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New Fluoro Derivatives of the Pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide System: Evaluation of Fluorine Binding Properties in the Benzodiazepine Site on γ-Aminobutyrric Acid Type A ($GABA_A$) Receptor. Design, Synthesis, Biological, and Molecular Modeling Investigation†

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In the search for potent ligands at the benzodiazepine site on the $GABA_A$ receptor, new fluoro derivatives of the pyrazolo[5,1-*c*][1,2,4]benzotriazine system were synthesized to evaluate the importance of the introduction of a fluorine atom in this system. Biological and pharmacological studies indicate that the substitution at position 8 with a trifluoromethyl group confers pharmacological activity due to potential metabolic stability in comparison to inactive 8-methyl substituted analogues. In particular, the compound 3-(2-methoxybenzyloxycarbonyl)-8-trifluoromethylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (**21**) emerges because of its selective anxiolytic profile without side effects. An analysis of all the newly synthesized compounds in our pharmacophoric map confirms the essential interaction points for binding recognition and the important areas for affinity modulation. The fluorine atom was able to form a hydrogen bond interaction only when it is not in position 3.

Introduction

GABA (γ-aminobutyrric acid[a]) is the major inhibitory neurotransmitter in the brain. The $GABA_A$ receptor is a ligand-gated ion channel (LGIC) whose involvement in various diseases (epilepsy, anxiety, panic disorder, cognitive impairment, pain) is well-known, making the gabaergic system a useful target for drug development.[1] Even though various subunits (α(1−6), β(1−3), γ(1−3), δ, ε, π, ρ, θ) have been identified, many combinations should be possible, whereas only a few have been shown to actually exist.[2] The most common form of $GABA_A$ receptor contains α, β, and γ subunits in different combinations. The site of benzodiazepine ligands is located between the α(1−3,5) and γ2 subunits. It is believed that the α-subunit is the main determinant of the variability in the benzodiazepine site's affinity and efficacy.[3] For many years, our research group has studied heterocyclic polyazotated polycondensed systems as useful scaffolds for the synthesis of compounds with a well-defined target.[4−7] In regard to the benzodiazepine site on the $GABA_A$ receptor (Bz site/$GABA_A$-R), various ligands with a pyrazolobenzotriazine core were synthesized, and in a more recent study, in comparison with the more frequently used Cook's model,[8,9] a pharmacophoric model was elaborated[10] as reported in Figure 1.

The notable results achieved prompted us to continue the synthesis of other derivatives of this tricyclic system to obtain useful compounds in the benzodiazepine receptor area. Here we report the synthesis and binding studies of new derivatives of the pyrazolo[5,1-*c*][1,2,4]benzotriazine system bearing one or more fluorine atom. The synthesis of the 3-fluorine derivatives is designed to complete the study on the 3-halogen series.[10,11] The insertion of the trifluoromethyl group in position 7 or 8 was used to evaluate whether the negligible pharmacological activity of 7- and 8-methyl derivatives was due to metabolic instability even though they showed high affinity binding.[12] Finally, the difluoromethoxy derivatives were synthesized to evaluate whether the oxygen atom at position 8 influences receptor interaction as in the 8-alkyloxyderivatives.[11] We then carried out in vivo tests on the most interesting ligands.

Our decision to synthesize fluorine derivatives arises from the increasing importance of fluorine in medicinal chemistry in recent years. In fact, many compounds reported in literature are endowed with remarkable biological and medicinal applications. The fluorine's small size, high electronegativity,

†Crystal structural data are available from the Cambridge Crystallographic Data Center CCDC 746814.

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[a] Abbreviations: $GABA_A$, GABA type A receptor; LGCIs, ligand-gated ion channels; Bz site/$GABA_A$-R, benzodiazepine site on $GABA_A$ receptor; SAR, structure−affinity relationships; NCS, *N*-chlorosuccinimmide; $Pd(PPh_3)_4$, tetrakis(triphenylphosphine)palladium (0); PTZ, pentylenetetrazole; Lp, lipophilic point; HBp, hydrogen bond interaction point; diazepam, 7-chloro-1-methyl-5-phenyl-3*H*-1,4-benzodiazepin-2(1*H*)-one; CGS 9896, 2-(4-chlorophenyl)pyrazolo[4,3-*c*]quinolin-3-one; CMC, carboxymethylcellulose; flu, flumazenil; PET, positron emission tomography; SPECT, single photon emission computed tomography.

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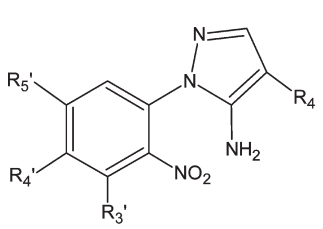
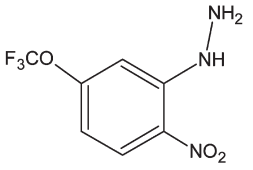
Table 1. Chemical Data for New Synthesized Compounds **1**−**6** and **33**



1-4, 6



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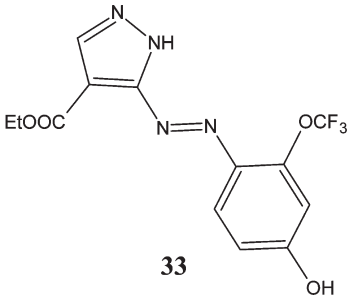


33

N°	R_4	$R_{3'}$	$R_{4'}$	$R_{5'}$	MF (MW)	yield (%)	mp °C (recryst solvent)
1	COOEt	CF_3	H	H	$C_{13}H_{11}N_4O_4F_3$ (344.26)	38	132−134 (ethanol)
2	COOEt	H	CF_3	H	$C_{13}H_{11}N_4O_4F_3$ (344.26)	45	117−118 (water/ethanol)
3	COOEt	H	H	CF_3	$C_{13}H_{11}N_4O_4F_3$ (344.26)	33	168−170 (ethanol)
4	COOH	CF_3	H	H	$C_{13}H_7N_4O_4F_3$ (316.26)	45	254−256 dec (ethanol)
5					$C_7H_6N_3O_3F_3$ (221.12)	56	70−72 (ethanol 80%)
6	COOEt	H	H	OCF_3	$C_{13}H_{11}N_4O_5F_3$ (360.4)	55	155−157(ethanol)
33					$C_{13}H_{11}N_4O_4F_3$ (344.12)	70	250−252 (ethanol)

and low polarizability are intriguing characteristics that can modify the chemical and physical parameters of the derivatives. A single atom of fluorine or perfluorinated groups can opportunely modulate the stability of compounds and affect the metabolism.[13−19] The development of procedures, methods or strategies for the synthesis of fluorine derivatives, and commercially available intermediates have made possible access to synthetic and design strategies to introduce fluorine into target molecules.[15,20] Moreover, fluorine derivatives could be useful tools for potential development of radioligands for brain imaging (positron emission tomography, PET or single photon emission computed tomography, SPECT) of neuropsychiatric diseases.

Chemistry

All chemical and physical data of the new compounds are reported in Tables 1 and 2. The synthetic approach to obtaining the desired fluorinated compounds is depicted in Schemes 1−6.

The 3-fluoro-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide, **7**, was obtained by reaction of the corresponding 3-unsubstituted compound[21] with an N−F fluorinating agent (F-TEDA-BF_4, 1-chloromethyl-4-fluoro-1,4-diazoniabicyclo-[2.2.2]octanebis(tetra-fluoroborate)).[22] The final complex mixture was purified by column chromatography. Finally, **7** was subjected to the nucleophilic substitution of the chlorine atom at position 8 in PTC conditions[11] to obtain the 3-fluoro-8-alkyloxy-/arylmethoxy derivatives **8**−**13**, see Scheme 1.

For synthesis of the trifluoromethyl derivatives (Scheme 2), the intermediates ethyl 5-aminopyrazole-4-carboxylate **1**−**3** were synthesized from the corresponding 3-, 4-, 5-trifluoromethyl-2-nitro-phenylhydrazine[23−25] and 2-cyano-3-ethoxypropenate, following a previously reported procedure.[21] The next cyclization to the pyrazolobenzotriazine system was obtained in standard conditions of 10% sodium hydroxide solution[21] and then were acidified with concentrated HCl. The 3-carboxy-7-trifluoromethyl- and the 3-carboxy-8-trifluoromethylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxides, **14** and **15**, were obtained from **2** and **3**, respectively. The 5-aminopyrazole **1** in the same condition did not cyclize to the pyrazolobenzotriazine system but afforded to the corresponding 5-amino-1-(2-nitro-3-trifluoromethylphenyl)-1*H*-pyrazole-4 carboxylic acid **4**.

Compounds 3-carboxy-7/8-trifluoromethylpyrazolo[5,1-*c*][1,2,4]benzotriazine, **14** and **15**, were respectively modified (Scheme 3) by decarboxylation with HCl conc (compounds **16** and **17**), by esterification (compounds **18**−**23**), or by transformation in ketones (compounds **24** and **25**). The insertion of a halogen atom (Scheme 4) at position 3 of compounds **16** and **17**, by reaction with bromine, NCS, or ICl, gave respectively the 3-halo derivatives **26**−**29**. This latter, **29**, the 3-iodo-8-trifluoromethylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide was in turn useful to achieve, by Suzuki coupling, the corresponding 3-heteroaryl derivatives **30**−**32**.

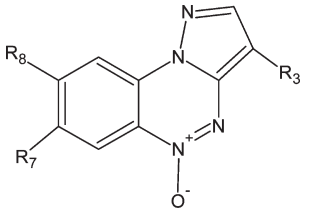
Finally, since in a our previous work,[11] we evidenced the importance of the alkoxy group at position 8 of pyrazolobenzotriazine system, the idea of obtaining derivatives bearing the 8-oxygen atom linked to the trifluoromethyl group was intriguing. To obtain the 8-trifluoromethoxypyrazolobenzotriazine system, it was necessary to synthesize the new 2-nitro-5-trifluoromethoxyphenylhydrazine, **5**, following two synthetic approaches as depicted in Scheme 5 (route a, route b).

Route a was the better synthetic pathway, using the 4-trifluoromethoxyaniline as starting material. The halogenations with NCS,[26] followed by diazotation and subsequent decomposition of the obtained diazonium salt with sodium nitrite and cuprous oxide,[27] gave the corresponding 2-chloro-4-trifluoromethoxynitrobenzene.[28] The next nucleophilic substitution of the chlorine atom by hydrazine hydrate afforded the desired 2-nitro-5-trifluoromethoxyphenylhydrazine in good yield.

In route b (Scheme 5), the 3-trifluoromethoxyaniline was used as starting material. Nitration, diazotation, and the next reduction[29] gave the desired compound **5**, but this route b was not exploited because of the low yields of the various reaction steps.

The next reaction of **5** with 3-ethoxy-2-cyanopropenate gave compound **6**, the ethyl 1-(2-nitro-5-trifluoromethoxyphenyl)-5-aminopyrazole-4-carboxylate. The attempt to obtain the corresponding 3-carboxy-8-trifluoromethoxypyrazolo-[5,1-*c*][1,2,4]benzotriazine 5-oxide in alkali medium was disappointing. In fact, although the intramolecular condensation between nitro and amino group occurred and the pyrazolobenzotriazine system was formed, liability of the CF_3−O bond was evidenced and the 8-hydroxyderivative, previously synthesized by another method,[21] was recovered.

Table 2. Chemical Data for New Synthesized Compounds **7**−**32**, **34**−**42**



N°	R_3	$R_{7/8}$	MF (MW)	yield (%)	mp °C (recryst solvent)
7	F	8-Cl	$C_9H_4N_4OClF$ (238.45)	28	204−205 (ethanol)
8	F	8-O-$CH_2C{\equiv}CH$	$C_{12}H_7N_4O_2F$ (258.40)	62	219−221 (ethanol)
9	F	8-O-CH_2-2-OCH_3Ph	$C_{17}H_{13}N_4O_3F$ (340.32)	45	205−206 (ethanol)
10	F	8-O-CH_2-2-OCF_3Ph	$C_{17}H_{10}N_4O_3F_4$ (394.30)	48	164−166 (ethanol)
11	F	8-O-CH_2-4-Py	$C_{15}H_{10}N_5O_2F_4$ (311.29)	39	264−265(ethanol)
12	F	8-O-CH_2-2-thienyl	$C_{14}H_9N_4O_2SF$ (316.42)	52	224−226 (ethanol)
13	F	8-O-CH_2-2-furyl	$C_{14}H_9N_4O_3F$ (300.25)	66	190−192 (ethanol)
14	COOH	7-CF_3	$C_{11}H_5N_4O_3F_3$ (298.19)	43	> 300 dec (ethanol)
15	COOH	8-CF_3	$C_{11}H_5N_4O_3F_3$ (298.19)	70	263−265 (ethanol)
16	H	7-CF_3	$C_{10}H_5N_4OF_3$ (254.18)	50	282−283 (ethanol)
17	H	8-CF_3	$C_{10}H_5N_4OF_3$ (254.18)	35	172−174 (ethanol)
18	$COOCH_2CH_3$	7-CF_3	$C_{13}H_9N_4O_3F_3$ (326.24)	42	223−224 (ethanol)
19	$COOCH_2CH_3$	8-CF_3	$C_{13}H_9N_4O_3F_3$ (326.24)	60	211−212 (ethanol)
20	$COOCH_2$-2-OCH_3-Ph	7-CF_3	$C_{19}H_{13}N_4O_4F_3$ (418.34)	50	205−206 (*i*-propyl alchol)
21	$COOCH_2$-2-OCH_3-Ph	8-CF_3	$C_{19}H_{13}N_4O_4F_3$ (418.34)	26	212−214 (*i*-propyl alcohol)
22	$COOCH_2$-2-thienyl	7-CF_3	$C_{16}H_9N_4O_3SF_3$ (394.34)	55	180−182 (*i*-propyl alcohol)
23	$COOCH_2$-2-thienyl	8-CF_3	$C_{16}H_9N_4O_3SF_3$ (394.34)	35	210−1° (*i*-propyl alcohol)
24	CO-2-OCH_3-Ph	8-CF_3	$C_{18}H_{13}N_4O_4F_3$ (406.18)	63	250−252 (ethanol)
25	CO-2-thienyl	8-CF_3	$C_{15}H_7N_4O_2SF_3$ (364.36)	27	230−231 (ethanol)
26	Br	7-CF_3	$C_{10}H_4N_4OF_3Br$(254.18)	73	202−203 (ethanol)
27	Cl	8-CF_3	$C_{10}H_4N_4OClF_3$ (289.71)	55	203−205 (ethanol)
28	Br	8-CF_3	$C_{10}H_4N_4OBrF_3$ (334.26)	80	223−225(ethanol 80%)
29	I	8-CF_3	$C_{10}H_4N_4OIF_3$ (380.1)	50	194−196 (ethanol)
30	2-thienyl	8-CF_3	$C_{14}H_7N_4OSF_3$ (336.30)	45	209−210 (*i*-propyl alcohol)
31	3-thienyl	8-CF_3	$C_{14}H_7N_4OSF_3$ (336.30)	26	207−208 (ethanol)
32	3-furyl	8-CF_3	$C_{14}H_7N_4O_2F_3$ (320.23)	47	222−224 (*i*-propyl alcohol)
34[a]	I	8-OH	$C_9H_5N_4OI$ (312.15)	32	225−226(ethanol 80%)
36	COOH	8-OH	$C_{10}H_6N_4O_3$ (230.20)	70	> 300 (ethanol)
37	$COOCH_2$-2-thienyl	8-OH	$C_{15}H_{10}N_4O_3S$ (326.34)	28	> 300 (ethanol)
38[a]	I	8-OCF_2H	$C_{10}H_5N_4OF_2I$ (362.10)	26	185−187 (ethanol)
39[a]	$COOCH_2CH_3$	8-OCF_2H	$C_{13}H_{10}N_4O_3F_2$ (308.25)	42	148−150 (ethanol)
40[a]	$COOCH_2$-2-thienyl	8-OCF_2H	$C_{16}H_{10}N_4O_3SF_2$ (376.35)	55	127−129 (ethanol)
41	I	8-OCF_2H	$C_{10}H_5N_4O_2F_2I$ (378.10)	75	170−172 (ethanol)
42	$COOCH_2CH_3$	8-OCF_2H	$C_{13}H_{10}N_4O_4F_2$ (324.25)	59	189−190 (ethanol)

