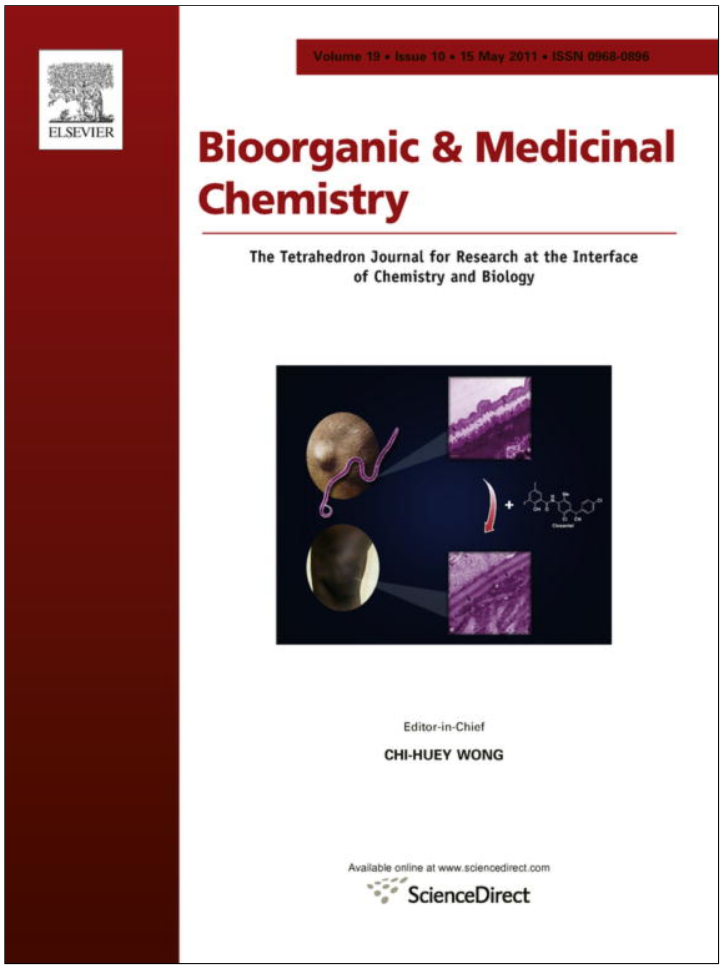

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New 3-, 8-disubstituted pyrazolo[5,1-*c*][1,2,4]benzotriazines useful for studying the interaction with the HBp-3 area (hydrogen bond point area) in the benzodiazepine site on the γ-aminobutyric acid type A ($GABA_A$) receptor

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ABSTRACT

The pharmacophoric model using ADLR procedure, based on pyrazolo[5,1-*c*][1,2,4]benzotriazine system, studied in our laboratory, allowed us to identify the essential interaction points (HBp-1, HBp-2, and Lp-1) and the important areas for affinity modulation (HBp-3 and Lp-2) for binding recognition at benzodiazepine (Bzs) site of $GABA_A$ receptors ($GABA_A$-Rs). In this work ADLR method is used to rationalize the affinity data of 23 new compounds and to improve the knowledge on HBp-3 area, hydrogen bond area. Among these new compounds emerge the pyrrolo derivatives (**18**, **25**, **28**, **34**, and **37**) for their good affinity value ($14.9 > K_i(nM) > 63.0$). In the orientations proposed by ADLR, the NH moiety of the pyrrole ring, independently of the position in the pyrazolobenzotriazine core, fits in HBp-3 area and points out the acceptor feature of this hydrogen bond area, already known as donor area. Unexpectedly, the oxygen atom of the furane ring does not form efficient hydrogen bond with the same area, probably for an imperfect distance. The size of substituent at position 8 is important but not necessary for the receptor recognition, in fact the interdependence between the features of the 3- and 8-substituent's is again verified, (i.e., compound **20** vs **32**).

1. Introduction

The γ-aminobutyric (GABA)-ergic system is the most important inhibitory mechanism in the adult mammalian brain.[1] The GABA neurotransmitter binds to $GABA_A$ receptors ($GABA_A$-Rs), members of the Cys-loop family of ligand gated ion channels (LGICs) and to metabotropic G-protein-coupled $GABA_B$ receptors.[2] $GABA_A$-Rs are pentameric trans-membrane proteins formed by a combination of 19 different subunits (α1–6, β1–3, γ1–3, δ, ε, θ, π, and ρ1–3) with the most common composition being of two single alpha subunits, two single beta subunits, and one gamma subunit $\alpha_2\beta_2\gamma$.[3] The combination of the isoforms α1–3, 5, β2/3, and γ2 form a subset of $GABA_A$-Rs (α1β2γ2, α2β3γ2, α3β3γ2, and α5β3γ2) modulated by benzodiazepines (Bzs) and by non-benzodiazepine ligands belonging to several chemical classes. The Bz binding site is located at the interface of an α and a γ subunit and its pharmacology is mainly determined by the isoforms of these two subunits. Subunit β, although needed to generate a functional GABA gated ion channel, does not affect the sensitivity to the Bz site. Using genetically engineered mice and comparing drug-induced behavioral responses in mutated and wild-type mice, with respect to diazepam,[4–6] $GABA_A$-R subtype functions have been identified. The α1β2γ2 receptors mediate the sedative, anterograde amnesic and anticonvulsant actions of diazepam.[7–9] The anxiolytic-like effect and the muscle relaxant activities are mediated by the α2βγ2 and α3βγ2 receptors even if the α3-containing $GABA_A$-Rs seem to be involved in the inhibitory input for the dopaminergic system.[10–12] The α5βγ2 receptors, that are preferentially localized in the hippocampus, seem to influence learning and memory.[1]

Currently, the unified pharmacophore/receptor model for ligands at the Bzs site on the $GABA_A$-Rs developed by Cook et al.,[13] has been updated[14] and correlated with a comparative model[15] to individuate residues relative to descriptors of the pharmacophore/receptor model.

To increase our understanding of the requirements of the Bzs site on $GABA_A$-R, we have developed a pharmacophoric model based on pyrazolo[5,1-*c*][1,2,4]benzotriazine ligands,[16] using ADLR procedure. This procedure allowed us to identify the essential

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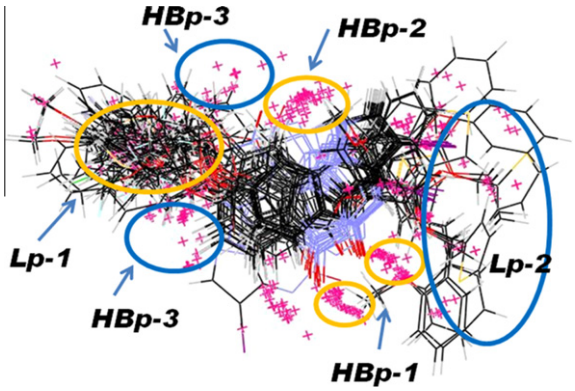


Figure 1. Overlapping of ligand conformations following the ADLR procedure: the figure shows the essential interaction points for binding recognition (yellow circles) and the important areas for affinity modulation (blue circles).

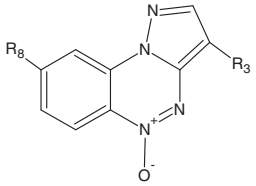
interaction points for binding recognition (HBp-1, HBp-2, and Lp-1) and the important areas for affinity modulation (HBp-3 and Lp-2) of the pyrazolobenzotriazine system, Figure 1.

At the same time, we elaborated structure–activity/affinity relationships for the pyrazolobenzotriazine system 3-, 8-disubstituted, identifying the heteroaryl groups, the thienylmethoxycarbonyl group[17] the iodine atom,[18] and the furoyl group[19] as better 3-substituents,[20] and, as 8-substituents, the groups endowed with different electronic/steric features (alkyl, thioalkyl, alkyloxy, halogen, aryloxy, and difluoromethoxy).[16,21–23] Among these compounds, it was very intriguing to modify the 8-aryloxy derivatives for which it was hypothesized that the 8-oxygen atom engaged a hydrogen bond interaction with the HBp-3 area.[16] In this paper we have decided to investigate the 8-aryl/heteroaryl derivatives,[16] maintaining at position 3 the better previously identified substituents. These modifications (introduction at position 8 of aryl/heteroaryl rings) permit us to evaluate whether the HBp-3 area is involved or if other receptor interactions can occur (π–π stacking, hydrophobic interaction…) and to measure the size and shape of lipophilic regions Lp-1 and Lp-2.

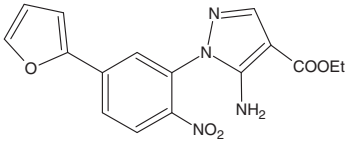
2. Chemistry

All compounds described here are listed in Table 1 (chemical data).

