 1H, H-9); 7.62 (d, 1H, H-2′ 3-furyl); 7.51 (dd, 1H, H-7); 7.42 (m, 1H, H-4′ 3-furyl); 6.57 (d, 1H, H-5′ 3-furyl); 5.30 (s, 2H, CH_2); 2.67 (s, 3H, CH_3). Anal. C, H, N.

6.1.5.20. 3-(2-Methoxyphenoxycarbonyl)-8-methylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 10c. From acid **1c**[18] method A. Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1730, 1570; ^{1}H NMR ($CDCl_3$) δ 8.68 (s, 1H, H-2); 8.45 (d, 1H, H-6); 8.28 (d, 1H, H-9); 7.54 (dd, 1H, H-7); 7.24 (m, 2H, H-4′ and H-5′ Ph); 7.02 (d, 2H, H-3′ and H-6′ Ph); 3.83 (s, 3H, OCH_3); 2.69 (s, 3H, CH_3). Anal. C, H, N.

6.1.6. General procedure for the synthesis of 3-heteroaryl derivatives 11a–g, 12c, and 12e–f. The cyclization to pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide system was obtained from the suitable 5-aminopyrazoles **IIa**, **IIb**, **IIc**, and **IIIc** in alkaline medium, 10% sodium hydroxide solution, according to a previously reported method.[10] For compounds **11d–g** the starting material was the previously described 1-(2-nitro-5-chlorophenyl)-4-(thien-3-yl)-5-amino pyrazole[10] that was suspended in 10% sodium hydroxide solution. In these conditions, the pyrazolobenzotriazine *N*-oxide system is formed and the chlorine atom undergoes the nucleophilic substitution yielding **11d** as precipitate and **11g** in solution, when treated with ethanol/sodium hydroxide; yielding **11e** when treated with methanol/sodium hydroxide and **11f** when treated with sodium thiomethoxide/ethanol.

6.1.6.1. 3-(Thien-3-yl)-8-bromopyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-oxide 11a. From **IIa**. Red crystals. TLC eluent: isopropyl ether/cyclohexane 8:3 v/v; IR ν^{-1} 1590; ^{1}H NMR ($CDCl_3$) δ 8.56 (d, 1H, H-9); 8.40 (d, 1H, H-6); 8.32 (s, 1H, H-2); 7.90 (dd, 1H, H-2′ 3-thienyl); 7.72 (dd, 1H, H-7); 7.64 (dd, 1H, H-5′ 3-thienyl); 7.44 (dd, 1H, H-4′ 3-thienyl). Anal. C, H, N.

6.1.6.2. 3-(Thien-3-yl)-8-iodiopyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-oxide 11b. From **IIb**. Red crystals. TLC eluent: isopropyl ether/cyclohexane 8:3 v/v; IR ν^{-1} 1578; ^{1}H NMR ($CDCl_3$) δ 8.78 (d, 1H, H-9); 8.32 (s, 1H, H-2); 8.23 (d, 1H, H-6); 7.90 (m, 2H, H-7 and H-2′ 3-thienyl); 7.65 (dd, 1H, H-5′ 3-thienyl); 7.44 (dd, 1H, H-4′ 3-thienyl). Anal. C, H, N.

6.1.6.3. 3-(Thien-3-yl)-8-methylpyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-oxide 11c. From **IIc**. Red crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1540; ^{1}H NMR ($CDCl_3$) δ 8.45 (d, 1H, H-6); 8.30 (s, 1H, H-2); 8.17 (d, 1H, H-9); 7.90 (dd, 1H, H-2′ 3-thienyl); 7.66 (dd, 1H, H-5′ 3-tienyl); 7.46 (m, 2H, H-7 and H-4′ 3-thienyl). Anal. C, H, N.

6.1.6.4. 3-(Thien-3-yl)-8-ethoxypyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-oxide 11d. From 1-(2-nitro-5-chlorophenyl)-4-(thien-3-yl)-5-aminopyrazole[10] in 10% sodium hydroxide/ethanol at 60 °C. Red crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1540; ^{1}H NMR ($CDCl_3$) δ 8.46 (d, 1H, H-6); 8.30 (s, 1H, H-2); 7.90 (dd, 1H, H-2′ 3-thienyl); 7.68 (m, 2H,

H-9 and H-5′ 3-tienyl); 7.44 (dd, 1H, H-4′ 3-thienyl); 7.14 (dd, 1H, H-7). Anal. C, H, N.

