Benzodiazepine Receptor Ligands. 4. Synthesis and Pharmacological Evaluation of 3-Heteroaryl-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxides

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The synthesis of new 3-heteroaryl-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxides and their binding activities at the central benzodiazepine receptor (BZR) are reported. The derivatives substituted at the 3-position with electron-rich five-membered rings, such as pyrrole **11**, 2-thiophene **13c**, or 3-thiophene **13d**, showed good affinity values for BZR. In in vivo tests the 3-(thien-3-yl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (**13d**) showed selective anticonvulsant activity.

Introduction

As part of our program directed toward the search for central nervous system (CNS) agents, the synthesis and evaluation of the affinity on the central benzodiazepine receptor (BZR) of numerous 2-, 3-, 7-, and 8-substituted pyrazolo[5,1-*c*][1,2,4]benzotriazines and corresponding 4- and 5-oxides have been reported.[1−4] From binding data we pointed out that some pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxides, bearing at the 3-position an ethoxycarbonyl group or bromine and at the 8-position a chlorine or small lipophilic group such as ethoxy or methyl, were new ligands for BZR with good affinity values (K_i range 35−93 nM) and with an efficacy trend typical of agonists and partial agonists (GR ratio 2.67−1.31).[2] In particular the 3-(ethoxycarbonyl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (**I**) was the derivative with the highest affinity (35.0 ± 2.0 nM) and agonist profile (GR 1.91). In general, modification of the ethoxycarbonyl group at the 3-position, such as a nitrile, carboxy, or carboxyamide group, or its replacement by a six-membered ring or a nitro group was found to be detrimental to receptor binding, suggesting the importance of an ester group for the receptor fitting. At present all results seem to indicate that the 3-substituent is critical for the BZR affinity.[2−4] On the basis of structure−activity relationship (SAR) data, we have proposed a pharmacophore/receptor model for these new BZR ligands. In this model the pyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-oxide binds to BZR with N-1 and N-4 by means of a hydrogen bond involving the H_2 and H_1 donor receptor sites, respectively. The substituent at the 3-position fits into the lipophilic region L_1/L_2. In particular, if this substituent is an ethoxycarbonyl group, as in the lead compound **I**, the lone pair orientation of the carbonyl oxygen of the estereal chain can reinforce the receptor binding by means of a three-centered hydrogen bond. The 5-oxide group also has an important role in reinforcing this hydrogen bond.

The lead compound **I**, selected for in vivo testing, showed poor anticonvulsant and anxiolytic activity and no myorelaxant properties.[2] These in vivo pharmacological results, in contrast with binding data, might be attributed to bioavailability problems or easy metabolic cleavage of the carboxylic ester to biologically inactive acid.

To obtain improved efficacy and greater selectivity in vivo, we decided on isosteric replacement of the ester function by 1,2,4-oxadiazole, 1,2,4-triazole, and isoxazole rings in the lead compound.[5−7] These small heterocycles, in which a nitrogen or oxygen takes the place of the carbonyl oxygen, might mimic an ester group and if tolerated in the 3-position could give metabolically more stable derivatives.

Moreover, we decided to replace the ester group with other heterocycles such as 2- or 3-thiophene and pyrrole which are different from the five-membered rings cited above for electronic and steric features. The synthesis of the new series of 3-heteroaryl-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxides seems very useful either for SAR or to further confirm our hypothesis about the critical role of the substituent at the 3-position of these ligands for binding at BZR.

Chemistry

All pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxides described here are listed in Table 1. To obtain 8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxides bearing in the 3-position, as isosteric replacement of the ester function, the triazolo and oxadiazole moiety, several synthetic pathways were followed[5] (see Scheme 1).