Table 1
Chemical data for new synthesized compounds



3-28, 30, 32-37, 40-42



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Compd[a]	R_3	R_8	Yield (%)	Mp °C (recryst. solvent)
3	H	2-OMePh	60	190–192 (ethanol)
4	H	4-OMePh	54	196–197 (ethanol)
5	H	4-Py	52	220–222 (ethanol)
6	H	2-Thienyl	65	200–202 (ethanol)
7	H	3-Thienyl	73	206–207 (ethanol)
8	H	2-Furyl	67	201–202 (ethanol)
9	H	1-Boc-2-pyrrolyl	70	196–198 (ethanol)
10	I	2-OMePh	40	212–214 (ethanol)
11	I	2-OHPh	35	250–251 (ethanol)
12	I	4-OMePh	45	234–236 (ethanol)
13	I	4-Py	27	267–269 (ethanol)
14	I	2-Thienyl	54	247–248 (ethanol)
15	I	3-Thienyl	45	258–259 (methoxyethanol)
16	I	2-Furyl	50	215–216 (ethanol)
17	I	1-Boc-2-pyrrolyl	45	207–208 (ethanol)
18	I	(1*H*)-2-Pyrrolyl	30	>300 (ethanol)
19	3-Thienyl	Ph	25	237–238 (ethanol)
20	3-Thienyl	4-OMePh	56	250–252 dec (chromatography)
21	3-Thienyl	2-Thienyl	30	229–230 (methoxyethanol)
22	3-Thienyl	3-Thienyl	25	233–234 (methoxyethanol)
23	3-Thienyl	2-Furyl	84	240–241 (ethanol)
24	3-Thienyl	1-Boc-2-pyrrolyl	45	196–198 dec (ethanol)
25	3-Thienyl	(1*H*)-2-Pyrrolyl	77	>300 (ethanol)
26	2-Furyl	3-Thienyl	37	249–250 (chromatography)
27	1-Boc-2-pyrrolyl	3-Thienyl	57	194–195 dec (ethanol)
28	(1*H*)-2-Pyrrolyl	3-Thienyl	72	219–220 (ethanol)
30	CO-2-furyl	I	30	233–234 (chromatography)
32	$COOCH_2$-2-thienyl	4-OMePh	21	200–202 (chromatography)
33	$COOCH_2$-2-thienyl	1-Boc-2-pyrrolyl	80	138–140 dec (ethanol)
34	$COOCH_3$	(1*H*)-2-Pyrrolyl	69	>300 (ethanol)
35	CO-2-furyl	4-OMePh	21	258–259 (chromatography)
36	CO-2-furyl	1-Boc-2-pyrrolyl	73	184–186 dec (ethanol)
37	CO-2-furyl	(1*H*)-2-Pyrrolyl	78	>300 (ethanol)
39	COOEt	2-furyl	90	140–142 (ethanol)
40	COOH	2-furyl	58	279–280 (ethanol)
41	$COOCH_2$-2-thienyl	2-Furyl	23	215–216 (ethanol)
42	CO-2-furyl	2-Furyl	35	230–233 dec (ethanol)

[a] Compound **1**–**2**, **29**, **31**, and **38** known, see chemical section for references. Compound **39**, ethyl 1-(2-nitro-5-fur-2-ylphenyl)-5-aminopyrazole-4-carboxylate.

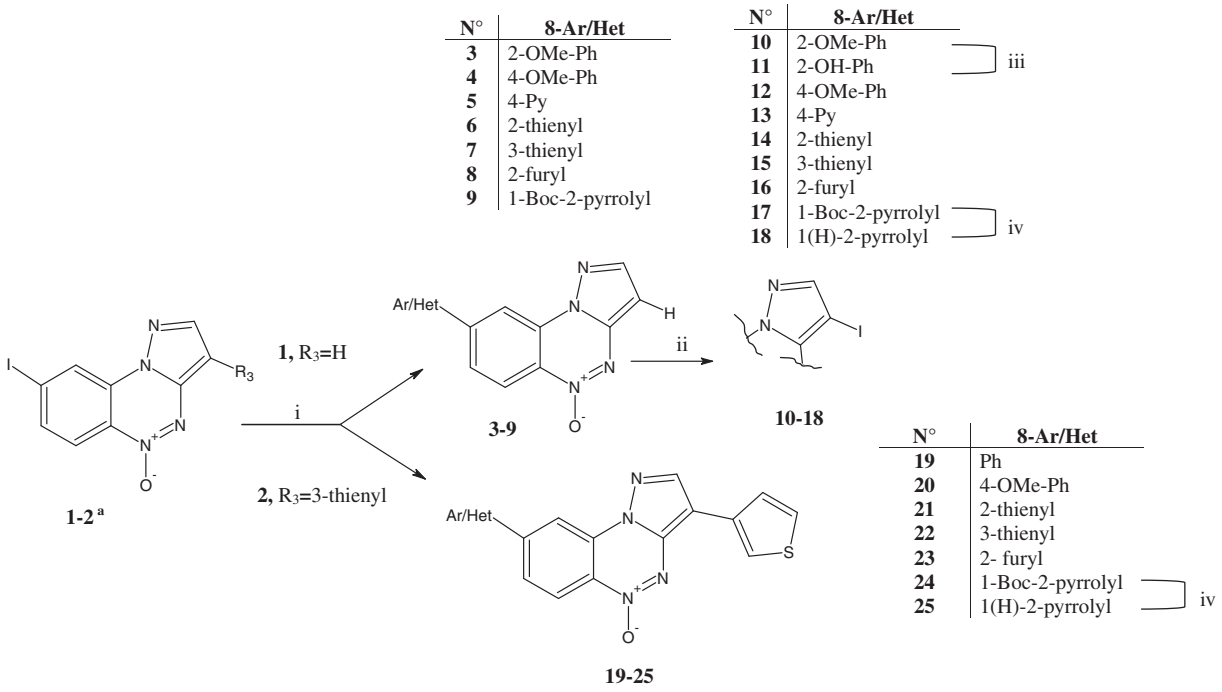
The Suzuki coupling reaction on suitable iodinate starting material was used for the synthesis of all 3-aryl-, 8-aryl-, and 3,8-diarylderivatives. As reported in Scheme 1, the 8-iodopyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide,[18] **1**, was used to obtain the 8-aryl derivatives **3–9**, using the suitable arylboronic acid.

The reaction with 2-furylboronic acid produced several problems, and for the synthesis of compound **8**, the most versatile and stable potassium 2-furantrifluoroborate in presence of the palladium catalyst, palladiumchloride (diphenylphosphine-ferrocene) $PdCl_2(dppf)\cdot CH_2Cl_2$ was used as the coupling partner.[24,25] (All the 3- and/or 8-(2-furyl) derivatives were synthesized following this procedure). Compounds **3–9** were in turn iodinated at position 3 with ceric ammonium nitrate (CAN) and iodine[26] to give derivatives **10**, **12–17** which are useful for structure–affinity relationships. The demethylation of derivative **10** with CH_2Cl_2/BBr_3 gave the corresponding 2-hydroxyphenyl derivative **11**, while the deprotection of **17** was realized with $NaOCH_3/CH_3OH$ in THF,[27] to give **18**. The starting material 3-(3-thienyl)-8-iodopyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide,[22] **2**, was instead used to obtain derivatives **19–24**. The deprotection of **24** was realized in the same previously described conditions,[27] and compound **25** was obtained.

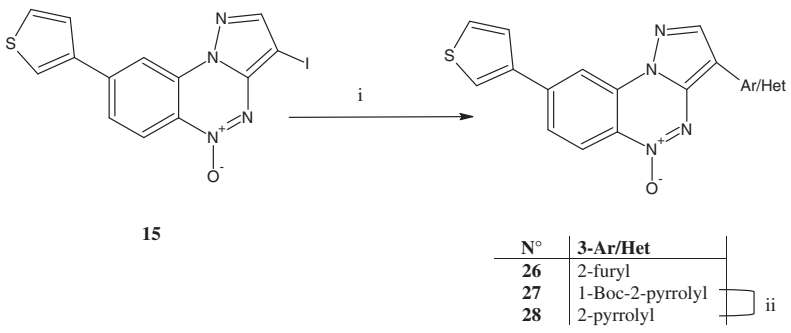
To obtain the 8-(3-thienyl)-3-heteroaryl substituted derivatives, **26–27** (Scheme 2), compound **15** was used as starting material for the Suzuki coupling. The deprotection of **27** afforded the pyrrole derivative **28**.

Scheme 3 depicts the synthetic pathway for obtaining derivatives **32–37**. The starting material is 3-carboxy-8-iodopyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide, **29**,[22] that was reacted by a Friedel–Craft reaction with thionyl chloride, $SnCl_4$, and furane[19] to obtain the new 3-(fur-2-ylcarbonyl)-8-iodopyrazolo[5,1-*c*]-[1,2,4]benzotriazine 5-oxide, **30**.

Following a described procedure, compound **29** was also used to obtain 3-(2-thienylmethoxycarbonyl)-8-iodopyrazolo[5,1-*c*]-[1,2,4]benzotriazine 5-oxide, **31**.[22] Both compounds **30** and **31** were subjected to Suzuki coupling and derivatives **32**, **33**, **35**, and **36** were synthesized. The treatment of derivative **33** with $NaOCH_3/CH_3OH$ caused, at the same time, pyrrole deprotection and trans-esterification, producing derivative **34**. In the same

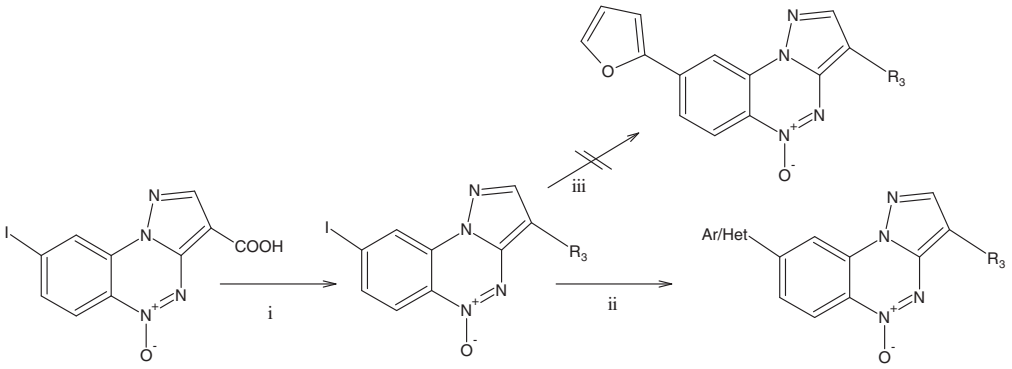


Scheme 1. For compound **1** see,[18] for compound **2** see[22] Reagents: (i) Suzuki conditions, Arylboronic acids, ethanol abs, $(PPh_3)_4Pd$ tetrakis(triphenylphosphine)palladium (0), sodium carbonate 2 M; for synthesis of derivatives **8** and **23**, potassium 2-furyltrifluoroborate, *n*-propanol, TEA, $PdCl_2(dppf)$; (ii) $I_2/CAN/CH_3CN$; (iii) for hydrolysis of compound **10**, CH_2Cl_2/BBr_3; (iv) for deprotection of *N*-Boc derivatives (**17** and **24**) THF/MeONa.