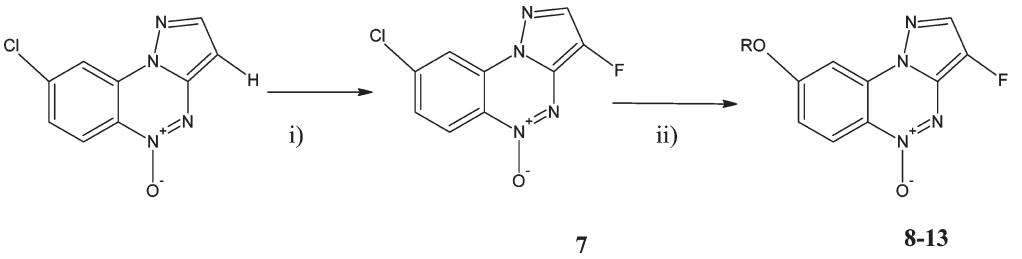
[a] 5-Deoxide derivative.

However, to try to obtain the desired compound 3-carboxy-8-trifluoromethoxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide, another attempt was made using a different synthesis strategy. A survey of the literature[30−32] indicated that the pyrazolo-[5,1-*c*][1,2,4]benzotriazine nucleus was also obtained by C-azo coupling of aromatic diazonium salt. In particular, diazotized aminopyrazole was reported to react with resorcinol to yield a diazo derivative that in turn could cycle, with elimination of water, to a pyrazolobenzotriazine system.[31,32] With this in mind, the 3-trifluoromethoxyphenol was reacted with the 4-ethoxycarbonylpyrazole-5-diazonium salt to obtain the desired intermediate 5-(2-hydroxy-4-trifluoromethoxyphenyl)azopyrazole-4-carboxylate. Because the obtained compound did not cycle under various condensation conditions (acetic acid, ethylenglycol), we investigated whether this lack of reactivity was due to a structural feature and thus performed RX analysis. From this study emerges unequivocally that the obtained compound was not the desired one because the C-azo coupling gave the regioisomer (ethyl 5-(2-trifluoromethoxy-4-hydroxyphenyl)azopyrazole-4-carboxylate) **33** that cannot eliminate water between the OH group and the NH-pyrazole because they are not adjacent. (see Scheme 5 and Figure 2).

Because the synthesis of 8-trifluoromethoxy compounds was problematic, we attempted to obtain the 8-difluoromethoxy derivatives. The introduction of the difluoromethoxy functionality was made by exploiting the 8-hydroxy group on the pyrazolobenzotriazine system. The reaction with a new difluorocarbene reagent, the 2-chloro-2,2-difluoroacetophenone[33] (Scheme 6), was made on the 5-deoxide derivatives, the new 3-iodo-, 3-ethoxycarbonyl-,[32] and 3-(2-thienylmethoxycarbonyl)-8-hydroxypyrazolo-[5,1-*c*][1,2,4]benzotriazine, **34**, **35**, and **37**, respectively. The reactions were complete, and the final compounds **38**−**40** were obtained.

Compound **37** was obtained by esterification of **36** in mild conditions (using trichloroacetonitrile and triphenylphosphine

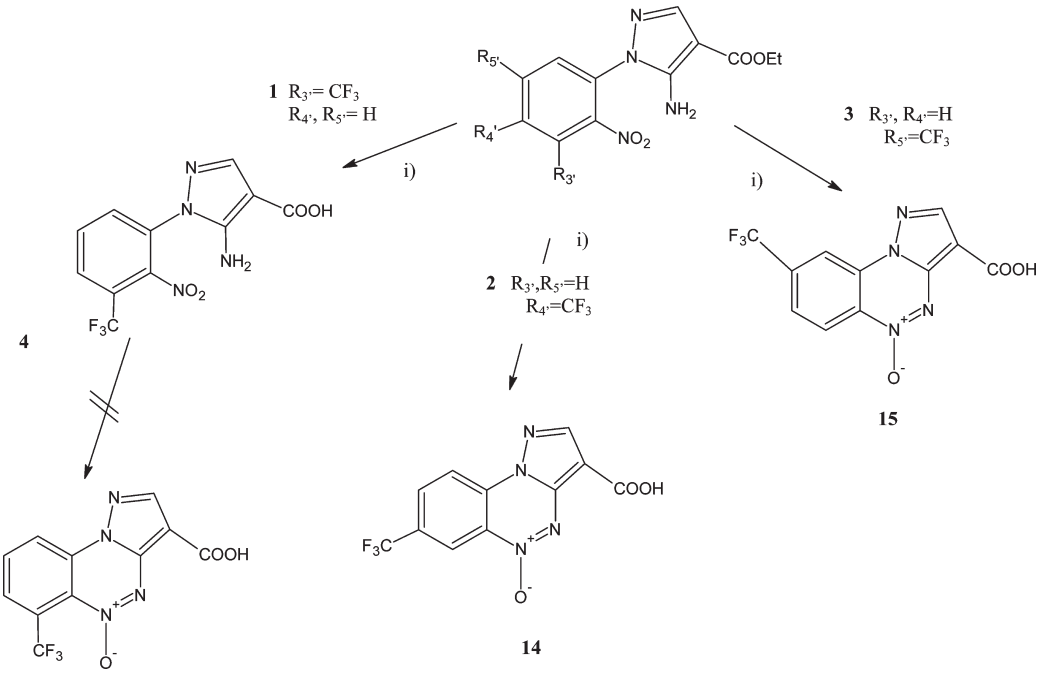
Scheme 1[a]



Compds	R
8	CH≡C–CH_2
9	2-OCH_3Ph–CH_2
10	2-OCF_3Ph–CH_2
11	4-Py–CH_2
12	2-Thienyl–CH_2
13	2-Furyl–CH_2

[a] (i) F-TEDA-BF_4, MeOH, room temperature, 25 h; (ii) ROH/NaOH 40% solution/$NBu_4^+Br^-/CH_2Cl_2$.

Scheme 2[a]



[a] (i) (a) NaOH 10% solution; (b) HCl conc.

to form the corresponding acid chloride[34] with the addition of the 2-thiophenmethanol). In turn, **36** was obtained by alkaline hydrolysis of the ethyl 8-hydroxypyrazolo[5,1-*c*][1,2,4]benzotriazine 3-carboxylate **35**.[32]

Only compounds **38** and **39** were oxidized with acetic anhydride/hydrogen peroxide to achieve the corresponding 5-oxide derivatives **41** and **42**. The attempt to oxidize compound **40** failed because a mixture of byproduct was recovered.

Results and Discussion

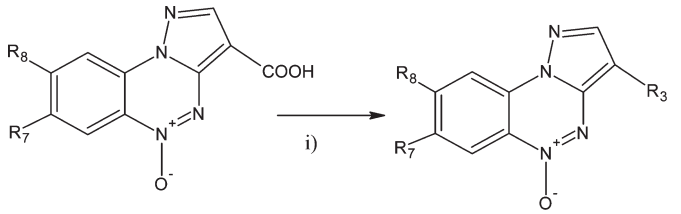
In Vitro and in Vivo Studies. The Bz site/$GABA_A$-R binding affinity of newly synthesized compounds was evaluated by their ability to displace [^{3}H]flumazenil (Ro15-1788) from its specific binding in bovine brain membrane and was expressed as K_i value only for those compounds inhibiting radioligand binding by more than 80% at fixed concentrations of 10 μM. The binding data (Table 3, compounds **7**−**13**, **18**−**32**, **38**−**42**) show that all compounds were able to bind to Bz site/$GABA_A$-R with good affinity, with only a few exceptions.

Most of the 3-fluoroderivatives (**7**−**13**) displayed affinity in the range of $3.19 \leq K_i \leq 380.8$ nM. The comparison between compounds **9** and **10** with ortho-substituents in the aromatic ring (OCH_3 in **9** and OCF_3 in **10**) is interesting. While compound **9** has the best affinity (K_i = 3.19 nM), compound **10** completely lost receptor recognition (I% = 27), thus we hypothesize a probable steric hindrance of the 8-substituent. On the other hand, by comparing these biological results with the corresponding data of 3-iodine analogues (K_i range 0.42−6.6 nM),[10] it is possible to assert that in the 3-fluorine series, the 8-substituent plays a critical role in the binding affinity.

Among the 8-trifluoromethyl derivatives (compounds **19**, **21**, **23**−**25**, **27**−**32**, range $5.8 \leq K_i$ (nM) ≤ 316), compounds **21** and **23** (the 3-(2-methoxybenzyloxycarbonyl)- and the 3-(2-thienylmethoxycarbonyl)-, respectively) had the best affinity (K_i = 5.8 and 10 nM, respectively). The fact that these compounds have a 2.5- and 10-fold lower binding affinity than the corresponding 8-methyl derivatives[12] (K_i = 2.3 nM and 1.41 nM, respectively) could be due to the larger volume of the trifluoromethyl than the methyl group.[15]

Compounds **24** and **25**, bearing an acyl group (2-methoxybenzoyl-, thien-2-carbonyl-) at position 3, have low affinity

Scheme 3[a]

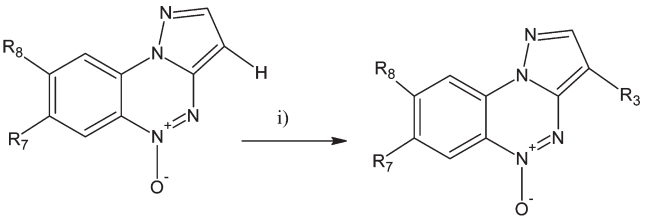


14, 15

Compds	R_3	R_7	R_8
16	H	CF_3	H
17	H	H	CF_3
18	$COOCH_2CH_3$	CF_3	H
19	$COOCH_2CH_3$	H	CF_3
20	$COOCH_2$-2-OCH_3Ph	CF_3	H
21	$COOCH_2$-2-OCH_3Ph	H	CF_3
22	$COOCH_2$-2-thienyl	CF_3	H
23	$COOCH_2$-2-thienyl	H	CF_3
24	CO-2-OCH_3Ph	H	CF_3
25	CO-2-thienyl	H	CF_3

[a] (i) HCl conc for compounds **16**, **17**; $SOCl_2$/EtOH for compounds **18**, **19**; NEt_3/ClCOOEt/THF, ROH for compounds **20**−**23**; toluene/tetrakis/Cs_2CO_3(2M), 2-methoxyphenylboronic acid/EtOH for compound **24**; $SOCl_2$, CH_2Cl_2/$SnCl_4$/thiophene for compound **25**.

Scheme 4[a]



Compds	R_3	R_7	R_8
16	H	CF_3	H
17	H	H	CF_3

Compds	R_3	R_7	R_8
26	Br	CF_3	H
27	Br	H	CF_3
28	Cl	H	CF_3
29	I	H	CF_3

ii) →

Compds.	R_3	R_7	R_8
30	2-thienyl	H	CF_3
31	3-thienyl	H	CF_3
32	3-furyl	H	CF_3

[a] (i) NCS/$CHCl_3$ or Br_2/$CHCl_3$ or ICl/$CHCl_3$; (ii) toluene/tetrakis/Na_2CO_3 (2M); 2-thienyl-, 3-thienyl-, 3-furylboronic acid/EtOH.