6.1.6.5. 3-(Thien-3-yl)-8-methoxypyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-oxide 11e. From 1-(2-nitro-5-chlorophenyl)-4-(thien-3-yl)-5-aminopyrazole[10] in 10% sodium hydroxide solution/methanol at 60 °C. Red crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR v^{-1} 1540; ^{1}H NMR ($CDCl_3$) δ 8.50 (d, 1H, H-6); 8.34 (s, 1H, H-2); 7.92 (dd, 1H, H-2′ 3-thienyl); 7.71 (dd, 1H, H-9); 7.68 (m, 1H, H-4′ 3-tienyl); 7.45 (m, 1H, H-5′ 3-thienyl); 7.16 (dd, 1H, H-7); 4.10 (s, 3H, OCH_3). Anal. C, H, N.

6.1.6.6. 3-(Thien-3-yl)-8-methylthiopyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 11f. From 1-(2-nitro-5-chlorophenyl)-4-(thien-3-yl)-5-aminopyrazole[10] in sodium thiomethoxide/ethanol at RT °. Red crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR v^{-1} 1540; ^{1}H NMR ($CDCl_3$) δ 8.41 (d, 1H, H-6); 8.32 (s, 1H, H-2); 8.03 (dd, 1H, H-9); 7.92 (dd, 1H, H-2′ 3-thienyl); 7.68 (m, 1H, H-4′ 3-tienyl); 7.45 (m, 1H, H-5′ 3-thienyl); 7.42 (dd, 1H, H-7); 2.70 (s, 3H, SCH_3). Anal. C, H, N.

6.1.6.7. 3-(Thien-3-yl)-8-hydroxypyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-oxide 11g. From 1-(2-nitro-5-chlorophenyl)-4-(thien-3-yl)-5-aminopyrazole[10] in 10% sodium hydroxide solution/ethanol at 60 °C as by-product. This compound was obtained after concentration of reaction solvent and acidification with hydrochloric acid. The residue was treated with ethanol/water and filtered. Red crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR v^{-1} 3400–2900, 1540; ^{1}H NMR (DMSO-d_6) δ 11.7 (br s, exch. 1H, OH); 8.65 (s, 1H, H-2); 8.30 (d, 1H, H-6); 7.90 (dd, 1H, H-2′ 3-thienyl); 7.70 (m, 2H, H-4′ and H-5′ 3-tienyl); 7.50 (d, 1H, H-9); 7.14 (dd, 1H, H-7). Anal. C, H, N.

6.1.6.8. 3-(Thien-2-yl)-8-methylpyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-oxide 12c. From **IIIc**. Red crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR v^{-1} 1550; ^{1}H NMR ($CDCl_3$) δ 8.44 (d, 1H, H-6); 8.28 (s, 1H, H-2); 8.18 (d, 1H, H-9); 7.64 (dd, 1H, H-5′ 2-tienyl); 7.44 (dd, 1H, H-7); 7.34 (dd, 1H, H-3′ 2-tienyl); 7.14 (dd, 1H, H-4′ 2-thienyl); 2.63 (s, 3H, CH_3). Anal. C, H, N.

6.1.6.9. 3-(Thien-2-yl)-8-methoxypyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-oxide 12e. From 1-(2-nitro-5-chlorophenyl)-4-(thien-2-yl)-5-aminopyrazole[10] in 10% sodium hydroxide solution/methanol. Red crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR v^{-1} 1540; ^{1}H NMR ($CDCl_3$) δ 8.50 (d, 1H, H-6); 8.30 (s, 1H, H-2); 7.71 (dd, 1H, H-9); 7.66 (dd, 1H, H-3′ 2-thienyl); 7.35 (dd, 1H, H-5′ 2-tienyl); 7.17 (dd, 1H, H-7); 7.15 (m, 1H, H-4′ 2-thienyl); 4.10 (s, 3H, OCH_3). Anal. C, H, N.

This compound is also obtained, with excellent yield, by the Suzuki-coupling reaction using as starting material 8-methoxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide, **15e** (see below), and 2-thiophen boronic acid following the same procedure described for compounds **13c–f** and **14c–d**.