Scheme 2. Reagents: (i) Suzuki conditions, potassium 2-furyltrifluoroborate, n-propanol, TEA, $PdCl_2(dppf)$ and *N*-(Boc)pyrrole-2-boronic acid, ethanol abs, $(PPh_3)_4Pd$ tetrakis(triphenylphosphine)palladium (0), sodium carbonate 2 M; (ii) for deprotection of *N*-Boc derivative (**27**) THF/MeONa.

41 R_3= $COOCH_2$(2-thienyl)
42 R_3=CO-2furyl

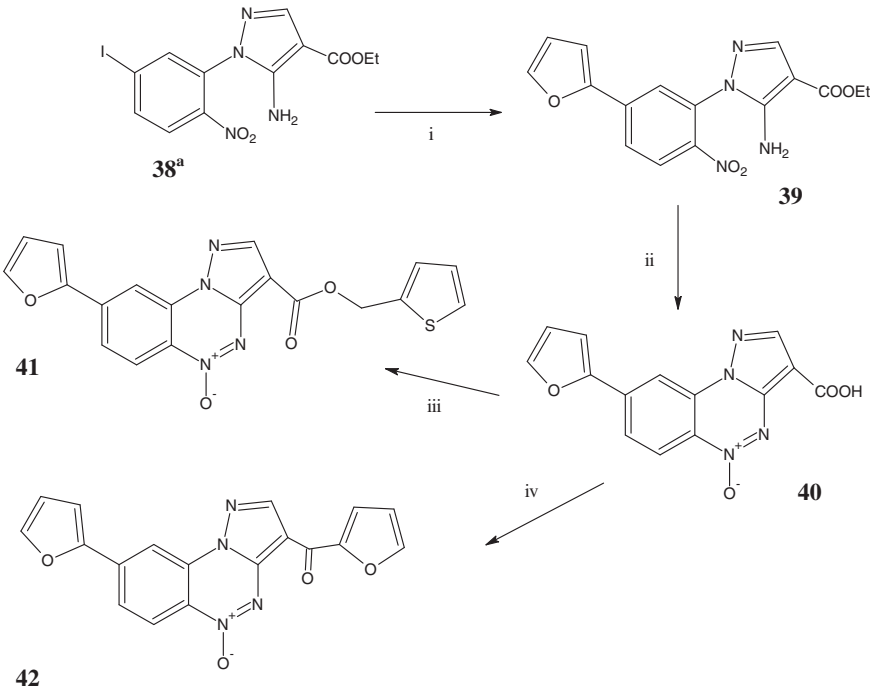


29[a]

30 R_3=CO-2furyl
31[a] R_3= $COOCH_2$(2-thienyl)

N°	R_3	8-Ar/Het	
32	$COOCH_2$-2-thienyl	4-OMe-Ph	
33	$COOCH_2$-2-thienyl	1-Boc-2-pyrrolyl	iv
34	$COOCH_3$	1(H)-2-pyrrolyl	
35	CO-2furyl	4-OMe-Ph	
36	CO-2furyl	1-Boc-2-pyrrolyl	iv
37	CO-2furyl	1(H)-2-pyrrolyl	

Scheme 3. For compound **29** and **31** see[22]: Reagents: (i) Friedel–Craft reaction on **29**, 3-carboxy-8-iodopyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide,[22] to obtain **30**: thionyl chloride/CH_2Cl_2/$SnCl_4$/furane; starting from **29**, following a procedure described in[22] to obtain **31**; (ii) Starting material **30** or **31**,[22] reacted in Suzuki conditions: 4-methoxyphenylboronic acid for compound **32** and **35**, *N*-Boc-2-pyrrolylboronic acid for compounds **33** and **36**, ethanol abs, tetrakis, sodium carbonate 2 M; (iii) potassium 2-furyltrifluoroborate, *n*-propanol, TEA, $PdCl_2$(dppf); (iv) for deprotection of *N*-Boc derivatives (**33** and **36**) THF/MeONa. In case of derivative **33** in this reaction condition a trans-esterification occurred, obtained compound **34**.



38[a]
39
40
41
42

Scheme 4. For **38**.[22] Reagents: (i) potassium 2-furantrifluoroborate, $PdCl_2$(dppf), TEA; (ii) 10% NaOH solution, diglyme; (iii) NEt_3, *i*-butylchloroformate, 2-thiofenemethanol; (iv) CH_2Cl_2, Cl_3CCN, PPh_3, $SnCl_4$, and furane.

reaction conditions, in derivative **36**, the removal of *N*-Boc group was selective and derivative **37** was obtained. The reaction of coupling of the furyl ring at position 8 on compounds **30** and **31**, with potassium 2-furantrifluoroborate gave very low yields and with a heavy work-up, thus the synthesis of compounds **41–42** was made with an alternative synthetic way, Scheme 4. The intermediate ethyl 1-(2-nitro-5-iodophenyl)-5-aminopyrazole-4-carboxylate **38**,[22] was used as starting material to introduce the 2-furyl group,

using again potassium 2-furantrifluoroborate and palladium chloride(diphenylphosphine ferrocene) $PdCl_2(dppf)\cdot CH_2Cl_2$ and **39** was obtained in good yield.

The next cyclization in alkali medium[28] gave the 3-carboxy-8-(2-furyl)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide **40**, that was esterified by the mixed anhydride method (triethyl amine/*i*-butyl-chloroformate and 2-thiophenmethanol), to yield compound **41**. The same compound **40** was transformed into corresponding acid chloride in mild conditions, by treatment with trichloroacetonitrile and triphenylphosphine in methylene chloride[29]; this acyl chloride was not isolated, but subjected to a Friedel–Craft reaction with $SnCl_4$ and furane to obtain derivative **42**.

3. Results and discussion

3.1. Biological results

The affinity at the Bz site on $GABA_A$-R of the new synthesized compounds was evaluated by their ability to displace [^{3}H]flumazenil (Ro 15-1788) from its specific binding in bovine brain membrane.[19] The affinity was expressed as K_i only for those compounds inhibiting radioligand binding by more than 80% at fixed concentrations of 10 μM.

From the binding results reported in Table 2, some observations arise. The new tested compounds show receptor recognition with a K_i range of 14.9–2480 nM. Compounds **3–9** as well as compound **40**, (Table 1) were not tested because previous reports indicate that these types of derivatives have no receptor recognition.[21]

Among the 3-iodopyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxides, bearing various aryl/heteroaryl rings at position 8, **10–18**, the features of ring moiety are very important. In fact, the optimization of the 8-phenyl derivative, **A27**,[16] (K_i = 966 nM), that is, compounds **10** and **12**, allow a threefold higher binding affinity: **10**, K_i = 305 nM and **12** K_i = 241 nM. These compounds have a methoxy group in the phenyl ring (*ortho*- and *para*-position, respectively) that could reinforce the interaction with receptor protein.[16]

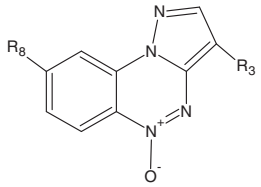
A more hydrophilic substituent at position 8 is detrimental for the binding as shown for compound **11** (K_i = 2480 nM), obtained from demethylation of **10** and for the 8-(4-pyridyl)-derivative (K_i = 2380 nM), compound **13**. The isosteric replacement of the 8-phenyl ring with five-membered heteroaryl rings 2-, 3-thienyl, 2-furyl and (1*H*)-2-pyrrolyl gave compounds **14–16** and **18**. The best affinity was shown by derivative (1*H*)-2-pyrrolyl, **18** (K_i = 25 nM), that contains a hydrogen bond donor atom (NH). Among the other heteroaryl rings, only the 8-(3-thienyl) derivative **15** is endowed with good binding affinity (K_i = 56 nM) compared to the 8-(2-thienyl), **14**, K_i = 202 nM and the 8-(2-furyl), **16**, K_i = 190 nM. These results suggest that the smaller size of the 8-substituents versus phenyl ring (K_i = 966 nM) permits better receptor interaction.

In the series of the 3-thienyl derivatives **(19–23, 25**) the introduction at position 8 of aromatic/heteroaromatic rings gave useful information. The 8-phenyl derivative **19** shows a poor binding affinity (K_i = 642 nM), as well as the 8-(4-OCH_3phenyl) derivative, **20**, K_i = 817 nM. When in position 8 was introduced a five-membered ring, 2-, 3-thienyl, 2-furyl and (1*H*)-2-pyrrolyl, **21–23** and **25**, the receptor recognition falls in the range of 370–19.4 K_i(nM), confirming again the importance of ring size. In particular, the need of hydrogen bond donor moiety (NH pyrrole) is evidenced as for compound **25** (K_i = 19.4 nM), while the presence of an acceptor moiety (furane oxygen) gave weak affinity (**23**, K_i = 209 nM). We thought that it might be useful to invert the substituents of positions 3 and 8 of compounds **23** and **25** to evaluate the influence of their interchangeability in receptor protein recognition, thus obtaining compounds **26** and **28**. The various orientations that the pyrazolobenzotriazine scaffold assumes, when selected by ADLR model, show that the substituents at position 3 and 8 can interact indiscriminately with the pharmacophoric points Lp-1 or Lp-2 in case of lipophilic moiety and HBp-1, HBp-2, or HBp-3 in case of a moiety able to form a hydrogen bond interaction.[16,23] This would explain the good affinity shown by 3-(2-furane derivative), **26** (K_i = 26.9 nM) compared to its isomer **23** (8-(2-furane derivative, K_i = 209 nM), that probably has less interaction strength. The two pyrrole derivative isomers, instead, have good affinity: 3-(1(*H*)-2-pyrrol-) derivative **28**, K_i = 48.3 nM and 8-(1(*H*)-2-pyrrol-) derivative **25**, K_i = 19.4 nM. (We are going to rationalize these results in the next molecular modeling section).