The starting material for synthesis of both derivatives was the 3-carbamoyl-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (**1**)[1] that by treatment with dimethylformamide−dimethyl acetal (DMF−DMA) or dimethylacetamide−dimethyl acetal (DMA−DMA)[5] yielded the intermediate acylamidine **2a** or **2b**, respectively. These intermediates were cyclized with hydrazine hydrate at

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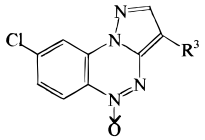
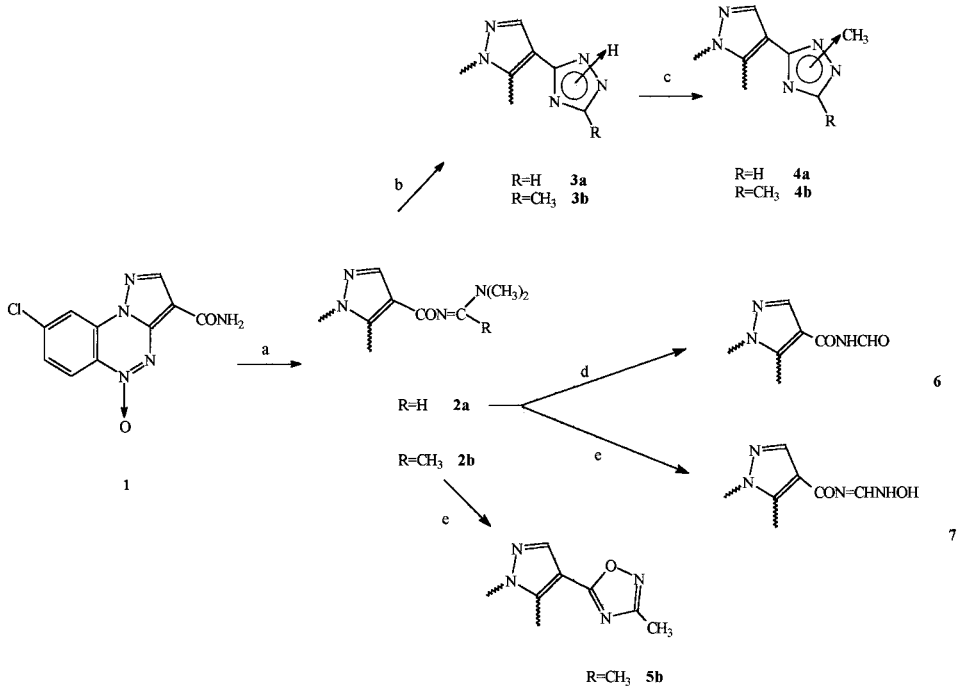
Table 1. Chemical Data for Pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxides



compd	R^3	formula (MW)	mp (°C) (recrystallization solvent) yield
2a	−CON=CH(NMe_2)	$C_{13}H_{11}N_6O_2Cl$ (318.75)	259−261 (washed by ether) 97%
2b	−CON=C(NMe_2)Me	$C_{14}H_{13}N_6O_2Cl$ (332.78)	234−235 (washed by ether) 73%
3a	−1,2,4-triazol-3-yl	$C_{11}H_6N_7OCl$ (287.69)	>300 (ethanol) 53%
3b	−1,2,4-triazol-5-Me-3-yl	$C_{12}H_8N_7OCl$ (301.72)	>300 (methoxyethanol) 20%
4a	−1,2,4-triazol-1(2)(4)-Me-3-yl	$C_{12}H_8N_7OCl$ (301.72)	273−276 (acetic acid) 20%
4b	−1,2,4-triazol-1(2)(4),5-diMe-3-yl	$C_{13}H_{10}N_7OCl$ (315.75)	279−280 (acetic acid, 50%) 25%
5b	−1,2,4-oxadiaxol-3-Me-5-yl	$C_{12}H_7N_6O_2Cl$ (302.70)	249−250 (methoxyethanol) 80%
6	−CONHCHO	$C_{11}H_6N_5O_3Cl$ (291.67)	271−273 (washed by water) 77%
7	−CON=CHNHOH	$C_{11}H_7N_6O_3Cl$ (306.69)	262−263 (methoxyethanol) 60%
9	−NHCOOtBu	$C_{14}H_{14}N_5O_3Cl$ (335.78)	199−200 (ethanol/water) 97%
10	$-NH_2$	$C_9H_6N_5OCl$ (235.65)	244−246 (ethanol) 57%
11	−pyrrol-1-yl	$C_{13}H_8N_5OCl$ (295.71)	210−211 (methoxyethanol) 90%
13c	−2-thienyl	$C_{13}H_7N_4OSCl$ (302.75)	225−226 (methoxyethanol) 58%
13c′ [a]	−2-thienyl	$C_{13}H_7N_4SCl$ (286.75)	203−204 (methoxyethanol) 35%
13d	−3-thienyl	$C_{13}H_7N_4OSCl$ (302.75)	220−221d (methoxyethanol) 80%
16	$-CH_2CONH_2$	$C_{11}H_8N_5O_2Cl$ (277.69)	244−245 (methoxyethanol) 75%
17	$-CH_2COOH$	$C_{11}H_7N_4O_3Cl$ (278.67)	224−225 (water) 90%
18	−C(CHO)=CHNMe_2	$C_{13}H_{12}N_5O_2Cl$ (305.75)	220−222 (washed with water) 60%
19	−C(CHO)=CHOH	$C_{12}H_7N_4O_2Cl$ (274.68)	212−213 (water) 50%
20	−4-isoxazolyl	$C_{12}H_6N_5O_2Cl$ (287.68)	266−267 (methoxyethanol) 35%