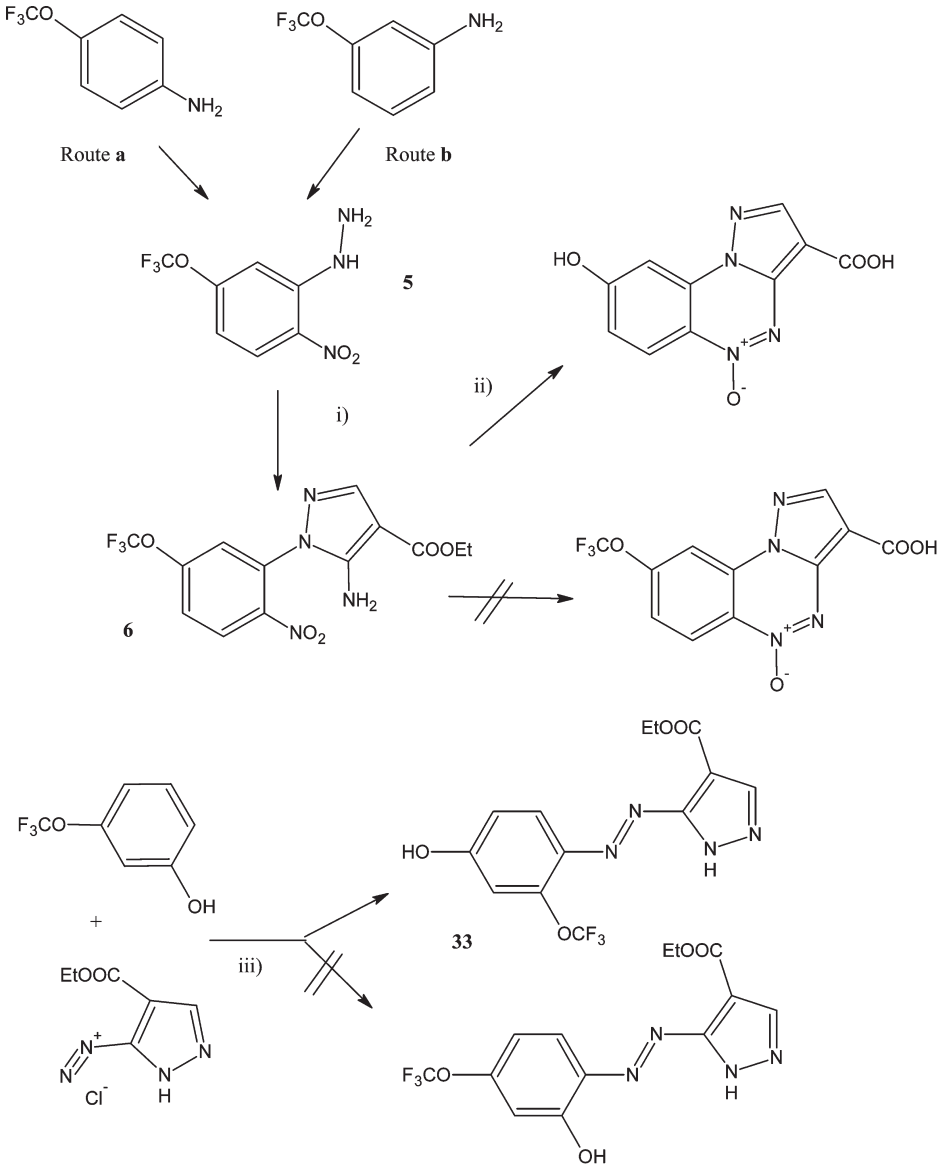
(K_i = 1543 and 164 nM) according to previous reports.[35] In the case of the 3-halogen series (**27**, 3-Cl; **28**, 3-Br; **29**, 3-I), the affinity increased up to 10-fold from chlorine to iodine: **27**, K_i = 175 nM, **28**, K_i = 139 nM, **29** K_i = 18 nM. Thus, the best substituent was the more lipophilic and the larger halogen atom, allowing us to hypothesize that these features were necessary for binding. The binding affinity for the 3-heteroaryl series (**30**, 2-thienyl; **31**, 3-thienyl; **32**, 3-furyl) was in the nanomolar range (**30**, K_i = 57 nM, **31**, K_i = 192 nM, and **32**, K_i = 112 nM).

The lack of binding affinity of the 7-trifluoromethyl derivatives (**18**, **20**, **22**, **26**; *I*% range 3−38%) shows that position 7 must not be occupied.

The 8-difluoromethoxy derivatives, **38**−**42**, were synthesized to evaluate if the oxygen atom at position 8 influences the receptor interaction. The affinity data indicate that the 8-difluoromethoxy substitution leads to higher affinity (K_i range 1.04−51.68 nM) than the 8-alkyloxy derivatives bearing the same substituents at position 3.[11,12] The presence of the *N*-oxide group slightly improves the binding affinity as seen by comparing compounds **41** vs **38** (K_i = 2.47 nM vs 5.01 nM) and **42** vs **39** (K_i = 5.75 nM vs 51.68 nM).

The difference in affinity between compounds **33** and **34** (3-ethoxycarbonyl- and the 3-(2-thienylmethoxycarbonyl)-derivative, K_i = 51.68 and 1.04 nM, respectively) is probably due to the better ability of the 3-(2-thienylmethoxycarbonyl)

Scheme 5[a]



5-(2-hydroxy-4-trifluoromethoxyphenyl)azopyrazole-4-carboxylate

[a] Route a: (1) NCS/CH_3CN;[26] (2) HCl/$NaNO_2$, $NaNO_2$/Cu_2O;[27,28] (3) N_2H_4 hydrate/ethanol. Route b: (1) acetic anhydride, HNO_3 conc, NaOH 10% solution; (2) AcOH/HCl/$NaNO_2$; (3) $SnCl_2$/HCl.[29] (i) 3-Ethoxy-2-cyanopropenate/EtOH; (ii) NaOH 10%; (iii) EtOH/sodium acetate.

group to engage in a strong lipophilic interaction, e.g. $\pi-\pi$ stacking, with the receptor protein as previously evidenced.[36]

Compounds **21**, **23**, and **41** were chosen for in vivo tests in mice. Six potential benzodiazepine actions were considered. Murine motor coordination and muscle relaxant effects were screened with the rota-rod test and the grip strength meter test; the anticonvulsant action was evaluated using the new drugs against pentylenetetrazole-induced convulsions (Table 4). The drugs were also tested for their ethanol-potentiating action (Table 5). Potential anxiolytic-like effects were screened using the light−dark choice test (Figure 3). Mouse learning and memory modulation were evaluated by the passive avoidance test (Figure 4); diazepam was used as the positive reference molecule (see Tables 4 and 5 and Figures 3 and 4).

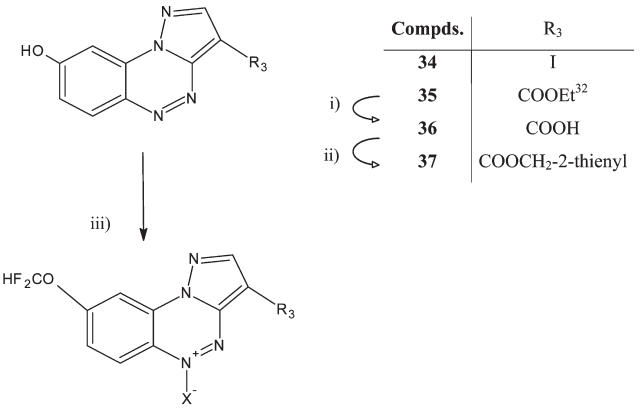
The behavioral effects of the 8-trifluoromethyl derivatives **21** and **23** were tested in comparison with diazepam with the aim of evaluating whether the in vivo inactivity of the corresponding 8-methyl derivatives[12] was due to the metabolic transformation of the methyl group. Of the 8-difluoromethoxyderivatives, compound **41** was chosen for in vivo evaluation.

According to the mouse rota-rod test and the grip strength meter test, neither **21** nor **23** induced any pharmacological effect on motor coordination or myorelaxation (Table 4), whereas the reference compound (diazepam 3 mg/kg ip) increased the number of falls from the rotating rod and induced muscle relaxant effect in a statistically significant way.

Protection from convulsions was evaluated in mice using pentylentetrazole (PTZ) as a chemical convulsant agent. Compounds **21**, **23** (10 and 30 mg/kg po) and **41** (10 mg/kg po) were devoid of any effect on PTZ-shock, whereas diazepam (1 mg/kg ip) completely protected against PTZ-induced shocks and convulsions (Table 4).

The effects of the newly synthesized molecules, **21**, **23**, and **41**, in comparison with diazepam on mouse anxiety, were

Scheme 6[a]



Compds.	R_3
34	I
35	$COOEt$[32]
36	COOH
37	$COOCH_2$-2-thienyl

Compds.	X	R_3
38	-	I
39	-	COOEt
40	-	$COOCH_2$2-thienyl
41[b]	O	I
42[b]	O	COOEt

[b] Acetic acid/acetic anhydride/H_2O_2 on **38** and **39**. [ab] (i) NaOH 40% solution refluxing conditions, HCl; (ii) $CCl_3CN/CH_2Cl_2/Ph_3P$, thiophenmethanol; (iii) 2-chloro-2,2-difluoroacetophenone, water/CH_3CN/K_2CO_3.

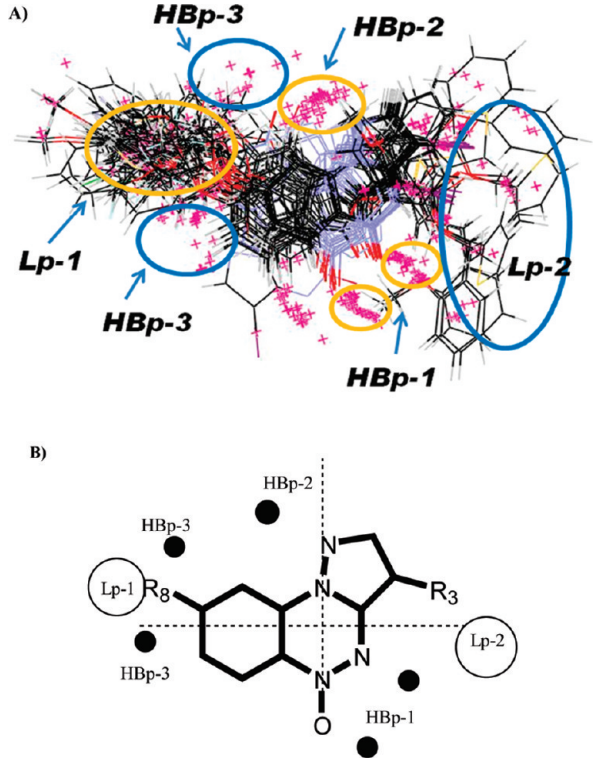


Figure 1. (A) Overlapping of ligand conformations considered in this study (82 compounds): in the figure are reported the essential interaction points for binding recognition (yellow circles) and the important areas for affinity modulation (blue circles). (B) One of the likely orientations that our system can adopt. It is evidenced that the lipophilic points Lp-1 and Lp-2 and hydrogen bond interaction points HBp-1 and HBp-2 coincide, respectively, with the L_{Di}, L-1/L-2, H1, and H2 sites of the Cook model.[8] Instead, the HBp-3 (corresponding to the acceptor hydrogen bond site A2 in Cook's model) probably is an acceptor/donor hydrogen bond area which is not essential for receptor recognition but modulates the affinity ligands.

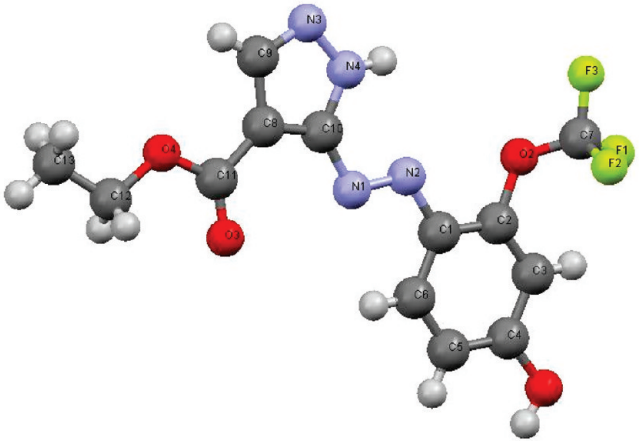
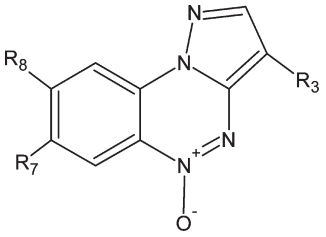


Figure 2. X-ray structure of compound **33**.