The (hetero)aryl rings at position 8 in the 3-ester derivatives, **32–34**, **41**, were chosen considering the influence, positive or negative, which they have in the two previous series. In the 3-ester series the negligible influence of the ring size at position 8 was seen, in fact the affinity value is in the same order of magnitude ($14.9 \leqslant K_i$(nM) $\leqslant 54.8$) for all compounds, except derivative **33**, (K_i = 395.4 nM). The presence of a six-membered ring (*para*-methoxyphenyl), as in derivative **32**, or five-membered ring, compound **41**, confers quite comparable affinity values, **32** K_i = 29.4 nM and **41** K_i = 54.8 nM. When position 8 was substituted with a 2-(1*H*)-pyrrolyl, as in the case of the 3-methoxycarbonyl derivative **34**, the affinity value was the best, K_i = 14.9 nM. The NH pyrrole protection, as in compound **33**, reduced the affinity value confirming the importance of the NH pyrrole in the binding. Thus, the importance of anchorage of the 3-ester group is again confirmed[17] and the affinity modulation due to substituent at position 8 is emphasized.[16]

The importance of the type of 8-substituent in the 3-furoyl derivatives, **35**, **37**, **42** that show binding affinity values in the

Table 2
BZR ligand affinity of new 8-aryl-/heteroarylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide 3-substituted



N°	R_3	R_8	K_i (nM) [a]
A27[b]	I	Ph	966 ± 95
10	I	2-OMePh	305 ± 29.3
11	I	2-OHPh	2480 ± 120
12	I	4-OMePh	241 ± 23.2
13	I	4-Py	2380 ± 190
14	I	2-Thienyl	202 ± 20
15	I	3-Thienyl	56 ± 4.9
16	I	2-Furyl	190 ± 22
18	I	(1*H*)-2-Pyrrolyl	25 ± 2.4
19	3-Thienyl	Ph	642 ± 62.6
20	3-Thienyl	4-OMePh	817.0 ± 16.7
21	3-Thienyl	2-Thienyl	370 ± 35.7
22	3-Thienyl	3-Thienyl	357 ± 33.9
23	3-Thienyl	2-Furyl	209 ± 20
25	3-Thienyl	(1*H*)-2-Pyrrolyl	**19.4 ± 2.8**
26	2-Furyl	3-Thienyl	**26.9 ± 2.5**
28	(1*H*)-2-pyrrolyl	3-Thienyl	**48.3 ± 6.1**
32	$COOCH_2$-2-thienyl	4-OMePh	**29.4 ± 1.8**
33	$COOCH_2$-2-thienyl	1-Boc-2-pyrrolyl	395.4 ± 39
34	COOMe	(1*H*)-2-Pyrrolyl	**14.9 ± 1.1**
41	$COOCH_2$-2-thienyl	2-Furyl	**54.8 ± 4.1**
35	CO-2-furyl	4-OMePh	238.67 ± 18
37	CO-2-furyl	(1*H*)-2-Pyrrolyl	**63.0 ± 5.5**
42	CO-2-furyl	2-Furyl	856.9 ± 4.2

[a] K_i value are means ± SEM of five determinations.
[b] See Ref. 16.

range of $63 \leqslant K_i(nM) \leqslant 856$ is again verified. Compound **37**, the 8-(1*H*)-2-pyrrolyl emerges for its good affinity, $K_i = 63$ nM.

From binding results it is possible assert that compounds bearing the (1*H*)-2-pyrrolyl moiety are very good ligands (**18**, **25**, **28**, **34**, and **37**) in each series, suggesting the critical role of a hydrogen bond donor (pyrrole NH) in receptor recognition. Good affinity values were also identified for compounds bearing acceptor atom moiety (**26**, **32**, and **41**) if only by having the suitable substituent in the complementary position. Moreover, the size of the substituent at position 8 is very important only in the 3-iodine-, 3-heteroaryl-, and 3-furoylderivative, while it is negligible in the 3-ester series.

3.2. Molecular modeling

The ADLR method[16,23] was used here to rationalize the affinity data and to improve our knowledge of the HBp-3 area. Among the compounds endowed with better binding affinity, emerge derivatives with the pyrrole moiety in position 8 or 3 (**18**, **25**, **34**, **37**, and **28**). In the overlapping performed by ADLR, the pyrrole ring fits in the HBp-3 area permitting the NH to form hydrogen bond interaction, independently of the position of the pyrrole moiety in the pyrazolobenzotriazine core (see Fig. 2).

When the pyrrole ring is replaced with furane (**16**, **23**, **42**, **41**, and **26**, Figs. 3 and 4) bearing a potential hydrogen bond acceptor atom (O), ADLR oriented the furane oxygen mainly toward the HBp-3 area. As depicted in Figure 3,compounds **16**, **23**, and **42** present the furane moiety in this mode, but the lower affinity value of compounds (K_i = 190, 209, and 856 nM, respectively) could be due to a reduced hydrogen bond force or no efficient interaction with LP-1 area (for **16** and **23**) and steric hindrance for **42**. Unexpectedly, compound **26** (isomer of compound **23)** presents a different orientation proposed by ADLR (Fig. 4).

The thiophene ring efficiently interacts with the lipophilic area Lp-1, justifying the best affinity (K_i = 26.9 nM). Compound **41** bearing an ester group (the 2-thienylmethoxycarbonyl group) at position 3 shows a good affinity value (K_i = 54.8 nM) according to the fact that the ester group contributes significantly to receptor anchorage as previously stated,[16] interacting with the lipophilic area Lp-1 or Lp-2 depending on the orientation (A or B), see Figure 5.

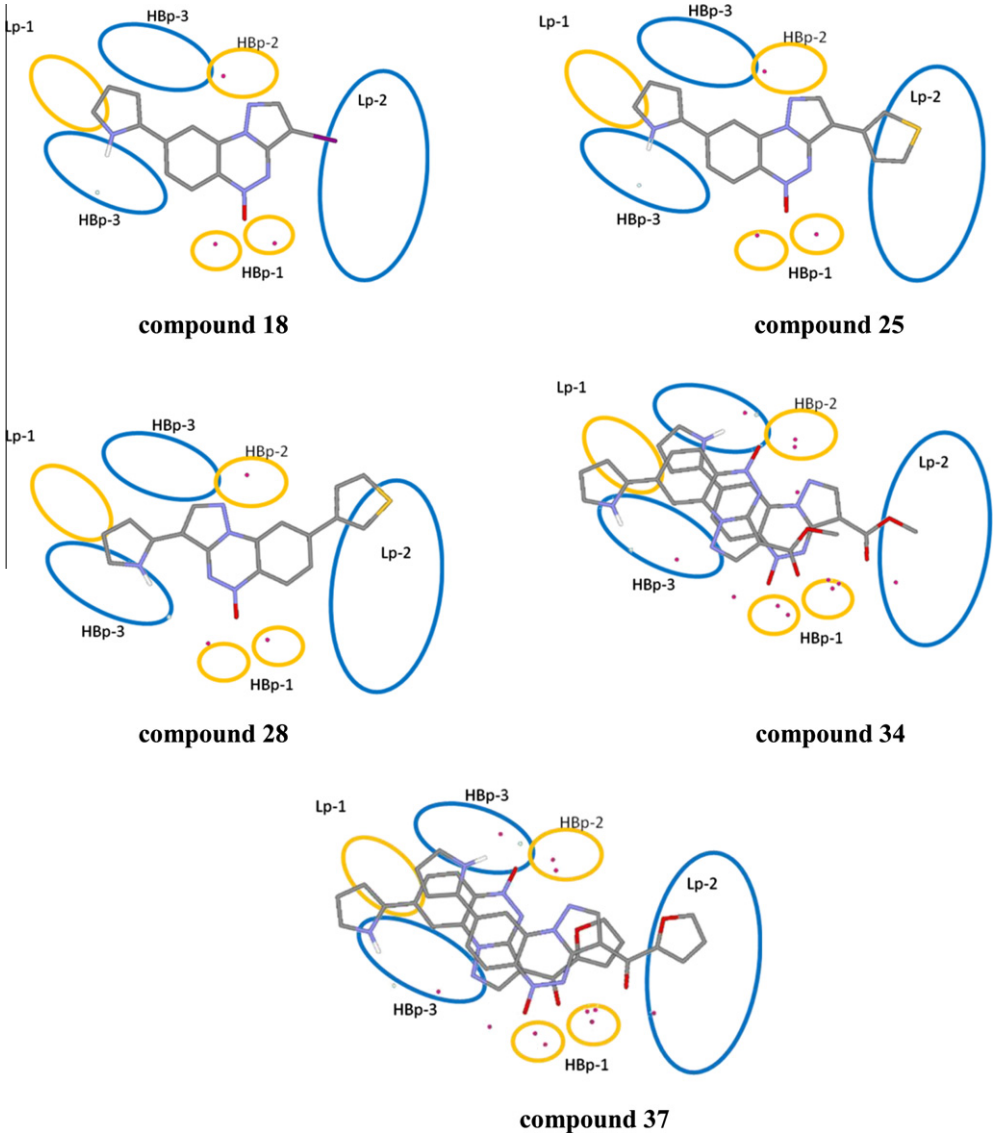


Figure 2. Compounds **18**, **25**, **28**, **34**, and **37** in the pharmacophoric model performed by ADLR. The pyrrole ring fits in the HBp-3 area permitting the NH moiety to form hydrogen bond interaction, independently of the position of the pyrrole moiety in the pyrazolobenzotriazine core.