6.1.6.10. 3-(Thien-2-yl)-8-methylthiopyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 12f. From 1-(2-nitro-5-chlorophenyl)-4-(thien-2-yl)-5-aminopyrazole[10] in sodium thiomethoxide/ethanol under N_2. Red crystals. TLC eluent: isopropyl ether/cyclohexane 8:3 v/v; IR v^{-1} 1540; ^{1}H NMR ($CDCl_3$) δ 8.40 (d, 1H, H-6); 8.29 (s, 1H, H-2); 8.02 (dd, 1H, H-9); 7.66 (dd, 1H, H-5′ 2-thienyl); 7.42 (dd, 1H, H-7); 7.35 (m, 1H, H-3′ 2-tienyl); 7.15 (m, 1H, H-4′ 2-thienyl); 2.70 (s, 3H, SCH_3). Anal. C, H, N.

This compound is also obtained, with excellent yield, by the Suzuki-coupling reaction using as starting material 8-methylthioypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide[18] and 2-thiophenboronic acid following the same procedure described for compounds **13c–f** and **14c–d**.

6.1.7. General procedure for the synthesis of 3-furyl derivatives 13c–f and 14c–d. Tetrakis-(triphenylphpsphine)palladium(0) (30 mg, 0.026 mmol) and the suitable 3-iodio derivatives (0.30 mmol), **16c–f** (see below), were combined in anhydrous toluene (4.0 mL). The 3- and 2-furanboronic acids (70 mg, 0.62 mmol) in absolute ethanol (2.5 mL) and aqueous sodium carbonate (2 M, 4 mL) were added and the reaction mixture was heated to reflux for 12 h and monitored by TLC. The red product was then extracted by methylene chloride and the organic layer was washed with water and dried over sodium sulfate. The evaporation of solvent gave the crude product that was recrystallized by suitable solvent.

6.1.7.1. 3-(Fur-3-yl)-8-methylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 13c. From **16c** and 3-furanboronic acid. Red crystals. TLC eluent: acetone/cyclohexane 1:4 v/v; IR v^{-1} 1530; ^{1}H NMR ($CDCl_3$) δ 8.44 (d, 1H, H-6); 8.20 (s, 1H, H-2); 8.17 (d, 1H, H-9); 8.07 (d, 1H, H-2′ 3-furyl); 7.53 (dd, 1H, H-4′ 3-furyl); 7.43 (dd, 1H, H7); 6.91 (dd, 1H, H-5′ 3-furyl); 2.64 (s, 3H, CH_3). Anal. C, H, N.

6.1.7.2. 3-(Fur-3-yl)-8-ethoxypyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-oxide 13d. From **16d** and 3-furanboronic acid. Red crystals. TLC eluent: isopropylether; IR v^{-1} 1530; ^{1}H NMR ($CDCl_3$) δ 8.44 (d, 1H, H-6); 8.18 (s, 1H, H-2); 8.08 (d, 1H, H-2′ 3-furyl); 7.64 (d, 1H, H-9); 7.52 (dd, 1H, H-5′ 3-furyl); 7.10 (dd, 1H, H7); 6.90 (dd, 1H, H-4′ 3-furyl); 4.20 (q, 2H, CH_2); 1.40 (t, 3H, CH_3). Anal. C, H, N.

6.1.7.3. 3-(Fur-3-yl)-8-methoxypyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-oxide 13e. From **16e** and 3-furanboronic acid. Red crystals. TLC eluent: isopropyl ether/cyclohexane 8:3 v/v; IR v^{-1} 1530; ^{1}H NMR ($CDCl_3$) δ 8.48 (d, 1H, H-6); 8.22 (s, 1H, H-2); 8.09 (d, 1H, H-2′ 3-furyl); 7.70 (d, 1H, H-9); 7.54 (m, 1H, H-5′ 3-furyl); 7.15 (dd, 1H, H7); 6.93 (m, 1H, H-4′ 3-furyl); 4.10 (s, 3H, OCH_3). Anal. C, H, N.