Table 3. BzR Ligand Affinity of New Compounds



N°	R_3	R_7	R_8	I% or K_i (nM)[a]
7	F		Cl	50% ± 0.18
8	F		$OCH_2C{\equiv}CH$	268.3 ± 8.32
9	F		OCH_2-2-OCH_3Ph	3.19 ± 0.79
10	F		OCH_2-2-OCF_3Ph	27% ± 2.30
11	F		OCH_2-4-Py	380.8 ± 35.0
12	F		OCH_2-2-thienyl	93.3 ± 0.10
13	F		OCH_2-2-furyl	129.34 ± 9.76
18	$COOCH_2CH_3$	CF_3		3% ± 0.31
19	$COOCH_2CH_3$		CF_3	316 ± 25.60
20	$COOCH_2$-2-OCH_3-Ph	CF_3		38% ± 3.50
21	$COOCH_2$-2-OCH_3-Ph		CF_3	5.8 ± 0.60
22	$COOCH_2$-2-thienyl	CF_3		34% ± 3.40
23	$COOCH_2$-2-thienyl		CF_3	10 ± 1.00
24	CO-2-OCH_3-Ph		CF_3	1543 ± 120
25	CO-2-thienyl		CF_3	164 ± 16.0
26	Br	CF_3		38% ± 3.50
27	Cl		CF_3	175 ± 15.0
28	Br		CF_3	139 ± 14.2
29	I		CF_3	18 ± 2.0
30	2-thienyl		CF_3	57 ± 3.70
31	3-thienyl		CF_3	192 ± 20
32	3-furyl		CF_3	112 ± 10
38[b]	I		OCF_2H	5.01 ± 0.20
39[b]	$COOCH_2CH_3$		OCF_2H	51.68 ± 5.89
40[b]	$COOCH_2$-2-thienyl		OCF_2H	1.04 ± 0.03
41	I		OCF_2H	2.47 ± 0.20
42	$COOCH_2CH_3$		OCF_2H	5.75 ± 0.40
Diaz[c]				10 ± 1.1

[a] Percent of inhibition of specific [^{3}H]Ro15-1788 binding at 10 μM concentration; K_i are means ± SEM of five determination. [b] 5-Deoxide derivative. [c] See ref 12.

studied using a light−dark box apparatus. Compound **21** (10 mg/kg po), had good anxiolytic activity, comparable to diazepam, while **23** (3, 10, and 30 mg/kg po) and **41** (10 mg/kg po) exhibited a slight anxiogenic effect (Figure 3). The anxiolytic-like effects of **21** (10 mg/kg po) and the anxiogenic

Table 4. Effects of the New Compounds in Comparison with Diazepam on Motor Coordination, Muscle Relaxant Effect, and Convulsion

		motor coordination rota-rod test (16 rpm)		myorelaxant activity grip-strength meter		anticonvulsant activity	
treatment[a]	mg/kg po	*n*	N° of falls in 30 min	*n*	test 35 min (g)	N° of mice (number of dead mice)	against PTZ-induced attacks (%)
CMC 1%[b]	0.1 mL	28	0.3 ± 0.1	43	58.3 ± 1.0	28(9)	7.1
diazepam	0.3 (ip)					14	71.4**
	1	9	0.3 ± 0.2	7	55.8 ± 3.7	9	100**
	3	11	0.9 ± 0.3*	5	0.5 ± 0.5**		
21	10	10	0.2 ± 0.1	10	51.7 ± 1.2	10(3)	0
	30	10	0.2 ± 0.1	10	48.5 ± 1.7	10(3)	0
23	10	10	0.1 ± 0.1	9	52.6 ± 1.9	10(4)	0
	30	10	0.2 ± 0.1	10	57.9 ± 1.6	8(1)	0
41	10		nt		nt	10	0

[a] New compounds were administered 30 min and diazepam (ip) 20 min before the test. *$P < 0.01$, **$P < 0.001$ versus control mice. [b] Carboxymethylcellulose 1%. Each value represents the mean ± SEM of treated-mice.

Table 5. Effect on Ethanol-Induced Sleeping Time

treatment[a]	mg/kg po	*n*	sleep time (s)
CMC 1%[b]	0.1 mL	18	59.2 ± 8.7
diazepam	0.3 (ip)	16	134.5 ± 7.9*
	1	10	140.5 ± 4.0*
21	3	8	36.2 ± 11.6
	10	8	54.3 ± 21.0
23	3	9	48.6 ± 7.0
	10	9	40.8 ± 11.7

[a] New compounds were administered 30 min and diazepam (ip) 20 min before the test. *$P < 0.001$ versus control mice. [b] Carboxymethylcellulose 1%. Each value represents the mean ± SEM of treated-mice.

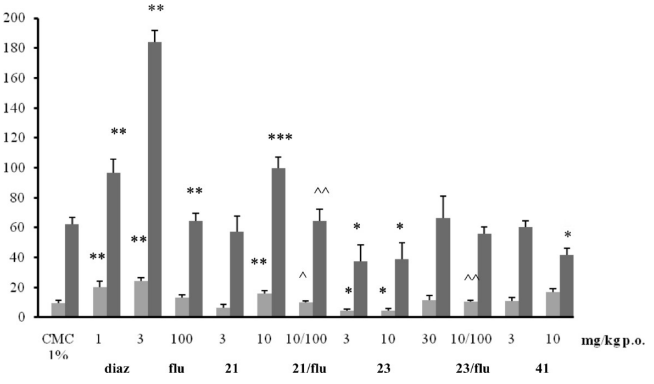


Figure 3. Effect of compounds **21**, **23**, and **41** in comparison with diazepam (diaz, ip) on mouse light−dark box test. The first column represents the number of transfers in 5 min, and the second column represents the time spent in light. Compounds and diazepam were administered 30 min and flumazenil (flu, ip) 40 min before the test. Each column represents the means ± SEM of 9−10 mice. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus control. ^$P < 0.05$, ^^$P < 0.01$ versus **21**-treated and **23**-treated mice.

effect of **23** (10 mg/kg po) were completely antagonized by flumazenil (100 mg/kg ip, dose at which flumazenil was able to antagonize the anxiolytic effect of diazepam).

To evaluate the effect of the newly synthesized compounds on learning and memory processes, mouse performance of passive avoidance test was investigated. In this assay, the parameters taken into consideration are training and retention latencies expressed in seconds. While the training latencies did not differ among the various groups, some retention latencies were significantly different from others. As can be observed in Figure 4, only compound **23** (3 and 10 mg/kg po) improved, in a statistically significant manner, mouse memory processes, whereas compounds **21** (10 mg/kg po) and **41** (3, 10, 30 mg/kg po) did not influence mouse performance. In the same experimental test, flumazenil (100 mg/kg po) showed a statistically significant increase in retention latency (Figure 4).

In the test of ethanol-induced sleeping time (Table 5), compounds **21** (3, 10 mg/kg po) and **23** (3, 10 mg/kg po) did not modify the duration of loss of righting reflex. As expected, the reference drug diazepam (diaz, ip) enhanced sedation in a statistically significant manner.

Pharmacophoric Model. Our previously reported pharmacophoric model[10] that illustrated the essential 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 a pyrazolobenzotriazine system (Figure 1) is used here to rationalize the affinity data. The compound orientations (one or more), reported in Figures 5−9, are the most likely and emerged from the method reported in the Experimental Protocols and Supporting Information.

The 3-fluoro derivatives (**7**−**13**) present three different orientations in our pharmacophoric model. For clarity, Parts A, B, and C of Figure 5 illustrate the three orientations of compound **9**, of which 5C is the most probable. In the first two orientations (Figure 5A,B), the HBp-1 or HBp-3 points engage respectively a hydrogen bond with a fluorine atom at position 3, confirming its ability to form this interaction.[18,37,38] The lipophilic areas, Lp-2 in orientation 5A and Lp-1/Lp-2 in orientation 5B (essential interaction points for binding recognition/modulation) are not involved.

Instead, in the third orientation (Figure 5C), all essential pharmacophoric points, HBp-1, HBp-2, and Lp-1, interact efficiently through the *N*-oxide group, the *N*1-pyrazole, and the 8-substituent, respectively. The 3-fluorine atom is located in the Lp-2 area, but because it is unable to form efficient lipophilic interactions ($F, \pi = 0.14$), the driving force for the binding affinity could be due to physical and chemical features of the 8-substituent that occupies the Lp-1 area. In fact, by comparing compounds **9** and **10** (**9**, $R_8 = OCH_2$-2-OCH_3Ph, and **10**, $R_8 = OCH_2$-2-OCF_3Ph), we can explain the different binding affinity in terms of steric hindrance (**9** $K_i = 3.19$ nM vs **10** $I\% = 27$). The *ortho*-OCF_3-phenyl ring interacts with a steric repulsive area near Lp-1 (Figure 5C,D).

Compounds having the CF_3 group in position 8 (**19**, **21**, **23**−**25**, **27**−**32**) show all pharmacophoric groups correctly set to form efficient hydrogen bonds and lipophilic interactions, thus explaining the good/fair affinity of the 8-CF_3 derivatives compared to the 7-CF_3 (**18**, **20**, **22**, and **26**). The

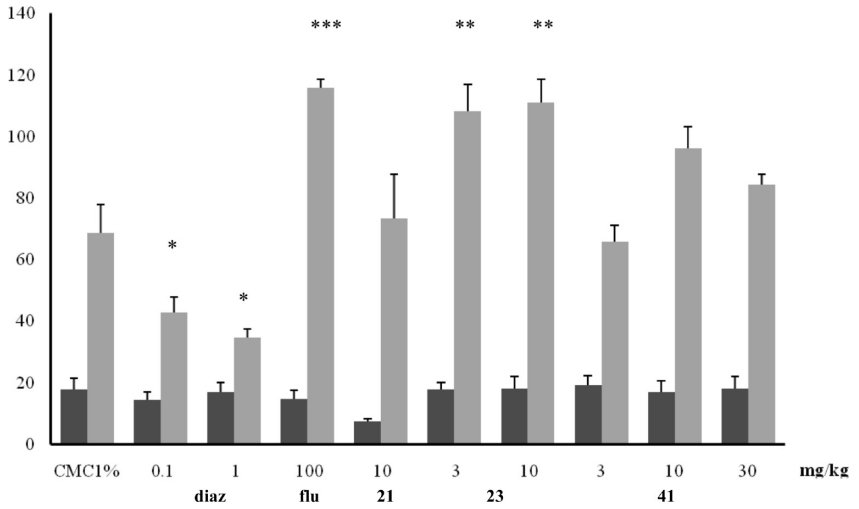


Figure 4. Effects of compounds **21**, **23**, and **41** in comparison with diazepam (diaz, ip) and flumazenil (flu, ip) on mouse passive avoidance test. The first column represents the training test, and the second column represents the retention latency. Mice were treated 30 min before the training test. The retention test was performed 24 h later. Each column represents the mean ± SEM of 9−11 mice. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus control.

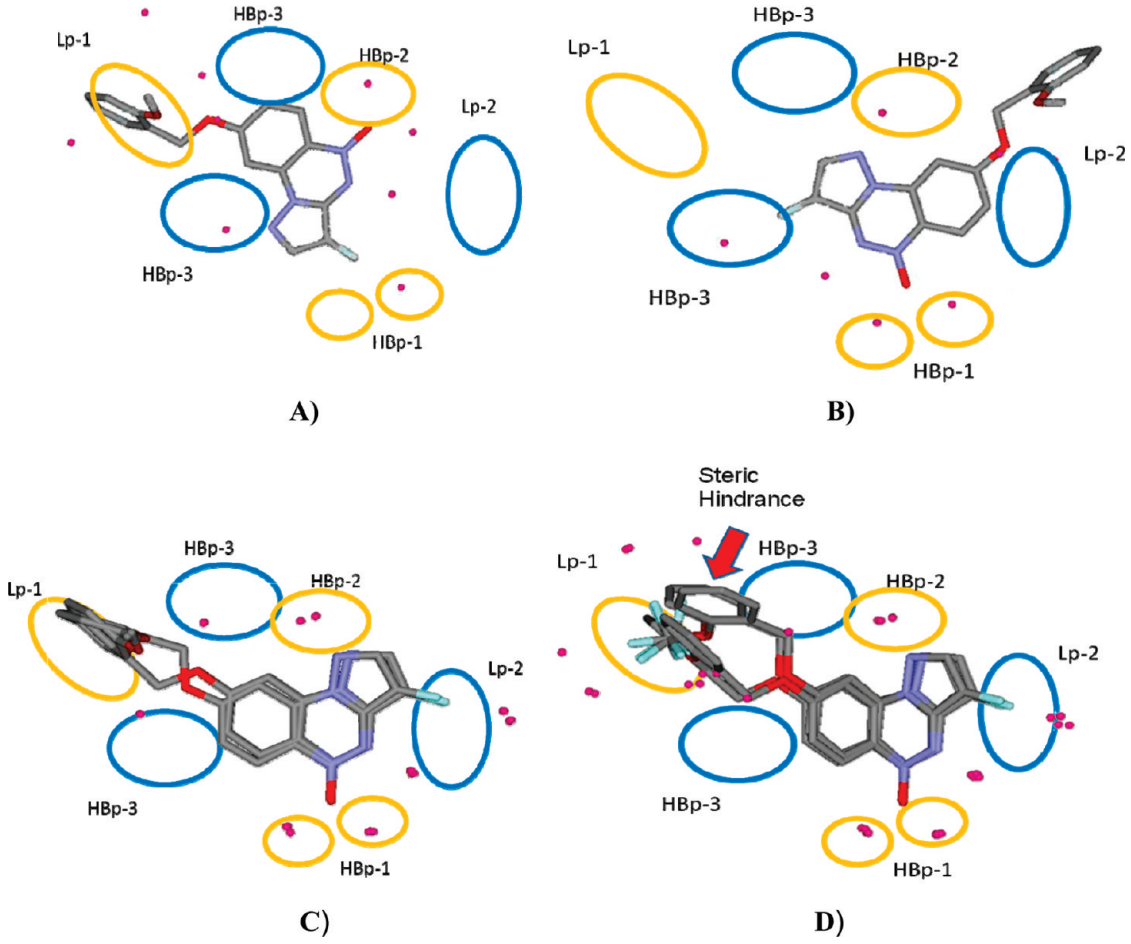


Figure 5. (A−C) Three orientations of compound **9**. Orientation depicted in **5C** is the most probable. (D) Compound **10** has a steric hindrance evidenced with red arrow. The essential interaction points are represented by yellow circles and the areas for affinity modulation with blue circles.

different affinity of **20** and **21** ($I\%$ = 38 and K_i = 5.8 nM) can be explained because the CF_3 group of **20** interacts with a steric hindrance near previously individualized Lp-1 (Figure 6A,B).[10]

The position of a trifluoromethyl group at the pyrazolobenzotriazine core is important even when a halogen atom (**26**−**29**) is present in position 3. Parts A and B of Figure 7 illustrate compounds **26** and **28** that fit into our pharmaco-

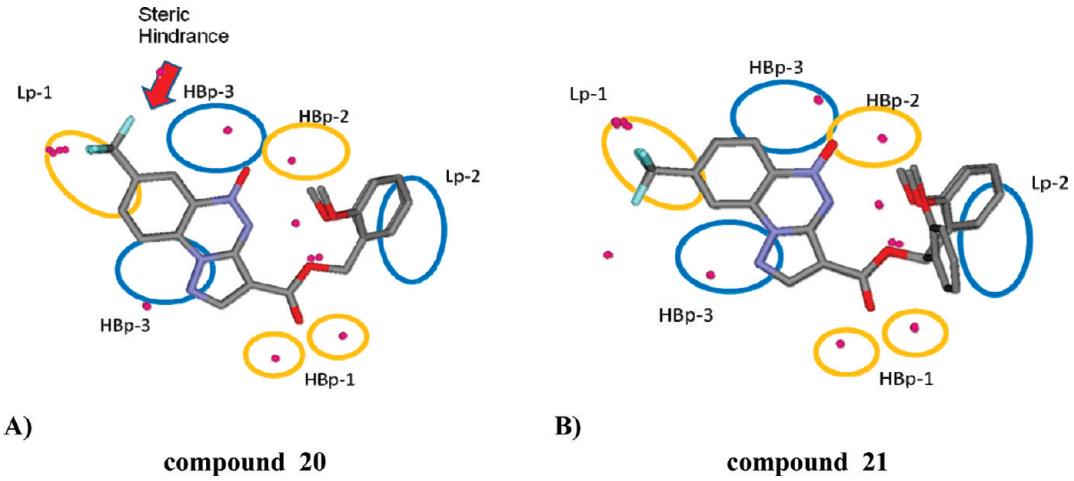


Figure 6. (A) The inactivity of compound **20** is due to the steric hindrance evidenced with the red arrow due to CF_3 group in position 7. (B) Compound **21** fits in the pharmacophoric model, with the 8-CF_3 group in the suitable position. The essential interaction points are represented by yellow circles and the areas for affinity modulation with blue circles.