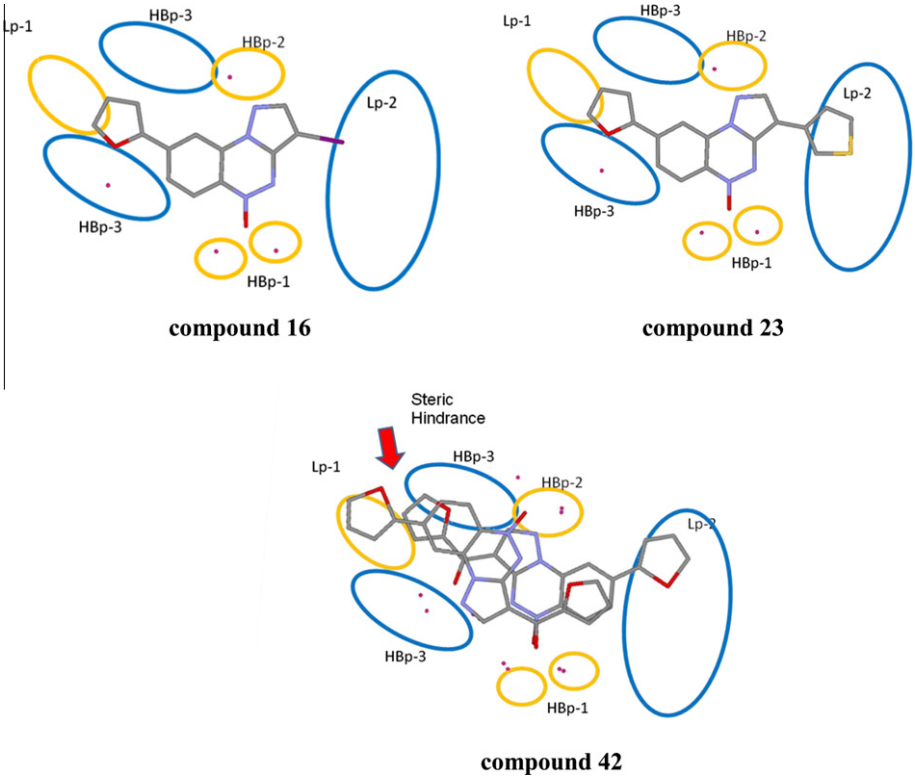


Figure 3. Compounds **16**, **23**, **42** in the pharmacophoric model performed by ADLR. The furane ring is oriented toward the HBp-3, but a reduced hydrogen bond force in the HBp-3 area or no efficient interaction with Lp-1 area (**16** and **23**) and a steric hindrance (**42**) are responsible of a low affinity (**16**, K_i = 190 nM; **23**, K_i = 209 nM; **42**, K_i = 856 nM).

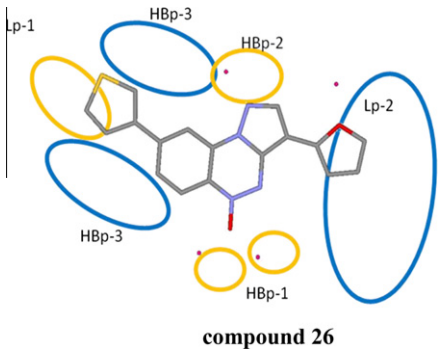


Figure 4. Compounds **26** in the pharmacophoric model performed by ADLR: unexpected orientation of the furane ring; the driving force of the binding is due to lipophilic interaction of thiophene ring.

The importance of the anchorage by the ester group is also evidenced in compound **32** (K_i = 29.4 nM), in which the presence of a large size group at position 8 is not binding for affinity, Figure 5.

4. Conclusion

In previous studies it was hypothesized that the 8-oxygen atom of the 8-aryloxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide derivatives engaged hydrogen bond interaction with the HBp-3 area.[16] On the other hand, the idea/hypothesis that this HBp-3 was a bifunctional area arose when the CGS 9698 compound, used to validate our pharmacophoric model with respect to Cook's 'unified pharmacophore/receptor model', showed hydrogen bond interaction with the same area. In this study, this hypothesis is confirmed since the NH group of the pyrrole ring engages a strong hydrogen bond interaction with HBp-3. The fact that the furane derivatives (**16**, **23**, and **42**) do not have a good binding, despite the ability to form a hydrogen bond interaction through the oxygen atom, could be due to an imperfect distance from hydrogen bond donor in the HBp-3 area.

In a preliminary study (data not published) derivatives bearing a phenylethylamine moiety (-$NHCH_2Ph$) at position 8 of pyrazolobenzotriazine system were endowed with very good binding affinity (in a range of 0.1 nM), thus the synthesis of 8-NH isoster of 8-OAr[16] will be the object of our next research project.

5. Experimental

5.1. Chemistry

Melting points were determined with a Gallenkamp apparatus and were uncorrected. Silica gel plates (Merck F_{254}) and silica gel 60 (Merck 70–230 mesh) were used for analytical and column chromatography, respectively. The structures of all compounds were supported by their IR spectra (KBr pellets in nujol mulls, Perkin–Elmer 1420 spectrophotometer) and ^{1}H NMR data (measured with a Bruker 400 MHz). Chemical shifts were expressed in δ ppm, using DMSO-d_6 or $CDCl_3$ as solvent. The chemical and physical data of new compounds are shown in Tables 1; all new compounds possess a purity $\geqslant$95%: microanalyses were performed with a Perkin–Elmer 260 analyzer for C, H, N.

5.2. General procedure for the synthesis of 3–7, 9, 19–22, 24, 27, 32–33, 35–36

Tetrakis(triphenylphosphine)palladium (0) (20 mg, 0.017 mmol) and the suitable iodopyrazolo benzotriazine (0.15 mmol) were combined in anhydrous tetrahydrofurane (5.0 mL). The starting materials were: compound **1**,[18] for the synthesis of derivatives

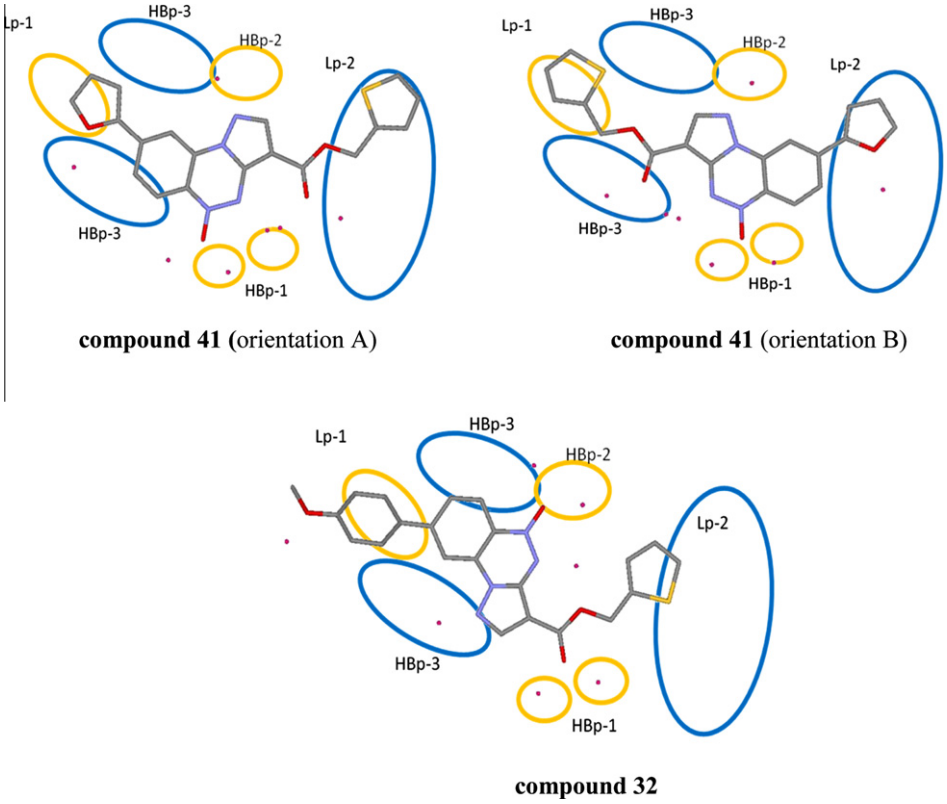


Figure 5. Compounds **41** (in two orientations A, B) and compound **32** in the pharmacophoric model performed by ADLR. Contribute of ester group to receptor anchorage.

3–7, 9; compound **2**,[22] for the synthesis of derivatives **19–22, 24**; compound **15** (see below) for the synthesis of derivative **27**; compound **30** for the synthesis of **35** and **36**; compound **31**,[22] for the synthesis of derivatives **32** and **33**. The solution of the suitable arylboronic acid (0.23 mmol, 1:1.5) in absolute ethanol and aqueous sodium carbonate (2 M, 2 mL) were added and the reaction was maintained at 25–70 °C (see the exact condition in each compound) and monitored by TLC. The final suspension was treated with water, filtrated to crude final product and purified by recrystallization by suitable solvent.

5.2.1. 8-(2-Methoxyphenyl)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (3)

From **1**,[18] and 2-methoxy phenylboronic acid, 4 h, room temperature. Yellow crystals; TLC eluent: diisopropyl ether/cyclohexane 8:3 v/v; ^{1}H NMR (DMSO-d_6) δ 8.48 (m, 2H, H-6 and H-9); 8.29 (d, 1H, H-2); 7.87 (dd, 1H, H-7); 7.53 (m, 2H, H-4 and H-6 phenyl); 7.25 (d, 1H, H-3 phenyl); 7.16 (t, 1H, H-5 phenyl); 6.92 (d, 1H, H-3); 3.85 (s, 3H, OCH_3). Anal. C, H, N.