6.1.7.4. 3-(Fur-3-yl)-8-methylthiopyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-oxide 13f. From **16f** and 3-furanboronic acid. Red crystals. TLC eluent: isopropyl ether/cyclo-

hexane 8:3 v/v; IR ν^{-1} 1530; ^{1}H NMR ($CDCl_3$) δ 8.40 (d, 1H, H-6); 8.21 (s, 1H, H-2); 8.08 (d, 1H, H-2′ 3-furyl); 8.02 (d, 1H, H-9); 7.54 (m, 1H, H-5′ 3-furyl); 7.41 (dd, 1H, H7); 6.93 (m, 1H, H-4′ 3-furyl); 2.70 (s, 3H, SCH_3). Anal. C, H, N.

6.1.7.5. 3-(Fur-2-yl)-8-methylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 14c. From **16c** and 2-furanboronic acid. Red crystals. TLC eluent: ethyl acetate/cyclohexane 1:3 v/v; IR ν^{-1} 1530; ^{1}H NMR ($CDCl_3$) δ 8.43 (d, 1H, H-6); 8.36 (s, 1H, H-2); 8.17 (d, 1H, H-9); 7.51 (d, 1H, H-3′ 2-furyl); 7.43 (dd, 1H, H-7); 6.97 (d, 1H, H-5′ 2-furyl); 6.54 (dd, 1H, H-4′ 2-furyl); 2.64 (s, 3H, CH_3). Anal. C, H, N.

6.1.7.6. 3-(Fur-2-yl)-8-ethoxypyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-oxide 14d. From **16d** and 2-furanboronic acid. Red crystals. TLC eluent: isopropylether; IR ν^{-1} 1530; ^{1}H NMR ($CDCl_3$) δ 8.44 (d, 1H, H-6); 8.38 (s, 1H, H-2); 7.64 (d, 1H, H-9); 7.48 (d, 1H, H-3′ 2-furyl); 7.12 (dd, 1H, H-7); 6.96 (m, 1H, H-5′ 2-furyl); 6.54 (m, 1H, H-4′ 2-furyl); 4.20 (q, 2H CH_2); 1.40 (t, 3H, CH_3). Anal. C, H, N.

6.1.7.7. 8-methoxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 15e. Compound **1e**, 3-carboxy-8-methoxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide, was treated with concd hydrochloric acid until the starting material disappeared by evaluation with TLC. The final solution was treated with ice and the precipitate was filtered and crystallized by a suitable solvent.

Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1560; ^{1}H NMR (DMSO-d_6) δ 8.50 (d, 1H, H-6); 8.10 (s, 1H, H-2); 7.73 (d, 1H, H-9); 7.17 (dd, 1H, H7); 6.47 (d, 1H, H-3); 4.08 (s, 3H, OCH_3). Anal. C, H, N.

6.1.8. General procedure for the synthesis of compounds 16c–f. Compounds 8-methylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide,[18] 8-ethoxy pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide,[17] 8-methoxypyrazolo[5,1-*c*][1,2,4] benzotriazine 5-oxide, **15e**, and 8-methylthiopyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide[18] were dissolved in chloroform and treated with an excess of iodine monochlorine (ICl) in chloroform. The reaction was monitored by TLC; the final solution was evaporated and the residue was treated with ethanol 80%, filtered, and recrystallized by suitable solvent.

6.1.8.1. 3-Iodio-8-methylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 16c. From 8-methylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide[18] and ICl. Dark yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1570; ^{1}H NMR ($CDCl_3$) δ 8.43 (d, 1H, H-6); 8.17 (d, 1H, H-9); 8.09 (s, 1H, H-2); 7.44 (dd, 1H, H-7); 2.64 (s, 3H, CH_3). Anal. C, H, N.

6.1.8.2. 3-Iodio-8-ethoxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 16d. From 8-ethoxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide[17] and ICl. Dark yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1560; ^{1}H NMR ($CDCl_3$) δ 8.45 (d, 1H, H-6); 8.10 (s, 1H, H-2); 7.65 (d, 1H, H-9); 7.15 (dd, 1H, H-7); 4.30 (q, 2H, CH_2); 1.50 (t, 3H, CH_3). Anal. C, H, N.

6.1.8.3. 3-Iodio-8-methoxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 16e. From **15e** and ICl. Dark yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1570; ^{1}H NMR ($CDCl_3$) δ 8.48 (d, 1H, H-6); 8.11 (s, 1H, H-2); 7.69 (d, 1H, H-9); 7.17 (dd, 1H, H-7); 4.10 (s, 3H, OCH_3). Anal. C, H, N.