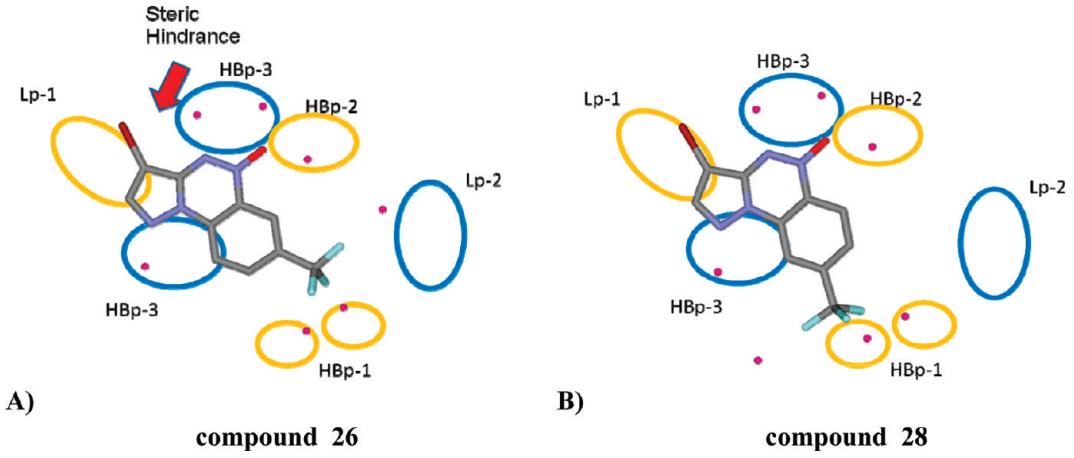


Figure 7. (A) The inactivity of compound **26** is due to the steric hindrance evidenced with the red arrow. (B) Compound **28** fits in the pharmacophoric model, with the 8-CF_3 group in the suitable position to engage the hydrogen bond with HBp-1 area. The essential interaction points are represented by yellow circles and the areas for affinity modulation with blue circles.

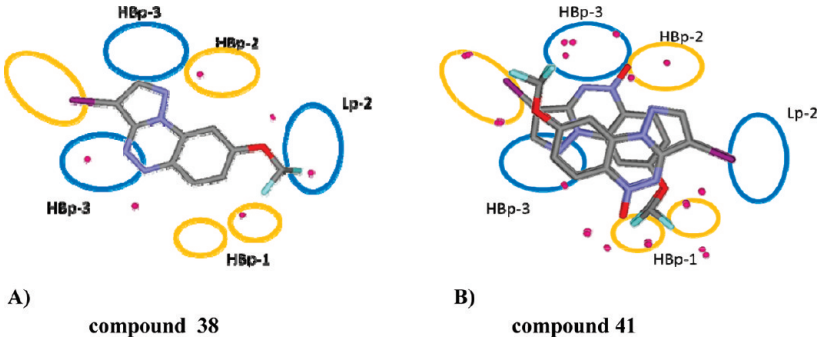


Figure 8. Compound **38** and **41** in the pharmacophoric model. (A) The *N*-deoxide derivative **38** interacts with all hydrogen bond areas HBp involving also the fluorine atom of difluoromethoxy group. The corresponding *N*-oxide derivative **41** (B) fits in the pharmacophoric model in two possible orientations. The essential interaction points are represented by yellow circles and the areas for affinity modulation with blue circles.

phore model. They present only one possible orientation in which the fluorine atom(s) of the trifluoromethyl group interact with the hydrogen bond point HBp-1 and the 3-halogen atom fits into the lipophilic area Lp-1. When the trifluoromethyl is in position 8, a right alignment for the hydrogen bond occurs, permitting the halogen atom to fit into the lipophilic area more efficiently. In the case of the 7-trifluoromethyl derivative, if an efficient hydrogen bond interaction between fluorine and HBp-1 occurs, the 3-halogen atom interacts with the probable steric repulsive area near Lp-1 and the receptor recognition fails.

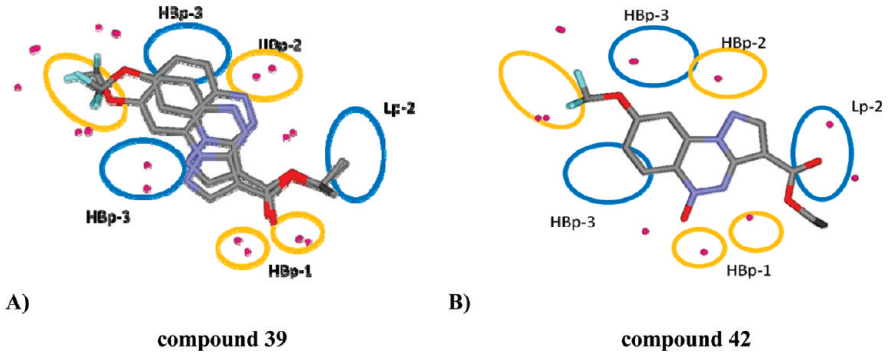


Figure 9. (A) The 5-*N*-deoxide derivative **39**, and in (B) compound **42**, the 5-*N*-oxide derivative in the pharmacophoric model. The essential interaction points are represented by yellow circles and the areas for affinity modulation with blue circles.

Concerning the 8-difluoromethoxy derivatives, molecular modeling suggests that the fluorine atoms (of 8-OCF_2H group) contribute significantly to receptor interaction for compounds **38** and **41** (3-iodo derivatives), while the oxygen atom of the same 8-substituent plays an important role in the binding for compounds **39** and **42** (3-ethoxycarbonyl derivatives). Compound **38** presents one possible orientation in which the fluorine atom(s) of the difluoromethoxy group cause hydrogen bond interaction with the HBp-1 area. Compound **41** presents, instead, two possible orientations in which the fluorine atom(s) of the difluoromethoxy group alternatively interact with the HBp-1 or HBp-3 and the iodine atom at position 3 fits with Lp-1 or Lp-2 (Figure 8A,B). The same hydrogen bond interaction is evidenced, also, for the oxygen atom of the 8-OCF_2H group in compounds **39** and **42**, in the assumed orientations (Figure 9A,B). The difference in the affinity shown by **39** and **42** (**39**, 5-*N*-deoxide, K_i = 51.68 nM, and **42**, 5-*N*-oxide K_i = 5.75 nM) may be due to the better alignment of **42** in the pharmacophoric map (Figure 9A,B). Thus, it emerges that the substituent at position 3 determines the interaction of the fluorine or oxygen atom of the $-OCF_2H$ group, within the HBp areas.

Conclusion

We have evaluated the binding properties of the fluorine atom(s) in the Bz site on $GABA_A$ receptors and their ability to render compounds more metabolically stable. These findings indicate that the fluorine atom is very important for recognition, engaging hydrogen bond interaction, when it is in position 8 inserted in the $-CF_3$ or $-OCF_2H$ groups. When the fluorine atom is in position 3, it does not cause any interaction and the driving force for recognition is the substituent in position 8.

The in vivo results on compounds **21**, the 3-(2-methoxybenzyloxycarbonyl)- and **23**, the 3-(2-thienylmethoxycarbonyl)-8-trifluoromethylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide, confirm the potential metabolic stability due to the CF_3 group versus the CH_3 group. In fact, these ligands, although having about 2−10-fold less affinity than corresponding 8-methyl derivatives,[12] present interesting pharmacological activity. Compound **21** has a selective anxiolytic-like profile (10 mg/kg) without impairing memory or modifying the duration of righting reflex loss. Compound **23** has an inverse-agonist profile because it shows anxiogenic and pro-mnemonic activity (3 and 10 mg/kg). These activities are mediated by acting on the benzodiazepine site on $GABA_A$ receptors because they are completely antagonized by flumazenil.

We have updated this pharmacophoric model, previously validated[10] by inserting two ligands (CGS 9698 and diazepam, already used by Cook[9]), and further confirmed the essential interaction points for binding recognition.

Experimental Protocols

Chemistry. Melting points were determined with a Gallenkamp apparatus and were uncorrected. Silica gel plates (Merk F_{254}) and Silica Gel 60 (Merk 70−230 mesh) were used for analytical and column chromatography, respectively. The structures of all compounds were supported by their IR spectra (KBr pellets in nujol mulls, Perkin-Elmer 1420 spectrophotometer) and ^{1}H NMR data (measured with a Bruker 400 MHz). Chemical shifts were expressed in δ ppm, using DMSO-d_6 or $CDCl_3$ as solvent. The coupling constant values ($J_{H6-H7,\ H7-H6}$; $J_{H7-H9,\ H9-H7}$) were in agreement with the assigned structure. The chemical and physical data of new compounds are shown in Tables 1 and 2. All new compounds possess a purity ≥95%;microanalyses were performed with a Perkin-Elmer 260 analyzer for C, H, N. Mass spectra (*m*/*z*) were recorded on a LTQ mass spectrometer (ThermoFisher, San Jose, CA, USA). The crystal structure of compound **33** was solved by means of single-crystal X-ray diffraction.

2-Nitro-5-trifluoromethoxyphenylhydrazine (5). A solution in THF (20 mL) of 2-chloro-4-trifluoromethoxynitrobenzene (270 mg, 1.12 mmol), synthesized by diazotation of the 2-chloro-4-trifluoromethoxyaniline[26] and subsequent decomposition of the obtained diazonium salt with sodium nitrite and cuprous oxide,[27] was added of hydrazine hydrate (1 mL) and maintained at refluxing temperature. The reaction was monitored by TLC (toluene/ethyl acetate/acetic acid 8:2:1 v/v/v), and when the starting material disappeared, the red solution was evaporated to dryness. The residue was recovered by ethanol 80% and recrystallized by the same solvent. Red crystals. ^{1}H NMR ($CDCl_3$) δ 9.00 (bs, 1H, NH exch), 8.15 (d, 1H, H-3), 7.52 (s, 1H, H-6), 6.51 (d, 1H, H-4), 3.85 (bs, 2H, NH_2 exch). Anal. C, H, N.

General Procedure for the Synthesis of 5-Aminopyrazoles 1−3, 6. A suspension of the suitable 3-, 4-, 5-trifluoromethyl-2-nitrophenylhydrazine[23−25] or 2-nitro-5-trifluoromethoxy phenylhydrazine, **5** (1 mmol), and 2-cyano-3-ethoxypropenate (1 mmol) in ethanol (50 mL) with HCl as acid catalyst, were refluxed until the starting material disappeared in TLC (toluene/ethyl acetate/acetic acid 8:2:1 v/v/v). The workup of the final solution gave the ethyl 1-(2-nitro-3-, 4-, 5-trifluoromethylphenyl)-5-aminopyrazol-4-carboxylate, **1−3**, and the ethyl 1-(2-nitro-5-trifluoromethoxyphenyl)-5-aminopyrazol-4-carboxylate **6**, that were recrystallized by suitable solvent.

Ethyl 1-(2-Nitro-3-trifluoromethylphenyl)-5-aminopyrazol-4-carboxylate (1). Yellow crystals. IR ν cm^{-1} 3300, 3200, 1687. ^{1}H NMR ($CDCl_3$) δ 7.90 (d, 1H, H-4 phenyl), 7.88 (s, 1H, H-3), 7.70 (d, 1H, H-6 phenyl), 7.58 (t, 1H, H-5 phenyl), 5.10 (bs, 2H, NH_2 exch), 4.35 (q, 2H, CH_2), 1.40 (t, 3H, CH_3). Anal. C, H, N.

Ethyl 1-(2-Nitro-4-trifluoromethylphenyl)-5-aminopyrazol-4-carboxylate (2). Yellow crystals. IR ν cm^{-1} 3400, 3300, 1687. ^{1}H NMR ($CDCl_3$) δ 8.25 (s, 1H, H-3 phenyl), 8.10 (d, 1H, H-5 phenyl), 7.80 (m, 2H, H-6 phenyl and H-3), 5.30 (bs, 2H, NH_2 exch), 4.35 (q, 2H, CH_2), 1.40 (t, 3H, CH_3). Anal. C, H, N.

Ethyl 1-(2-Nitro-4-trifluoromethylphenyl)-5-aminopyrazol-4-carboxylate (3). Yellow crystals. IR ν cm^{-1} 3380, 3300, 1687. ^{1}H NMR ($CDCl_3$) δ 8.15 (dd, 1H, H-3 phenyl), 7.95 (m, 2H, H-4 and H-6 phenyl), 7.82 (s, 1H, H-3), 5.30 (bs, 2H, NH_2 exch), 4.35 (q, 2H, CH_2), 1.40 (t, 3H, CH_3). Anal. C, H, N.

Ethyl 1-(2-Nitro-4-trifluoromethyoxyphenyl)-5-aminopyrazol-4-carboxylate (6). Yellow crystals. IR ν cm^{-1} 3380, 3300, 1687. ^{1}H NMR ($CDCl_3$) δ 8.13 (dd, 1H, H-3 phenyl), 7.82 (s, 1H, H-3), 7.49 (m, 2H, H-4 and H-6 phenyl), 5.25 (bs, 2H, NH_2 exch), 4.40 (q, 2H, CH_2), 1.40 (t, 3H, CH_3). Anal. C, H, N.