5.2.2. 8-(4-Methoxyphenyl)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (4)

From **1**,[18] and 4-methoxyphenylboronic acid, 3 h, room temperature. Yellow crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; ^{1}H NMR (DMSO-d_6) δ 8.48 (m, 2H, H-6 and H-9); 8.31 (d, 1H, H-2); 8.05 (dd, 1H, H-7); 7.92 (d, 2H, H-2 and H-6 phenyl); 7.15 (d, 2H, H-3 and H-5 phenyl); 6.92 (d, 1H, H-3); 3.85 (s, 3H, OCH_3). Anal. C, H, N.

5.2.3. 8-(4-Pyridyl)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (5)

From **1**,[18] and 4-pyridin boronic acid, 3 h, reflux temperature. Yellow crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; ^{1}H NMR (DMSO-d_6) δ 8.85 (m, 2H, H-2 and H-6 pyridine); 8.70 (m, 2H, H-6 and H-9); 8.18 (d, 1H, H-2); 7.90 (dd, 1H, H-7); 7.70 (m, 2H, H-3 and H-5 pyridine); 6.82 (d, 1H, H-3). Anal. C, H, N.

5.2.4. 8-(Thien-2-yl)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (6)

From **1**,[18] and 2-thiophen boronic acid, 3 h, 70 °C. Yellow crystals; TLC eluent: diisopropyl ether/cyclohexane 8:3 v/v; ^{1}H NMR ($CDCl_3$) δ 8.59 (d, 1H, H-9); 8.57 (d, 2H, H-6); 8.14 (d, 1H, H-2); 7.87 (dd, 1H, H-7); 7.69 (d, 1H, H-5 thiophene); 7.57 (d, 1H, H-3 thiophene); 7.22 (m, 1H, H-4 thiophene); 6.79 (d, 1H, H-3). Anal. C, H, N.

5.2.5. 8-(Thien-3-yl)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (7)

From **1**,[18] and 3-thiophen boronic acid, 1.5 h, room temperature. Yellow crystals; TLC eluent: diisopropyl ether/cyclohexane 8:3 v/v; ^{1}H NMR ($CDCl_3$) δ 8.49 (m, 2H, H-6 and H-9); 8.05 (d, 1H, H-2); 7.78 (m, 2H, H-7 and H-2 thiophene); 7.51 (m, 1H, H-4 thiophene); 7.45 (m, 1H, H-5 thiophene); 6.69 (d, 1H, H-3). Anal. C, H, N.

5.2.6. 8-(*N*-Boc-pyrrol-2-yl)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (9)

From **1**,[18] and *N*(Boc)pyrrole-2-boronic acid 3 h, 60–70 °C, in toluene. Red crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; ^{1}H NMR ($CDCl_3$) δ 8.45 (m, 2H, H-6 and H-9); 8.02 (d, 1H, H-2); 7.73 (dd, 1H, H-7); 7.40 (m, 1H, H-3 pyrrole); 6.65 (d, 1H, H-3); 6.50 (m, 1H, H-5, pyrrole); 6.30 (m, 1H, H-4 pyrrole); 1.40 (s, 9H, $(CH_3)_3$). Anal. C, H, N.

5.2.7. 3-(Thien-3-yl)-8-phenylpyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-oxide (19)

From **2**,[22] and phenylboronic acid, 8 h, 50–60 °C. Red crystals; TLC eluent: diisopropyl ether/cyclohexane 8:3 v/v; ^{1}H NMR ($CDCl_3$) δ 8.62 (d, 1H, H-6); 8.59 (d, 1H, H-9); 8.36 (s, 1H, H-2); 7.93 (d, 1H,

H-2 thiophene); 7.88 (dd, 1H, H-7); 7.80 (d, 2H, H-2 and H-6 phenyl); 7.68 (d, 1H, H-4 thiophene); 7.55 (m, 3H, H-3, H-4 and H-5 phenyl); 7.46 (m, 1H, H-5 thiophene). Anal. C, H, N.

5.2.8. 3-(Thien-3-yl)-8-(4-Methoxyphenyl)pyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-oxide (20)

From **2**,[22] and 4-methoxyphenylboronic acid, 4 h, 60–70 °C. Red crystals; TLC eluent: diisopropyl ether/cyclohexane 8:3 v/v; ^{1}H NMR (DMSO-d_6) δ 8.75 (s, 1H, H-2); 8.47 (m, 2H, H-6 and H-9); 8.05 (dd, 1H, H-7); 8.00 (d, 1H, H-2 thiophene); 7.93 (d, 2H, H-2′ and H-6′ phenyl); 7.79 (d, 1H, H-4 thiophene); 7.72 (m, 1H, H-5 thiophene); 7.15 (d, 2H, H-3′ and H-5′ phenyl). Anal. C, H, N.

5.2.9. 3-(Thien-3-yl)-8-(thien-2-yl)pyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-oxide (21)

From **2**,[22] and 2-thiophenboronic acid, 2 h, room temperature. Red crystals; TLC eluent: diisopropyl ether/cyclohexane 8:3 v/v; ^{1}H NMR ($CDCl_3$) δ 8.58 (m, 2H, H-6 and H-9); 8.36 (s, 1H, H-2); 7.93 (m, 1H, H-2, 3-thiophene); 7.85 (dd, 1H, H-7); 7.68 (m, 2H, H-4, 3-thiophene and H-3, 8-thiophene); 7.58 (dd, 1H, H-5, 8-thiophene); 7.46 (dd, 1H, H-5, 3-thiophene); 7.21 (dd, 1H, H-4, 8-thiophene). Anal. C, H, N.

5.2.10. 3-(Thien-3-yl)-8-(thien-3-yl)pyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-oxide (22)

From **2**,[22] and 3-thiophenboronic acid, 3 h, 80 °C, in toluene. Red crystals; TLC eluent: diisopropyl ether/cyclohexane 8:3 v/v; ^{1}H NMR ($CDCl_3$) δ 8.59 (m, 2H, H-6 and H-9); 8.36 (s, 1H, H-2); 7.93 (m, 1H, H-2, 3-thiophene); 7.86 (m, 2H, H-7 and H-2, 8-thiophene); 7.69 (dd, 1H, H-4, 3-thiophene); 7.61 (dd, 1H, H-4, 8-thiophene); 7.54 (dd, 1H, H-5, 8-thiophene); 7.46 (dd, 1H, H-5, 3-thiophene). Anal. C, H, N.

5.2.11. 3-(Thien-3-yl)-8-(*N*-Boc-pyrrol-2-yl)pyrazolo[5,1-*c*]-[1,2,4]benzotriazine 5-oxide (24)

From **2**,[22] and *N*(Boc)pyrrole-2-boronic acid, 3 h, 60–70 °C, in toluene. Red crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; ^{1}H NMR (DMSO-d_6) δ 8.73 (s, 1H, H-2); 8.40 (d, 1H, H-6), 8.21 (d, 1H, H-9); 7.99 (m, 1H, H-2, 3-thiophene); 7.75 (m, 3H, H-7 and H-5, 3-thiophene and H-3 pyrrole); 7.57 (m, 1H, H-4, 3-thiophene); 6.70 (m, 1H, H-5, pyrrole); 6.51 (m, 1H, H-4 pyrrole); 1.40 (s, 9H, $(CH_3)_3$). Anal. C, H, N.

5.2.12. 3-(*N*-Boc-pyrrol-2-yl)-8-(thien-3-yl)pyrazolo[5,1-*c*]-[1,2,4]benzotriazine 5-oxide (27)

From **15** (see below) and *N*(Boc)pyrrole-2-boronic acid, 3 h, 60–70 °C, in toluene. Red crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; ^{1}H NMR (DMSO-d_6) δ 8.51 (d, 1H, H-9); 8.38 (m, 1H, H-2 thiophene); 8.36 (d, 1H, H-6); 8.32 (s, 1H, H-2); 8.18 (dd, 1H, H-7); 7.80 (m, 1H, H-5, thiophene); 7.70 (m, 1H, H-4 thiophene); 7.39 (m, 1H, H-3 pyrrole); 6.40 (m, 1H, H-5, pyrrole); 6.30 (m, 1H, H-4 pyrrole); 1.30 (s, 9H, $(CH_3)_3$). Anal. C, H, N.

5.2.13. 3-(2-Thienylmethoxycarbonyl)-8-(4-methoxy-phenyl)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (32)

From **31**,[22] and 4-methoxyphenylboronic acid, 8 h, room temperature. Yellow crystals; TLC eluent: toluene/ethyl acetate 8:3 v/v; ^{1}H NMR ($CDCl_3$) δ 8.68 (s, 1H, H-2); 8.51 (d, 1H, H-9); 8.49 (d, 1H, H-6); 8.12 (dd, 1H, H-7); 7.95 (d, 2H, H-2 and H-6 phenyl); 7.60 (d, 1H, H-5 thiophene); 7.30 (d, 1H, H-3 thiophene); 7.60 (d, 2H, H-3 and H-5 phenyl); 7.08 (m, 1H, H-5 thiophene); 5.52 (s, 2H, CH_2); 3.80 (s, 3H, CH_3). Anal. C, H, N.

5.2.14. 3-(2-Thienylmethoxycarbonyl)-8-(*N*-Boc-pyrrol-2-yl)-pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (33)

From **31**,[22] and *N*(Boc)pyrrole-2-boronic acid, 3 h, refluxing temperature, in toluene. Yellow crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; ^{1}H NMR (DMSO-d_6) δ 8.67 (s, 1H, H-2); 8.40 (d, 1H, H-6), 8.23 (d, 1H, H-9); 7.82 (dd, 1H, H-7); 7.60 (m, 1H, H-5 thiophene); 7.55 (m, 1H, H-3 pyrrole); 7.30 (m, 1H, H-3, 3-thiophene); 7.01 (m, 1H, H-4 thiophene); 6.71 (m, 1H, H-5, pyrrole); 6.42 (m, 1H, H-4 pyrrole); 5.51 (s, 2H, CH_2); 1.40 (s, 9H, $(CH_3)_3$). Anal. C, H, N.