6.1.8.4. 3-Iodio-8-methylthiopyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 16f. From 8-methylthiopyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide[18] and ICl. Dark yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1560; ^{1}H NMR ($CDCl_3$) δ 8.40 (d, 1H, H-6); 8.11 (s, 1H, H-2); 8.01 (d, 1H, H-9); 7.43 (dd, 1H, H-7); 2.65 (s, 3H, SCH_3). Anal. C, H, N.

6.1.9. General procedure for synthesis of compounds, 3cR, 4aR and 11cR. These compounds were obtained by following a previously described procedure of reduction with triethyl phosphite (1.5 mL) in toluene[18] (5.0 mL) at refluxing temperature for 6–8 h, starting from compounds **3c**, **4a**, and **11c**.

6.1.9.1. 3-Benzyloxycarbonyl-8-methylpyrazolo[5,1-*c*][1,2,4]benzotriazine 3cR. From **3c**. Yellow crystals. TLC eluent: toluene/ethyl acetate 8:3 v/v; IR ν^{-1} 1711; ^{1}H NMR ($CDCl_3$) δ 8.66 (s, 1H, H-2); 8.63 (d, 1H, H-6); 8.32 (d, 1H, H-9); 7.66 (dd, 1H, H-7); 7.58 (dd, 2H, H-2′ and H-6′ Ph); 7.40 (m, 3H, H-3′, H-4′ and H-5′ Ph); 5.51 (s, 2H, CH_2); 2.72 (s, 3H, CH_3). Anal. C, H, N.

6.1.9.2. 3-(2-Methoxybenzyloxycarbonyl)-8-bromopyrazolo[5,1-*c*][1,2,4]benzotriazine 4aR. From **4a**. Yellow crystals. TLC eluent: isopropyl ether/cyclohexane 8:3 v/v; IR ν^{-1} 1720; ^{1}H NMR ($CDCl_3$) δ 8.72 (d, 1H, H-9); 8.68 (s, 1H, H-2); 8.62 (d, 1H, H-6); 7.960 (dd, 2H, H-7); 7.62 (d, 1H, H-3′ Ph); 7.34 (t, 1H, H-5′ Ph); 7.02 (t, 1H, H-4′ Ph); 6.92 (d, 1H, H-6′ Ph); 5.60 (s, 2H, CH_2); 3.87 (s, 3H, OCH_3). Anal. C, H, N.

6.1.9.3. 3-(Thien-3-yl)-8-methylpyrazolo[5,1-*c*][1,2,4]-benzotriazine 11cR. From **11c**. Dark orange crystals. TLC eluent: acetone/cyclohexane 1:4 v/v; ^{1}H NMR ($CDCl_3$) δ 8.52 (d, 1H, H-6); 8.42 (s, 1H, H-2); 8.26 (d, 1H, H-9); 8.22 (dd, 1H, H-2′ 3-thienyl); 7.88 (dd, 1H, H-5′ 3-thienyl); 7.58 (dd, 1H, H-7); 7.50 (dd, 1H, H-4′ 3-thienyl). 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.[38,39] 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.[40] [^{3}H]Ro15-1788 binding studies were performed as previously reported.[10] Clonal mammalian cell lines, expressing relatively high levels of $GABA_A$ receptor subtypes ($\alpha_1\beta_2\gamma_2$, $\alpha_1\beta_2\gamma_2$, $\alpha_3\beta_2\gamma_2$, and $\alpha_5\beta_3\gamma_2$), were maintained as previously described[41] 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.[41] [^{3}H]Ro15-1788 binding assays to transfected cell membranes were carried out as previously described.[41] In brief, the cell line membranes were incubated in a volume of 500 μl, which contained [^{3}H]Ro15-1788 at a concentration of 1–2 nM and test compound in the 10^{-9}–10^{-5} M range. Nonspecific binding was defined by 10^{-5} M diazepam. Assays were incubated to equilibrium for 1 h at 4 °C. The compounds were dissolved in DMSO, the level of which did not exceed 1% and which was maintained constant in all tubes. At least six different concentrations of each compound were used. The data of $n = 5$ experiments carried out in 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.[42]

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 12/12 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.

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-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 (Sigma), and Zolpidem (Tocris) were the drugs used. All drugs, except for 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 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 and Murray. *P* values of less than 0.05 were considered significant.[43]

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