1-(2-Nitro-3-trifluoromethylphenyl)-5-aminopyrazol-4-carboxylic Acid (4). A suspension of compound **1** (0.2 mmol) in a 10% solution of sodium hydroxide (15 mL) was warmed at 60 °C for 3 days. The final suspension was made slightly acid, and the final compound was recovered. The recrystallization with ethanol gave the pure compounds. Yellow crystals. IR ν cm^{-1} 3380, 3300, 1687. ^{1}H NMR (DMSO-d_6) δ 12.00 (s, 1H, COOH), 8.05 (d, 1H, H-4), 7.85 (d, 1H, H-6 phenyl), 7.65 (t, 1H, H-5 phenyl), 7.68 (s, 1H, H-3), 6.38 (s, 2H, NH_2 exch). Anal. C, H, N.

3-Fluoro-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide (7). From 8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide[21] (100 mg, 0.45 mmol) and Selectfluor (F-TEDA-BF_4, 1-chloromethyl-4-fluoro-1,4-diazoniabicyclo[2.2.2]octanebis(tetra-fluoroborate) (2 equiv)) in methanol (6 mL) for 24 h at room temperature. The final suspension was evaporated and washed with water, and the raw precipitate was purified by column chromatography (eluent toluene/ethyl acetate/acetic acid 8:2:1 v/v/v). Yellow crystals. ^{1}H NMR ($CDCl_3$) δ 8.49 (d, 1H, H-6), 8.36 (d, 1H, H-9), 8.02 (d, 1H, H-2, J_{H-F} = 3.84 Hz), 7.60 (dd, 1H, H-7). MS(ESI) *m*/*z*: 239.09 ([M + H]$^+$). Anal. C, H, N.

General Procedure for the Synthesis of Compounds 8−13. A mixture of compound **7** (0.45 mmol), 10 mL of dichloromethane, 5 mL of 40% sodium hydroxide solution, 0.1 mol of tetrabutylammonium bromide, and suitable alcohol in large excess (5 mL) was vigorously stirred at 30−50 °C and monitored by TLC (eluent toluene/ethyl acetate/acetic acid 8:2:1 v/v/v). The organic layer was then separated and the aqueous layer extracted twice with 10 mL of dichloromethane. The combined organic extracts were evaporated, and the residue was recovered with 1−2 mL of ethanol and recrystallized by suitable solvent.

3-Fluoro-8-(2-propynyloxy)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide (8). From **7** and propargyl alcohol. Yellow crystals. ^{1}H NMR (DMSO-d_6) δ 8.43 (d, 1H, H-2, J_{H-F} = 3.84 Hz), 8.38 (d, 1H, H-6), 7.78 (d, 1H, H-9), 7.32 (dd, 1H, H-7), 5.17 (d, 2H, CH_2O), 3.78 (t, 1H, CH). Anal. C, H, N.

3-Fluoro-8-(2-methoxybenzyloxy)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide (9). From **7** and 2-methoxybenzyl alcohol. Yellow crystals. ^{1}H NMR ($CDCl_3$) δ 8.45 (d, 1H, H-6), 8.00 (d, 1H, H-2, J_{H-F} = 3.84 Hz), 7.85 (d, 1H, H-9), 7.49 (d, 1H, H-6′ phenyl), 7.38 (t, 1H, H-4′ phenyl), 7.23 (dd, 1H, H-7), 7.05 (t, 1H, H-5′ phenyl), 6.98 (d, 1H, H-3′ phenyl), 5.35 (s, 2H, CH_2O), 3.90 (s, 3H, OCH_3). Anal. C, H, N.

3-Fluoro-8-(2-trifluoromethoxybenzyloxy)pyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-Oxide (10). From **7** and 2-trifluoromethoxybenzyl alcohol. Yellow crystals. ^{1}H NMR (DMSO-d_6) δ 8.44 (d, 1H, H-2, J_{H-F} = 3.84 Hz), 8.38 (d, 1H, H-6), 7.81 (d, 1H, H-9), 7.76 (d, 1H, H-3′ phenyl), 7.58 (t, 1H, H-5′ phenyl), 7.48 (m, 1H, H-6′ and H-4′ phenyl), 7.36 (dd, 1H, H-7), 5.48 (s, 2H, CH_2O). Anal. C, H, N.

3-Fluoro-8-(pyridine-4-ylmethoxy)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide (11). From **7** and pyridine-4-methanol. Yellow crystals. ^{1}H NMR (DMSO-d_6) δ 8.65 (d, 2H, H-3′ and H-5′ Py), 8.44 (d, 1H, H-2, J_{H-F} = 3.84 Hz), 8.38 (d, 1H, H-6), 7.79 (d, 1H, H-9), 7.57 (d, 2H, H-2′ and H-6′ Py), 7.42 (dd, 1H, H-7), 5.52 (s, 2H, CH_2O). Anal. C, H, N.

3-Fluoro-8-(thien-2-ylmethoxy)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide (12). From **7** and thiophen-2-methanol. Yellow crystals. ^{1}H NMR (DMSO-d_6) δ 8.44 (d, 1H, H-2, J_{H-F} = 3.84 Hz), 8.36 (d, 1H, H-6), 7.81 (d, 1H, H-9), 7.62 (d, 1H, H-5′ thienyl), 7.35 (m, 2H, H-7 and H-3′ thienyl), 7.08 (t, 1H, H-4′ thienyl), 5.65 (s, 2H, CH_2O). Anal. C, H, N.

3-Fluoro-8-(fur-2-ylmethoxy)pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide (13). From **7** and furan-2-methanol (furfuryl alcohol). Yellow crystals. ^{1}H NMR (DMSO-d_6) δ 8.42 (d, 1H, H-2, J_{H-F} = 3.84 Hz), 8.36 (d, 1H, H-6), 7.82 (d, 1H, H-9), 7.75 (d, 1H, H-5′ furyl), 7.35 (dd, 1H, H-7), 6.70 (d, 1H, H-3′furyl), 6.51 (m, 1H, H-4′ furyl), 5.45 (s, 2H, CH_2O). Anal. C, H, N.

General Procedure for the Synthesis of Compounds 14 and 15. A suspension of 1.0 mmol of suitable 5-aminopyrazole **2**, **3** in 10% solution of sodium hydroxide (15 mL) was stirred at 30−40 °C. The reaction was monitored by TLC (eluent toluene/ethyl acetate/acetic acid 8:2:1 v/v/v), the final suspension was acidified with concentrated hydrochloric acid; crude acids, **14** and **15**, were recovered by filtration and recrystallized by ethanol.

3-Carboxy-7-trifluoromethylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide (14). From the 5-aminopyrazole **2**. Yellow crystals. IR ν cm^{-1} 2700−2600, 1680, 1550. ^{1}H NMR (DMSO-d_6) δ 13.00 (bs, 1H, COOH), 8.72 (d, 1H, H-6), 8.68 (s, 1H, H-2), 8.60 (d, 1H, H-9), 8.46 (dd, 1H, H-8). Anal. C, H, N.

3-Carboxy-8-trifluoromethylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide (15). From the 5-aminopyrazole **3**. Yellow crystals. IR ν cm^{-1} 2700−2600, 1680, 1550. ^{1}H NMR (DMSO-d_6) δ 13.00 (bs, 1H, COOH), 8.65 (m, 3H, H-2, H-6 and H-9), 8.12 (dd, 1H, H-7). Anal. C, H, N.

General Procedure for the Synthesis of Compounds 16−17. Compounds **14** or **15** (0.5 mmol) were suspended in concentrated hydrochloric acid (15 mL) at refluxing temperature. After 5 h,, the reaction was stopped, the suspension was extracted with chloroform, and the organic layer was dried with anhydrous sodium sulfate. Evaporation under vacuum gave yellow−orange residue recuperated by ethanol.

7-Trifluoromethylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide (16). From **14**; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v. IR ν cm^{-1} 1550. ^{1}H NMR ($CDCl_3$) δ 8.85 (d, 1H, H-6), 8.55 (d, 1H, H-9), 8.20 (m, 2H, H-2 and H-8), 6.80 (d, 1H, H-3). Anal. C, H, N.

8-Trifluoromethylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide (17). From **15**; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v. IR ν cm^{-1}1550. ^{1}H NMR ($CDCl_3$) δ 8.72 (m, 2H, H-6 and H-9), 8.18 (d, 1H, H-2), 7.88 (dd, 1H, H-7), 6.82 (d, 1H, H-3). Anal. C, H, N.

General Procedure for the Synthesis of Compounds 18−23. The starting acids **14** and **15** were transformed into ester derivatives (**18−23**) by means of two methods, A and B.

Method A. The ethyl esters **18** and **19** were obtained from corresponding acids **14** and **15** (0.5 mmol) by means of synthesis of the corresponding 3-carbonyl chloride, which was, in turn, suspended in anhydrous ethyl alcohol (15 mL). The precipitate was filtered and purified by recrystallization.

Method B. All other esters (**20−23**) were obtained by treating the corresponding acids (0.5 mmol) (**14**, **15**) in tetrahydrofurane (THF), with triethyl amine (1:3.5) in an ice bath for 30 min. The suspension was supplemented with ethylchlorocarbonate (1:2) and maintained from 0 °C to room temperature under stirring for 1 h to permit the anhydride to form. 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 that 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 using a suitable solvent.

3-Ethoxycarbonyl-7-trifluoromethylpyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-Oxide (18). From **14** and ethanol (method A). Yellow crystals. TLC eluent: toluene/ethyl acetate 8:2 v/v.

IR ν cm^{-1} 1720, 1550. ^{1}H NMR ($CDCl_3$) δ 8.85 (d, 1H, H-6), 8.60 (m, 2H, H-2 and H-9), 8.22 (dd, 1H, H-8), 4.45 (q, 2H, CH_2), 1.45 (t, 3H, CH_3). Anal. C, H, N.

3-Ethoxycarbonyl-8-trifluoromethylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide (19). From **15** and ethanol (method A). Yellow crystals. TLC eluent: toluene/ethyl acetate 8:2 v/v. IR ν cm^{-1} 1720, 1550. ^{1}H NMR ($CDCl_3$) δ 8.78 (d, 1H, H-9), 8.72 (d, 1H, H-6), 8.58 (s, 1H, H-2), 7.94 (dd, 1H, H-7), 4.45 (q, 2H, CH_2), 1.45 (t, 3H, CH_3). Anal. C, H, N.

3-(2-Methoxybenzyloxycarbonyl)-7-trifluoromethylpyrazolo-[5,1-*c*][1,2,4]benzotriazine 5-Oxide (20). From **14** and 2-methoxybenzyl alcohol (method B). Yellow crystals. TLC eluent: toluene/ethyl acetate 8:2 v/v. IR ν cm^{-1} 1720, 1550. ^{1}H NMR ($CDCl_3$) δ 8.85 (d, 1H, H-6), 8.60 (m, 2H, H-2 and H-9), 8.22 (dd, 1H, H-8), 7.56 (d, 1H, H-6′ phenyl), 7.34 (t, 1H, H-4′ phenyl), 7.02 (t, 1H, H-5′ phenyl), 6.92 (d, 1H, H-3′), 5.45 (s, 2H, CH_2), 3.85 (s, 3H, OCH_3). Anal. C, H, N.

3-(2-Methoxybenzyloxycarbonyl)-8-trifluoromethylpyrazolo-[5,1-*c*][1,2,4]benzotriazine 5-Oxide (21). From **15** and 2-methoxybenzyl alcohol (method B). Yellow crystals. TLC eluent: isopropyl ether/cyclohexane 8:3 v/v. IR ν cm^{-1} 1720, 1550. ^{1}H NMR ($CDCl_3$) δ 8.78 (d, 1H, H-9), 8.70 (d, 1H, H-6), 8.58 (s, 1H, H-2), 7.94 (dd, 1H, H-7), 7.58 (d, 1H, H-6′ phenyl), 7.35 (t, 1H, H-4′ phenyl), 7.02 (t, 1H, H-5′ phenyl), 6.95 (d, 1H, H-3′ phenyl), 5.50 (s, 2H, CH_2), 3.80 (s, 3H, OCH_3). Anal. C, H, N.

3-(2-Thienylmethoxycarbonyl)-7-trifluoromethylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide (22). From **14** and thiophen-2-methanol (method B). Yellow crystals. TLC eluent: toluene/ethyl acetate 8:2 v/v. IR ν cm^{-1} 1720, 1550. ^{1}H NMR ($CDCl_3$) δ 8.87 (d, 1H, H-6), 8.59 (m, 2H, H-2 and H-9), 8.24 (dd, 1H, H-8), 7.36 (dd, 1H, H-5′ thienyl), 7.24 (d, 1H, H-3′ thienyl), 7.02 (m, 1H, H-4′ thienyl), 5.60 (s, 2H, CH_2). Anal. C, H, N.

3-(2-Thienylmethoxycarbonyl)-8-trifluoromethylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide (23). From **15** and thiophen-2-methanol (method B). Yellow crystals. TLC eluent: isopropyl ether/cyclohexane 8:3 v/v. IR ν cm^{-1} 1720, 1550. ^{1}H NMR ($CDCl_3$) δ 8.78 (d, 1H, H-9), 8.70 (d, 1H, H-6), 8.58 (s, 1H, H-2), 7.94 (dd, 1H, H-7), 7.38 (dd, 1H, H-5′ thienyl), 7.24 (d, 1H, H-3′ thienyl), 7.02 (m, 1H, H-4′ thienyl), 5.60 (s, 2H, CH_2). Anal. C, H, N.