5.2.15. 3-(Fur-2-ylcarbonyl)-8-(4-methoxyphenyl)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (35)

From **30** (see below), and 4-methoxyphenylboronic acid, 12 h, room temperature. Yellow crystals; TLC eluent: toluene/ethyl acetate 8:3 v/v; ^{1}H NMR ($CDCl_3$) δ 8.91 (s, 1H, H-2); 8.55 (d, 1H, H-9); 8.40 (d, 1H, H-6); 8.18 (m, 2H, H-7 and H-5 furane); 7.98 (d, 2H, H-2 and H-6 phenyl); 7.61 (d, 1H, H-3 furane); 7.18 (d, 2H, H-3 and H-5 phenyl); 6.82 (m, 1H, H-4 furane); 3.80 (s, 3H, CH_3). Anal. C, H, N.

5.2.16. 3-(Fur-2-ylcarbonyl)-8-(*N*-Boc-pyrrol-2-yl)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (36)

From **30** and *N*(Boc)pyrrole-2-boronic acid, 3 h, refluxing temperature, in tetrahydrofurane. Yellow crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; ^{1}H NMR (DMSO-d_6) δ 8.91 (s, 1H, H-2); 8.42 (d, 1H, H-6), 8.30 (d, 1H, H-9); 8.12 (d, 1H, H-5 furane); 7.82 (dd, 1H, H-7); 7.62 (m, 1H, H-3 furane); 7.58 (m, 1H, H-3 pyrrole); 6.82 (m, 1H, H-4, furane); 6.72 (m, 1H, H-5, pyrrole); 6.42 (m, 1H, H-4 pyrrole); 1.40 (s, 9H, $(CH_3)_3$). Anal. C, H, N.

5.3. 3-(Fur-2-ylcarbonyl)-8-iodopyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-oxide (30)

The starting material **29**,[22] (0.22 mmol) was reacted with 2.5 mL of thionyl chloride and maintained at refluxing temperature for 1 h. The final solution was evaporated in vacuo and the corresponding 3-carbonylchloride intermediate was utilized as that in the next Friedel–Craft reaction. Dichloromethane (10 mL), $SnCl_4$ (0.2 mL), and furane (0.2 mL) were added to the carbonylchloride residue and the reaction was heated at 40–50 °C, monitoring by TLC (eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v). The reaction was quenched with HCl 1:1, diluted with dichloromethane and the organic phase separated. The dichloromethane phase was washed with a saturated solution of sodium bicarbonate, dried over sodium sulfate anhydrous and evaporated. The final residue was purified by chromatography column (eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v) and **30** was eluted as the fast band. Yellow crystals; ^{1}H NMR (DMSO-d_6) δ 8.91 (s, 1H, H-2); 8.78 (d, 1H, H-9); 8.18 (m, 3H, H-7, H-6 and H-5 furane); 7.61 (d, 1H, H-3 furane); 6.82 (m, 1H, H-4 furane).

5.4. General procedure for the synthesis of derivatives 8, 23, 26, and 39

A solution of potassium 2-furantrifluoroborate (60 mg, 0.34 mmol), $PdCl_2(dppf)\cdot CH_2Cl_2$ (20 mg), triethylamine (0.1 mL) and the suitable starting materials: **1**,[18] **2**,[22], **15**, **38**,[22] (0.2 mmol) in *n*-propyl alcohol (10 mL) was stirred at reflux for 3 h. The reaction was monitored by TLC (eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v) and when the staring material disappeared, was cooled to room temperature, and diluted with water (15 mL). The appropriate work up of the final suspension gave the desired final products **8**, **23**, **26, and 39**.

5.4.1. 8-(Fur-2-yl)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (8)

From **1**,[18] the final suspension was extracted with ethyl acetate and dried over sodium sulfate anhydrous. Evaporation of solution gave orange residue. ^{1}H NMR (DMSO-d_6) δ 8.45 (m, 2H, H-6 and H-9); 8.20 (d, 1H, H-2); 8.08 (m, 2H, H-7 and H-5 furane); 7.58 (m, 1H, H-3 furane); 6.92 (d, 1H, H-3); 6.80 (m, 1H, H-4 furane). Anal. C, H, N.

5.4.2. 3-(Thien-3-yl)-8-(fur-2-yl)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (23)

From **2**,[22] the final suspension was extracted with ethyl acetate (10 mL × 3), dried over sodium sulfate anhydrous and evaporated in vacuo and the residue recrystallized. Red crystals; ^{1}H NMR (DMSO-d_6) δ 8.78 (s, 1H, H-2); 8.49 (m, 2H, H-6 and H-9); 8.08 (d, 1H, H-7); 8.02 (s, 1H, H-2, thiophene); 8.00 (d, 1H, H-5 furane); 7.77 (m, 1H, H-4, thiophene); 7.72 (m, 1H, H-5, thiophene); 7.58 (d, 1H, H-3 furane); 6.78 (m, 1H, H-4 furane). Anal. C, H, N.

5.4.3. 3-(Fur-2-yl)-8-(thien-3-yl)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (26)

From **15**. The precipitate was filtered and purified by silica gel chromatography (eluting with toluene/ethyl acetate/acetic acid 8:2:1 v/v/v). Red crystals; ^{1}H NMR (DMSO-d_6) δ 8.60 (s, 1H, H-2); 8.58 (d, 1H, H-9); 8.45 (m, 2H, H-6 and H-2 thiophene); 8.12 (dd, 1H, H-7); 7.88 (m, 1H, H-5, thiophene); 7.80 (m, 2H, H-4 thiophene and H-5 furane); 6.85 (d, 1H, H-3 furane); 6.68 (m, 1H, H-4 furane). Anal. C, H, N.

5.4.4. Ethyl 1-(2-nitro-5-(fur-2-yl)phenyl)-5-aminopyrazol-4-carboxylate (39)

From **38**,[22] dark-yellow crystals; ^{1}H NMR (DMSO-d_6) δ 8.21 (d, 1H, H-3′); 8.01 (dd, 1H, H-4′); 7.97 (d, 1H, H-6′); 7.93 (s, 1H, H-5 furane); 7.70 (s, 1H, H-3); 7.41 (d, 1H, H-3 furane); 6.73 (m, 1H, H-4 furane); 6.57 (br s, 2H, NH_2, exch.); 4.22 (q, 2H, CH_2); 1.35 (t, 3H, CH_3). Anal. C, H, N.

5.5. General procedure for the synthesis of derivatives 10 and 12–17

A solution of appropriate starting material **3–9** (0.30 mmol) in acetonitrile (10 mL) was added of iodine (0.18 mmol) and cerium ammonium nitrate (CAN, 0.18 mmol) and maintained at room temperature for 45 min. The final red solution was evaporated under reduced pressure and the residue was washed with a 10% sodium hydroxide solution, the precipitate filtered and recrystallized by suitable solvent.

5.5.1. 3-Iodo-8-(2-methoxyphenyl)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (10)

From **3**. Orange crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; ^{1}H NMR (DMSO-d_6) δ 8.46 (d, 1H, H-6); 8.41 (d, 1H, H-9); 8.34 (s, 1H, H-2); 7.87 (dd, 1H, H-7); 7.53 (m, 2H, H-4 and H-6 phenyl); 7.25 (d, 1H, H-3 phenyl); 7.16 (t, 1H, H-5 phenyl); 3.85 (s, 3H, OCH_3). Anal. C, H, N.

5.5.2. 3-Iodo-8-(4-methoxyphenyl)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (12)

From **4**. Orange crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; ^{1}H NMR (DMSO-d_6) δ 8.46 (m, 2H, H-2 and H-6); 8.40 (d, 1H, H-9); 8.05 (dd, 1H, H-7); 7.92 (d, 2H, H-2 and H-6 phenyl); 7.13 (d, 2H, H-3 and H-5 phenyl); 3.85 (s, 3H, OCH_3). Anal. C, H, N.

5.5.3. 3-Iodo-8-(4-pyridyl)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (13)

From **5**. Orange crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; ^{1}H NMR (DMSO-d_6) δ 8.79 (d, 2H, H-2 and H-6 pyridine); 8.64 (d, 1H, H-9); 8.55 (d, 1H, H-6); 8.44 (s, 1H, H-2); 8.15 (dd, 1H, H-7); 7.98 (m, 2H, H-3 and H-5 pyridine). Anal. C, H, N.

5.5.4. 3-Iodo-8-(thien-2-yl)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (14)

From **6**. Orange crystals; TLC eluent: diisopropyl ether/cyclohexane 8:3 v/v; ^{1}H NMR ($CDCl_3$) δ 8.58 (m, 2H, H-6 and H-9); 8.14 (s, 1H, H-2); 7.88 (dd, 1H, H-7); 7.69 (d, 1H, H-5 thiophene); 7.57 (d, 1H, H-3 thiophene); 7.22 (m, 1H, H-4 thiophene). Anal. C, H, N.

5.5.5. 3-Iodo-8-(thien-3-yl)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (15)

From **7**. Orange crystals; TLC eluent: diisopropyl ether/cyclohexane 8:3 v/v; ^{1}H NMR ($CDCl_3$) δ 8.57 (m, 2H, H-6 and H-9); 8.14 (s, 1H, H-2); 7.88 (m, 2H, H-7 and H-2 thiophene); 7.59 (m, 1H, H-4 thiophene); 7.45 (m, 1H, H-5 thiophene). Anal. C, H, N.

5.5.6. 3-Iodo-8-(Fur-2-yl)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (16)

From **8**. Orange crystals; TLC eluent: diisopropyl ether/cyclohexane 8:3 v/v; ^{1}H NMR (DMSO-d_6) δ 8.47 (m, 3H, H-6, H-2, and H-9); 8.05 (dd, 1H, H-7); 7.98 (m, 1H, H-5 furane); 7.52 (m, 1H, H-3 furane); 6.79 (m, 1H, H-4 furane). Anal. C, H, N.

5.5.7. 3-Iodo-8-(*N*-Boc-pyrrol-2-yl)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (17)

From **9**. Orange crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; ^{1}H NMR (DMSO-d_6) δ 8.44 (m, 3H, H-2, H-6 and H-9); 7.85 (d, 1H, H-7); 7.79 (m, 1H, H-3 pyrrole); 6.72 (m, 1H, H-5, pyrrole); 6.42 (m, 1H, H-4 pyrrole); 1.40 (s, 9H, $(CH_3)_3$). Anal. C, H, N.

5.6. 3-Iodo-8-(2-hydroxyphenyl)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (11)

A solution of **10** (0.24 mmol) in dichloromethane (15 mL) was reacted with BBr_3 (0.3 mL) at refluxing temperature until the starting material disappeared in TLC (eluent: toluene/ethyl acetate/acetic acid 8:2:1). Then the final solution was treated with a 10% sodium hydroxide solution and the organic layer eliminated. The aqueous phase, after acidification (HCl 1:1) was extracted with ethyl acetate, dried and evaporated dryness obtained a yellow residue that was purified by recrystallization. ^{1}H NMR (DMSO-d_6) δ 10.2 (br s, 1H, OH); 8.55 (d, 1H, H-9); 8.46 (d, 1H, H-6); 8.34 (s, 1H, H-2); 7.95 (dd, 1H, H-7); 7.50 (d, 1H, H-3 phenyl); 7.35 (t, 1H, H-5 phenyl); 7.01 (d, 1H, H-6 phenyl); 7.00 (t, 1H, H-4 phenyl). Anal. C, H, N.

5.7. General procedure for the synthesis of derivatives18, 25, 28, 34 and 37

To a mixture of starting material **17**, **24**, **27**, **33**, and **36** (0.15 mmol) and anhydrous THF (2 mL) was added a solution of sodium methoxide in methanol (2 mL of a 0.43 M solution). The mixture was allowed to stir 1 h at room temperature and then treated with water. The resulting suspension was worked up and the row product recrystallized by suitable solvent.

In case of starting material **33** a trans-esterification occurred and the final product was the 3-methyl ester derivative **34**.

5.7.1. 3-Iodo-8-(pyrrol-2-yl)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (18)

From **17**. Red crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; ^{1}H NMR (DMSO-d_6) δ 12.00 (br s, 1H, NH, exch.); 8.43 (s, 1H, H-2); 8.40 (d, 1H, H-9); 8.25 (d, 1H, H-6); 8.00 (dd, 1H, H-7); 7.10 (m, 1H, H-3 pyrrole); 7.05 (m, 1H, H-5, pyrrole); 6.30 (m, 1H, H-4 pyrrole). Anal. C, H, N.

5.7.2. 3-(Thien-3-yl)-8-(pyrrol-2-yl)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (25)

From **24**. Red crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; ^{1}H NMR (DMSO-d_6) δ 12.00 (br s, 1H, NH, exch.); 8.73 (s, 1H, H-2); 8.50 (d, 1H, H-9); 8.35 (d, 1H, H-6); 8.00 (m, 2H, H-7 and H-2, 3-thiophene); 7.78 (m, 1H, H-5, 3-thiophene); 7.70 (m, 1H, H-5, 3-thiophene); 7.12 (m, 1H, H-5, pyrrole); 7.01 (m, 1H, H-4 pyrrole); 6.30 (m, 1H, H-3 pyrrole). Anal. C, H, N.

5.7.3. 3-(Pyrrol-2-yl)-8-(thien-3-yl)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (28)

From **27**. Red crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; ^{1}H NMR (DMSO-d_6) δ 11.35 (br s, 1H, NH, exch.); 8.51 (m, 2H, H-6 and H-2); 8.42 (m, 2H, H-9 and H-2 thiophene); 8.01 (dd, 1H, H-7); 7.83 (m, 1H, H-5, thiophene); 7.70 (m, 1H, H-4 thiophene); 6.90 (m, 1H, H-3 pyrrole); 6.60 (m, 1H, H-5, pyrrole); 6.18 (m, 1H, H-4 pyrrole). Anal. C, H, N.

5.7.4. 3-Methoxycarbonyl-8-(pyrrol-2-yl)pyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-oxide (34)

From **33**, in reaction conditions a trans-esterification occurred. Red crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; ^{1}H NMR (DMSO-d_6) δ 12.1 (br s, 1H, NH, exch.); 8.67 (s, 1H, H-2); 8.55 (d, 1H, H-9); 8.38 (d, 1H, H-6), 8.08 (dd, 1H, H-7); 7.18 (m, 1H, H-3 pyrrole); 7.08 (m, 1H, H-5, pyrrole); 6.30 (m, 1H, H-4 pyrrole); 3.90 (s, 3H, CH_3). Anal. C, H, N.

5.7.5. 3-(Fur-2-ylcarbonyl)-8-(pyrrol-2-yl)pyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-oxide (37)

From **36**. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; ^{1}H NMR (DMSO-d_6) δ 12.00 (br s, 1H, NH, exch.); 8.91 (s, 1H, H-2); 8.60 (d, 1H, H-9); 8.38 (d, 1H, H-6), 8.12 (m, 2H, H-7 and H-5 furane); 7.62 (m, 1H, H-3 furane); 7.15 (m, 1H, H-3 pyrrole); 7.08 (m, 1H, H-5, pyrrole); 6.80 (m, 1H, H-4 furane); 6.30 (m, 1H, H-4 pyrrole). Anal. C, H, N.

5.8. 3-Carboxy-8-(fur-2-yl)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (40)

A solution of **39** (0.5 mmol) in diglyme (5 mL) was added of 25 mL of 10% sodium hydroxide solution and was kept at 50–60 °C until the starting material disappeared, monitoring by TLC. The final suspension was treated with hydrochloric acid and the filtration of the precipitate afforded to the acid derivative **40**. The crude products were then recrystallized by suitable solvent. Yellow crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; ^{1}H NMR (DMSO-d_6) δ 12.9 (br s, 1H, OH, exch.); 8.61 (s, 1H, H-3); 8.51 (d, 1H, H-9); 8.48 (d, 1H, H-6); 8.15 (dd, 1H, H-7); 8.03 (d, 1H, H-5 furane); 7.60 (d, 1H, H-3 furane); 6.80 (m, 1H, H-4 furane). Anal. C, H, N.

5.9. 3-(2-Thienylmethoxycarbonyl)-8-(fur-2-yl)pyrazolo[5,1-*c*]-[1,2,4]benzotriazine 5-oxide (41)

A suspension of the acid **40** (0.337 mmol) in anhydrous THF and triethyl amine (1:3.5) was maintained at 0 °C in ice bath for 30′. To the suspension was added *i*-butylchloroformate (0.2 mL) and maintained under stirring for 1 h, from 0 °C to room temperature, to permit the anhydride to form. The 2-thiophen methanol was added in excess (0.2 mL) and the reaction was monitored by TLC (eluent: toluene/ethyl acetate 8:2). The final suspension was diluted with water and extracted with ethyl acetate which was in turn washed with sodium hydrogen carbonate and, after the normal work up, the residue was purified by chromatography column. Yellow crystals. ^{1}H NMR (DMSO-d_6) δ 8.68 (s, 1H, H-2); 8.52 (d, 1H, H-9); 8.49 (d, 1H, H-6); 8.14 (dd, 1H, H-7); 8.03 (d, 1H, H-5 furane); 7.60 (m, 2H, H-5 thiophene and H-3 furane); 7.30 (m, 1H, H-3, 3-thiophene); 7.06 (m, 1H, H-4 thiophene); 6.80 (m, 1H, H-4 furane); 5.55 (s, 2H, CH_2). Anal. C, H, N.

5.10. 3-(Fur-2-ylcarbonyl)-8-(fur-2-yl)pyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-oxide (42)

To a mixture of acid **40** (0.20 mmol) and trichloroacetonitrile (0.60 mmol) in dichloromethane (10 mL) was added triphenylphosphine (0.60 mmol) in dichloromethane (1.0 mL) dropwise at room temperature. The reaction mixture was stirred at room temperature for 2 h until the starting material disappeared in TLC (eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v). The reaction was then treated with $SnCl_4$ (0.1 mL) followed furane (0.1 mL). After 30′ the solution was treated with water and the organic layer was separated, dried and evaporated to dryness. The residue was recovered by diisopropyl ether and recrystallized by suitable solvent. Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; ^{1}H NMR (DMSO-d_6) δ 8.91 (s, 1H, H-2); 8.58 (d, 1H, H-9); 8.50 (d, 1H, H-6), 8.16 (dd, 1H, H-7); 8.12 (m, 1H, H-5, 2-furoyl); 8.06 (m, 1H, H-5 furane); 7.62 (m, 2H, H-3, 2-furoyl and furane); 6.83 (m, 2H, H-4, 2-furoyl and furane). Anal. C, H, N.

5.11. Radioligand binding assay

[^{3}H]Ro15-1788 (specific activity 78.8 Ci/mmol) was obtained from Perkin–Elmer.[30,31] All the other chemicals, which were of reagent grade, were obtained from commercial suppliers.

Bovine cerebral cortex membranes were prepared as previously described. 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.[32] [^{3}H]Ro 15-1788 binding studies were performed as previously reported.[20] 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.[33]

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.04.009.

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