3-(2-Methoxybenzoyl)-8-trifluoromethylpyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-Oxide (24). Compound **15** (0.4 mmol) was + reacted with 2.0 mL of thionyl chloride and maintained at 60 °C for 30 min. The final solution was evaporated in vacuum, and the intermediate 3-carbonylchloride was suspended in 8 mL of absolute toluene. 2-Methoxyphenylboronic acid (0.2 mmol), cesium carbonate (0.5 mmol), and an excess of triphenylphosphine palladium (0) (tetrakis, 10 mg) were added. The mixture was maintained at refluxing temperature under nitrogen for 2 days. The final suspension was diluted with ethyl acetate and washed with water and a saturated solution of sodium bicarbonate. The ethyl acetate solution was then dried over sodium sulfate and evaporated under pressure. The residue was recrystallized by ethanol. Yellow crystals. TLC eluent: isopropyl ether/cyclohexane 8:3 v/v. IR ν cm^{-1} 1680, 1550. ^{1}H NMR ($CDCl_3$) δ 8.78 (d, 1H, H-9), 8.68 (d, 1H, H-6), 8.50 (s, 1H, H-2), 7.92 (dd, 1H, H-7), 7.58 (m, 2H, H-6′ and H-4′ phenyl), 7.12 (t, 1H, H-5′ phenyl), 6.95 (d, 1H, H-3′ phenyl), 3.80 (s, 3H, OCH_3). Anal. C, H, N.

3-(Thien-2-ylcarbonyl)-8-trifluoromethylpyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-Oxide (25). Compound **15** (0.3 mmol) was reacted with 2.0 mL of thionyl chloride and maintained at 60 °C for 30 min. The final solution was evaporated in vacuum, and the intermediate 3-carbonylchloride was suspended in a solution of 15 mL of methylene chloride and 0.9 mmol of anhydrous tin tetrachloride; the dark-yellow solution was supplemented, after 5 min, with thiophene (1.2 mmol) and the reaction monitored by TLC (eluent toluene/ethyl acetate/acetic acid 8:2:1 v/v/v). The obtained suspension became red, and when the starting material disappeared, the reaction was quenched by treatment with HCl 1:1, diluted with methylene chloride, and the organic layer separated. This layer was washed with a saturated solution of sodium bicarbonate, dried over anhydrous sodium sulfate, and evaporated. The residue was recovered with isopropyl ether and recrystallized. Yellow crystals. IR ν cm^{-1} 1680, 1550. ^{1}H NMR ($CDCl_3$) δ 8.82 (d, 1H, H-9), 8.72 (d, 1H, H-6), 8.68 (s, 1H, H-2), 8.02 (dd, 1H, H-3′ thienyl), 7.94 (dd, 1H, H-7), 7.78 (d, 1H, H-5′ thienyl), 7.25 (m, 1H, H-4′ thienyl). Anal. C, H, N.

General Procedure for the Synthesis of Compounds 26−29. A solution in chloroform (10 mL) of starting material **16** or **17** (0.5 mmol) was supplemented with an excess of bromine (1.0 mL) to obtain compounds **26** and **28**, with *N*-chlorosuccinimide (NCS, 100 mg) and a catalytic amount of benzoyl peroxide to obtain compound **27**, and with iodine monochloride (1:2) chloroform solution to obtain compound **29**. The final solution was evaporated to dryness and the residue purified by recrystallization.

3-Bromo-7-trifluoromethylpyrazolo[5,1-*c*][1,2,4]pyrazolo-benzotriazine 5-Oxide (26). From **16** and bromine. TLC eluent: toluene/ethyl acetate 8:2:1 v/v/v. IR ν cm^{-1} 1550. ^{1}H NMR ($CDCl_3$) δ 8.85 (d, 1H, H-6), 8.50 (d, 1H, H-9), 8.18 (dd, 1H, H-8), 8.10 (s, 1H, H-2). Anal. C, H, N.

3-Bromo-8-trifluoromethylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide (27). From **17** and bromine. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v. IR ν cm^{-1} 1550. ^{1}H NMR ($CDCl_3$) δ 8.70 (m, 2H, H-6 and H-9), 8.15 (d, 1H, H-2), 7.89 (dd, 1H, H-7). Anal. C, H, N.

3-Chloro-8-trifluoromethylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (28). From **17** and NCS. TLC eluent: isopropyl ether/cyclohexane 8:3 v/v. IR ν cm^{-1} 1550. ^{1}H NMR ($CDCl_3$) δ 8.70 (m, 2H, H-6 and H-9), 8.12 (d, 1H, H-2), 7.89 (dd, 1H, H-7). Anal. C, H, N.

3-Iodo-8-trifluoromethylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (29). From **17** and ICl. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v. IR ν cm^{-1} 1550. ^{1}H NMR ($CDCl_3$) δ 8.70 (m, 2H, H-6 and H-9), 8.18 (d, 1H, H-2), 7.89 (dd, 1H, H-7). Anal. C, H, N.

General Procedure for the Synthesis of Compounds 30−32. Tetrakis (triphenylphosphinepalladium(0), 30 mg, 0.026 mmol) and compound **29** (0.30 mmol) were combined in anhydrous toluene (4.0 mL). The suitable heteroarylboronic acid (2-, 3-thiophen boronic acid and 3-furanboronic acid, 0.60 mmol) in absolute ethanol (2.5 mL) and aqueous sodium carbonate (2M, 4 mL) were added, and the reaction mixture was heated to reflux for 12 h. The red product was then extracted by dichloromethane, and the organic layer was washed with water and dried over anhydrous sodium sulfate. The evaporation of the solvent gave the crude product that was recrystallized by suitable solvent.

3-(Thien-2-yl)-8-trifluoromethylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide (30). Dark-red crystals. IR ν cm^{-1} 1550. ^{1}H NMR ($CDCl_3$) δ 8.68 (m, 2H, H-6 and H-9), 8.35 (s, 1H, H-2), 7.85 (dd, 1H, H-7), 7.68 (dd, 1H, H-3′ thienyl), 7.40 (d, 1H, H-5′ thienyl), 7.18 (m, 1H, H-4′ thienyl). Anal. C, H, N.

3-(Thien-3-yl)-8-trifluoromethylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide (31). Dark-red crystals; IR ν cm^{-1} 1550. ^{1}H NMR ($CDCl_3$) δ 8.70 (m, 2H, H-6 and H-9), 8.39 (s, 1H, H-2), 7.94 (dd, 1H, H-2′ thienyl), 7.85 (dd, 1H, H-7), 7.68 (dd, 1H, H-4′ thienyl), 7.48 (d, 1H, H-5′ thienyl). Anal. C, H, N.

3-(Fur-3-yl)-8-trifluoromethylpyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide (32). Dark-red crystals. IR ν cm^{-1} 1550. ^{1}H NMR ($CDCl_3$) δ 8.70 (m, 2H, H-6 and H-9), 8.28 (s, 1H, H-2), 8.10 (dd, 1H, H-2′ furyl), 7.85 (dd, 1H, H-7), 7.58 (dd, 1H, H-5′ furyl), 6.92 (d, 1H, H-4′ furyl). Anal. C, H, N.

Ethyl 5-(2-Trifluoromethoxy-4-hydroxyphenyl)azopyrazole-4-carboxylate (33). A solution of diazotized ethyl 3(5)-amino-1*H*-pyrazole-4-carboxylate (commercially available) (2.0 mmol) was slowly added to a solution in ethanol (10 mL) of 3-trifluoromethoxyphenol (1.0 mmol) containing sodium acetate (500 mg) at 0 °C with stirring. After complete addition, the reaction mixture was stirred for a further hour and then 100 g of ice were added. An orange−red precipitate was formed that was collected by filtration and recrystallized by ethanol. IR ν cm^{-1} 3178, 1693, 1572. ^{1}H NMR

(DMSO-d_6) δ 13.70 (bs, 1H, OH, exch), 11.00 (bs, 1H. NH, exch), 8.37 (s, 1H, H-3 pyrazole), 7.72 (d, 1H, H-6 phenyl), 6.98 (m, 2H, H-3 and H-5 phenyl), 4.20 (q, 2H, CH_2), 1.20 (t, 3H, CH_3). MS(ESI) m/z: 343.0, 344.1 ($[M + H]^+$). Crystallographic data for compound **33** are reported in the Supporting Information. Anal. C, H, N.

3-Iodo-8-hydroxypyrazolo[5,1-*c*][1,2,4]benzotriazine (34). From starting material 3-iodo-8-hydroxypyrazolo[5,1,-*c*][1,2,4]benzotriazine 5-oxide,[11] following a previously described procedure, was reduced with triethylphosphite (TEP, 1.0 mL) in toluene (10 mL)[7] at refluxing temperature for 8 h. Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v. IR ν cm^{-1} 3700−3600. ^{1}H NMR (DMSO-d_6) δ 11.90 (bs, 1H, OH, exch), 8.50 (m, 2H, H-6 and H-2), 7.59 (d, 1H, H-9), 7.30 (dd, 1H, H-7). Anal. C, H, N.

Ethyl 8-Hydroxypyrazolo[5,1-*c*][1,2,4]benzotriazine-3-carboxylate (35). This compound was synthesized following a described procedure.[32] Melting point and ^{1}H NMR data are according to the literature.

8-Hydroxypyrazolo[5,1-*c*][1,2,4]benzotriazine-3-carboxylic Acid (36). Compound **35** (0.70 mmol) was reacted with 20% sodium hydroxide solution at refluxing temperature. The final acid was recovered by filtration after cooling and acidification with concentrated hydrochloric acid. Yellow crystals. TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v. IR ν cm^{-1} 3700−3600, 1680. ^{1}H NMR (DMSO-d_6) δ 12.90 (bs, 1H, COOH, exch), 12.40 (s, 1H, OH, exch), 8.68 (s, 1H, H-2), 8.55 (d, 1H, H-6), 7.77 (d, 1H, H-9), 7.43 (dd, 1H, H-7). Anal. C, H, N.

3-(2-Thienylmethoxycarbonyl)-8-hydroxypyrazolo[5,1-*c*][1,2,4]-benzotriazine (37). A solution of compound **36** (0.40 mmol) in dichloromethane (5 mL) was supplemented with a suspension of trichloroacetonitrile (TCA, 0.80 mmol) and triphenylphospine (0.80 mmol) in dichloromethane (5 mL). The reaction was stirred until the acid disappeared, and 2-thiophenemethanol (0.2 mL, large excess) was added and maintained at refluxing temperature, with stirring, monitored by TLC (eluent: tolurne/ethyl acetate/acetic acid 8:2:1 v/v/v). The mixture was washed with water, and the organic layer was then dried over anhydrous sodium sulfate. The evaporation gave a brown residue that crystallized after being exposed to a few milliliters of ethanol. The raw product was recrystallized by ethanol. Yellow crystals. IR ν cm^{-1} 3700−3600, 1720. ^{1}H NMR (DMSO-d_6) δ 12.02 (s, 1H, OH, exch), 8.78 (s, 1H, H-2), 8.58 (d, 1H, H-6), 7.65 (d, 1H, H-9), 7.60 (dd, 1H, H-5′ thienyl), 7.40 (dd, 1H, H-7), 7.32 (d, 1H, H-3′ thienyl), 7.08 (m, 1H, H-4′ thienyl), 5.60 (s, 2H, CH_2). Anal. C, H, N.

General Procedure for the Synthesis of Compounds 38−40. Chlorodifluoro-acetophenone (1.0 mmol) was added to a mixture of compounds **34**, **35**, or **36** (0.20 mmol), potassium carbonate (2.0 mmol), acetonitrile (2 mL), and water (2.0 mL) and maintained at room temperature. The reaction was warmed to 70−80 °C and monitored by TLC. Then the mixture was extracted with ethyl acetate, and the organic layer was dried over anhydrous sodium sulfate and evaporated to dryness. The crude product was purified by chromatography column or by recrystallization.

3-Iodo-8-difluoromethoxypyrazolo[5,1-*c*][1,2,4]benzotriazine (38). From starting material **34** and purified by chromatography (eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; fast band running). Yellow crystals. ^{1}H NMR (DMSO-d_6) δ 8.78 (d, 1H, H-6), 8.61 (s, 1H, H-2), 8.10 (d, 1H, H-9), 7.77 (t, 1H, CHF_2, J_{H-F} 73 Hz), 7.66 (dd, 1H, H-7). Anal. C, H, N.

Ethyl 8-Difluoromethoxypyrazolo[5,1-*c*][1,2,4]benzotriazine-3-carboxylate (39). From starting material **35**, purified by recrystallization. Yellow crystals. ^{1}H NMR (DMSO-d_6) δ 8.86 (m, 2H, H-2 and H-6), 8.18 (d, 1H, H-9), 7.80 (t, 1H, CHF_2, J_{H-F} 73 Hz), 7.75 (dd, 1H, H-7), 4.40 (q, 2H, CH_2), 1.40 (t, 3H, CH_3). MS(ESI) m/z: 262.9, 308.9, 310.0 ($[M + H]^+$). Anal. C, H, N.

3-(2-Thienylmethoxycarbonyl)-8-difluoromethoxypyrazolo[5,1-*c*]-[1,2,4]benzotriazine (40). From starting material **37** and purified by recrystallization. Yellow crystals. IR ν cm^{-1}, 1720. ^{1}H NMR (DMSO-d_6) δ 8.80 (s, 1H, H-2), 8.77 (d, 1H, H-6), 8.09 (d, 1H, H-9), 7.71 (t, 1H, CHF_2, J_{H-F} 73 Hz) 7.67 (dd, 1H, H-7), 7.51 (dd, 1H, H-5′ thienyl), 7.25 (d, 1H, H-3′ thienyl), 6.98 (m, 1H, H-4′ thienyl), 5.60 (s, 2H, CH_2). Anal. C, H, N.

General Procedure for the Synthesis of Compounds 41−42. A solution of compound **38** or **39** (0.150 mmol) in acetic acid (7.0 mL) and acetic anhydride (4.0 mL) was added, with caution, to hydrogen peroxide (35 wt % in water, 4.0 mL). The mixture was refluxed and stirred for 2−3 h and monitored by TLC (eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v). The final solution was treated with ice, and the suspension formed was extracted by ethyl acetate; this was dried, evaporated to dryness, and the residue was recovered with a few milliliters of ethanol and purified by recrystallization.

3-Iodo-8-difluoromethoxypyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide (41). From starting material **38**. Orange crystals. ^{1}H NMR (DMSO-d_6) δ 8.49 (d, 1H, H-6), 8.41 (s, 1H, H-2), 8.00 (d, 1H, H-9), 7.72 (t, 1H, CHF_2, J_{H-F} 73 Hz), 7.50 (dd, 1H, H-7). Anal. C, H, N.

3-Ethoxycarbonyl-8-difluoromethoxypyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-oxide (42). From starting material **39**. Yellow crystals. ^{1}H NMR (DMSO-d_6) δ 8.68 (s, 1H, H-2), 8.54 (d, 1H, H-6), 8.06 (d, 1H, H-9), 7.76 (t, 1H, CHF_2, J_{H-F} 73 Hz), 7.58 (dd, 1H, H-7), 4.34 (q, 2H, CH_2), 1.34 (t, 3H, CH_3). Anal. C, H, N.

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

Bovine cerebral cortex membranes were prepared as previously described.[39,40] 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.[41] [^{3}H]Ro 15-1788 binding studies were performed as previously reported.[42] 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, USA), which provided IC_{50}, K_i, and SEM values for tested compounds, the K_i values being calculated from the Cheng and Prusoff equation.[43]

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

Rota-Rod Test. The integrity of the animals' motor coordination was assessed using a rota-rod apparatus (Ugo Basile, Varese, Italy) at a rotating speed of 24 rpm The number of falls from the rod over 30 s, 25 min after drug administration, were counted.

Grip-Strength Meter Test. The grip strength meter measures forelimb grip-strength in rodents. The apparatus is formed by a Perspex base on which is located a grasping trapeze. The mouse instinctively grabs the trapeze, when raised by trail, trying to stop this involuntary backward movement until the pulling force overcomes the animal's grip strength. After the animal loses its grip, the peak preamplifer automatically stores the peak pull force and shows it on a liquid crystal display.

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 cm × 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.

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

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

Drugs. Diazepam (Valium 10, Roche), flumazenil (Roche), pentylenetetrazole (PTZ) (Sigma), and zolpidem (Tocris) were used. All drugs except PTZ were suspended in 1% carboxymethylcellulose sodium salt and sonicated immediately before use. PTZ was dissolved in isotonic (NaCl 0.9%) saline solution and injected sc. All benzodiazepine receptor ligands were administered by 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 volume of 10 mL/kg by the po, ip, or sc routes.

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

Geometry Optimization of Structures. Geometry optimizations were achieved with the cff91 force field (consistent force field) of the DISCOVER module of the Insight II[44] program by applying the Conjugate Gradients algorithm with a convergence criterion of 0.001 kcal/mol.

Conformational Research (Simulated Annealing). We carried out a stochastic process by a simulated annealing procedure, in vacuum.

The calculations were carried out with the DISCOVER module of the Insight II program using the cff91 force field. A multiple-step procedure was used. The molecule was energetically minimized. The minimized system was used as the initial structure for the subsequent molecular dynamics (MD) simulation. The structures were heated gradually to 900 K and cooled gradually, after 5 ps, until 300 K. Finally, the structure was energetically minimized. This procedure was repeated 200 times for every molecule (therefore 200 conformations were collected for every molecule).

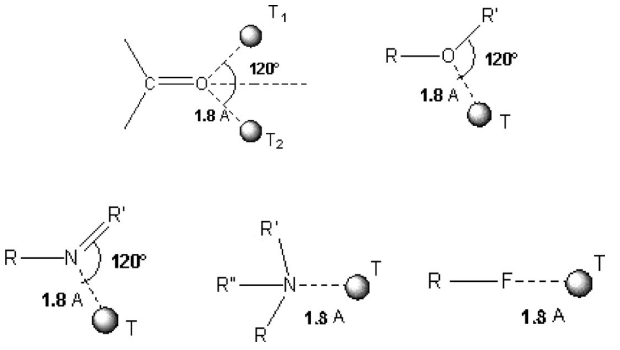
Procedure for Obtaining the Pharmacophoric Model. We have developed a procedure capable of predicting a combination of pharmacophoric points by taking into account the number of conformers needed to describe the conformational space which represents all the reasonable 3D conformational structures for all considered molecules. We call this original "homemade" procedure ADLR (Acceptor, Donor, Lipophilic and Ring). This procedure groups the molecules in clusters based on common combinations of pharmacophoric points (same number, type, and position).

We consider the chemical groups present in the molecules and we define a "pharmacophoric point" (Pp) the mapping of a chemical group to its corresponding chemical interaction type. ADLR considers the hydrogen bond (HBAcceptor and HBDonor) lipophilic and aromatic (to simplify, ring) interaction and the four Pps are defined as follows:

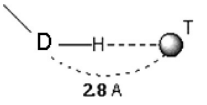
(a) Ring (R): the geometric center of atoms forming the aromatic moiety.

(b) Lipophilic (L): the geometric center calculated among the clusters of lipophilic atoms. Lipophilic atoms are carbon atoms sp^2 and sp^3, sulfur, halogen as bromine and iodine.

(c) Acceptor (A): the point in which it is necessary to have the respective target atom (hydrogen) to form an efficient hydrogen bond with the acceptor atoms (N, O, and F). It is possible to have various types of acceptor atoms. In the case of R−C═O, two points (T1 and T2) were considered, with a distance of 1.8 Å from the oxygen atom and the angle (COT) of 120°. In the case of R−O−R′, R−N═R′ was considered one point (T) located 1.8 Å from oxygen or nitrogen forming an angle of 120° between ROT or R′OT or R′NT. In the case of −N < , one point (T) was considered located 1.8 Å from the nitrogen atom orthogonal to the plane formed by R, R′, and R″. If the acceptor atom is the fluorine, the point T is located 1.8 Å from fluorine and the angle CFT is 180°.



(d) Donor (D): the point in which it is necessary to have the target atom (N, O) to form an efficient hydrogen bond with hydrogen of the donor atom (NH or OH). The Pp is located 2.8 Å from the hydrogen bond donor atom (N or O), with a DHT (D generic donor) angle of 180°.



Conformations for each molecule are collected in clusters based on the similarity of the Pp position. For each cluster, the mean and the standard deviation of the distances among the Pps of the conformers is calculated. All of these distance means generate the "*matrix of the distances*". The combination of the mean of the distances of Pps, for each cluster of conformers, is called "*structure*". Each molecule is represented by one or more "*structures*".

The similarity among the "*structures*" is evaluated on the bases of the similarity of Pp couples. Each Pp couple of one *structure* is compared with all other Pp couples of the other *structures*.

The couples are similar if they have the followings characteristics:

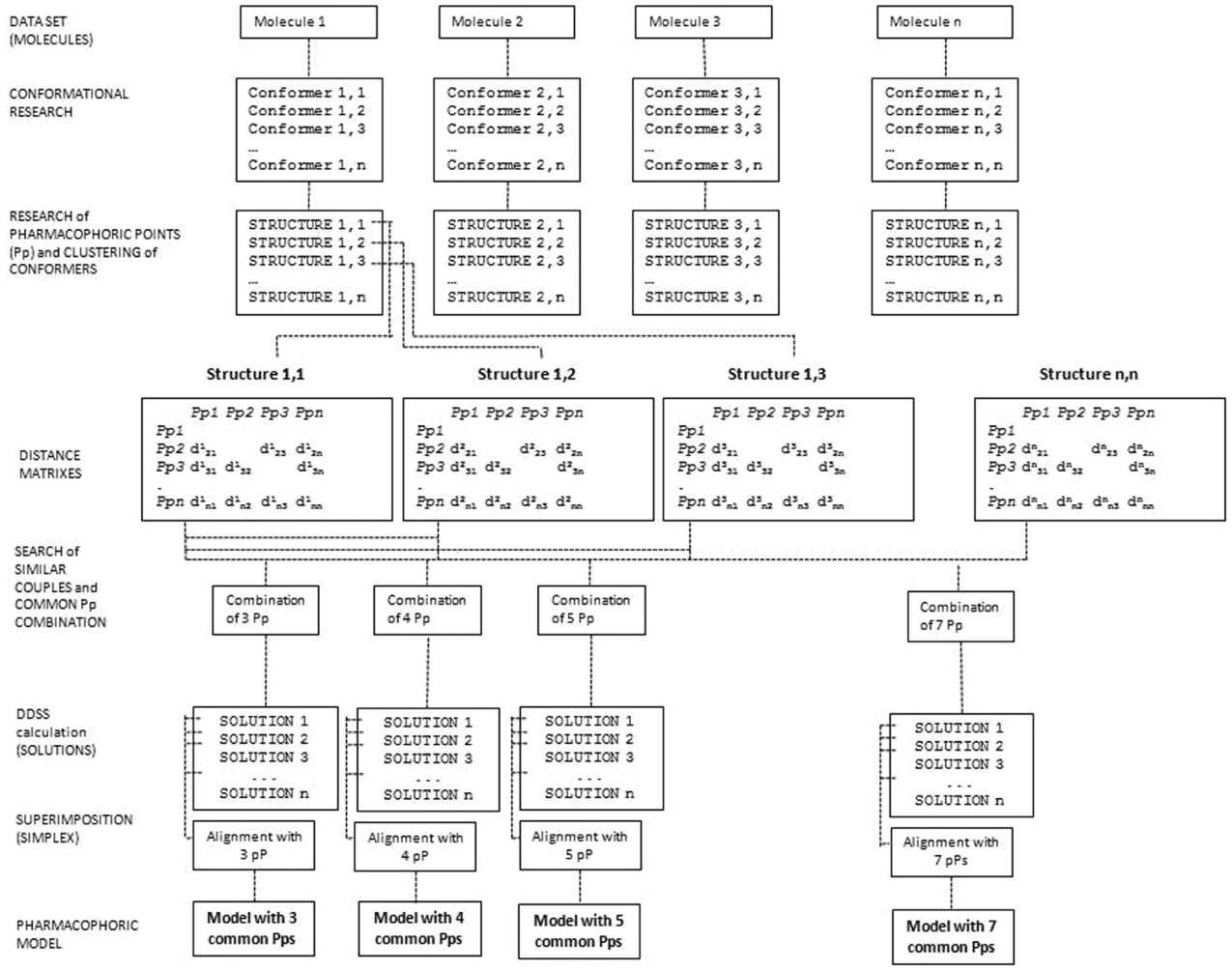
(1) Similar features of the Pps (acceptor, donor, lipophilic, ring).

(2) The mean of distance of the *ij* Pp couple of *structure 2* (d^2_{ij}) is in the range of the mean of distance of the Pp couple *ij* of *structure 1* (d^1_{ij}), that is:if $d^2_{ij} > d^1_{ij}$, the couple *ij* of *structure 2* is "similar" to Pp couple *ij* of *structure 1* if $(d^2_{ij} - SD^2_{ij} - Tol)/2 < (d^1_{ij} + SD^1_{ij} + Tol)/2$or:if $d^2_{ij} < d^1_{ij}$, the Pp couple *ij* of *structure 2* is "similar" to couple *ij* of *structure 1* if $(d^2_{ij} + SD^2_{ij} + Tol)/2 > (d^1_{ij} - SD^1_{ij} - Tol)/2$.

SD^1_{ij} represent the standard deviation of distance of the Pp couple *ij* of *structure 1*, and SD^2_{ij} is the standard deviation of distance of the Pp couple *ij* of *structure 2*. "Tol" represent a tolerance value (the default value is 0 Å).

ADLR searches common combinations of 3, 4, 5, 6, or 7 Pps among all *structures*. For example, two *structures* have in common a combination of n ($3 \le n \le 7$) Pps, if they have a $n!/k!\ (n - k)!$ number of similar couples in common (n is the number of Pps, k is 2 (the two element of the couple)). So for three Pps ($n = 3$), three "similar" couples in common are needed, for four Pps ($n = 4$), six "similar" couples in common are needed, and so on.

Chart 1



The similarity among combinations of Pps of different *structures* is calculated as the sum of square of mean distances difference, among the couples of Pps of both *structures* (distance difference sum square, DDSS). The DDSS and similarity are inversely proportional.

In general,

$$\mathrm{DDSS} = \sum_{n}(d_{ij}^{1} - d_{ij}^{2})^2/n$$

where N represents the number of pairs for combination, d^1_{ij} represents the distance between the Pp_i and the Pp_j of the *structure 1*, and d^2_{ij} represents the distance between the Pp_i and the Pp_j of the *structure 2*, etc.

For each cluster of conformers, the conformer that is closest to the average conformation is selected. All the selected conformers are aligned with the conformer of the reference molecule.

Alignment is optimized using the Simplex method that minimizes the sum of the squares of the distance differences among the Pp positions compared to the Pp positions of the reference *structure*. Alignment with the Simplex method originates from a number of 56 positions (vertexes) identified by six variables that represent three rotations (around axes x, y, and z) and three translations (along the axes x, y, and z). These initial positions are calculated following an experimental design.

Alignment by the Simplex method is preceded, for all the selected conformers, by a first alignment positioning the first Pp in the origin of the Cartesian axes and the second along the x axis (with coordinates y and z equal to 0).

The whole procedure for obtaining the pharmacophoric model is schematically reported (Chart 1):

Crystallographic Analyses. The data were collected at 293 (2)K on Xcalibur3 4-circle diffractometer using a graphite monochromator, Mo Kα radiation. A reference frame was monitored every 50 frames to control the stability of the crystal and the system revealed no intensity decay. The data set was corrected for Lorentz, polarization effects, and absorption correction was made by multiscan method using the CrysAlis Software package. The structure was solved using direct method with SIR97 software, and the refinement was carried out using the SHELXL-97 software package. All non-hydrogen atoms were located from the initial solution or from subsequent electron density difference maps during the initial course of the refinement. After locating the non-hydrogen atoms, the models were refined against F2, first using isotropic and finally anisotropic thermal displacement parameters. The hydrogen atoms were treated with the riding coordinate method.

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Supporting Information Available: Crystallographic data for compound **33** and a thermal ellipsoid figure. An example of research of Pps common combinations. This material is available free of charge via the Internet at http://pubs.acs.org.

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