[a] This compound is a 5-deoxy derivative: 3-(thien-2-yl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine.

Scheme 1[a]



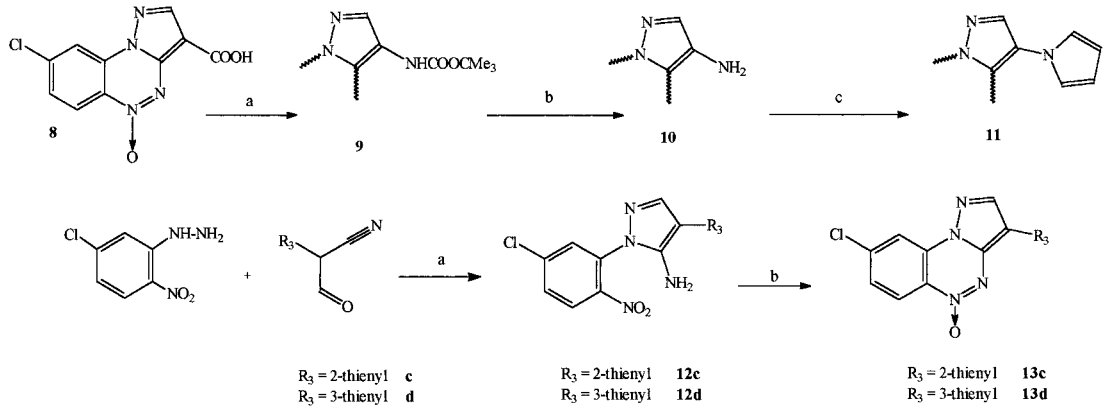
[a] (a) DMF−DMA or DMA−DMA; (b) hydrazine hydrate; (c) CH_3I; (d) acetic acid/H_2O; (e) $NH_2OH{\cdot}HCl$.

90−100 °C to obtain the 3-(1,2,4-triazol-3-yl) derivatives **3a** and **3b**, which were then alkylated by methyl iodide/dimethylformamide on N-1(2)(4) atom to yield the methyl derivatives **4a** and **4b**.

The same intermediate acylamidines, **2a** and **2b**, were also used to obtain the 3-(1,2,4-oxadiazol-5-yl) derivatives by treatment with hydroxylamine hydrochloride. In this case only compound **2b** yielded the desired oxadiazole derivative **5b**, while compound **2a** gave only the corresponding 3-*N*-hydroxylaminomethyleneamide **7** (see Scheme 1), whose cyclization failed either in acetic anhydride or in $POCl_3$. If compound **2a** is treated with H_2O/AcOH, the 3-*N*-formylamide derivative **6** is obtained, by the obvious hydrolysis of the acylamidine group. Both these products (**6** and **7**) were included in the SAR for the BZR because they bear at the 3-position substituents with particular electronic and polarity features.

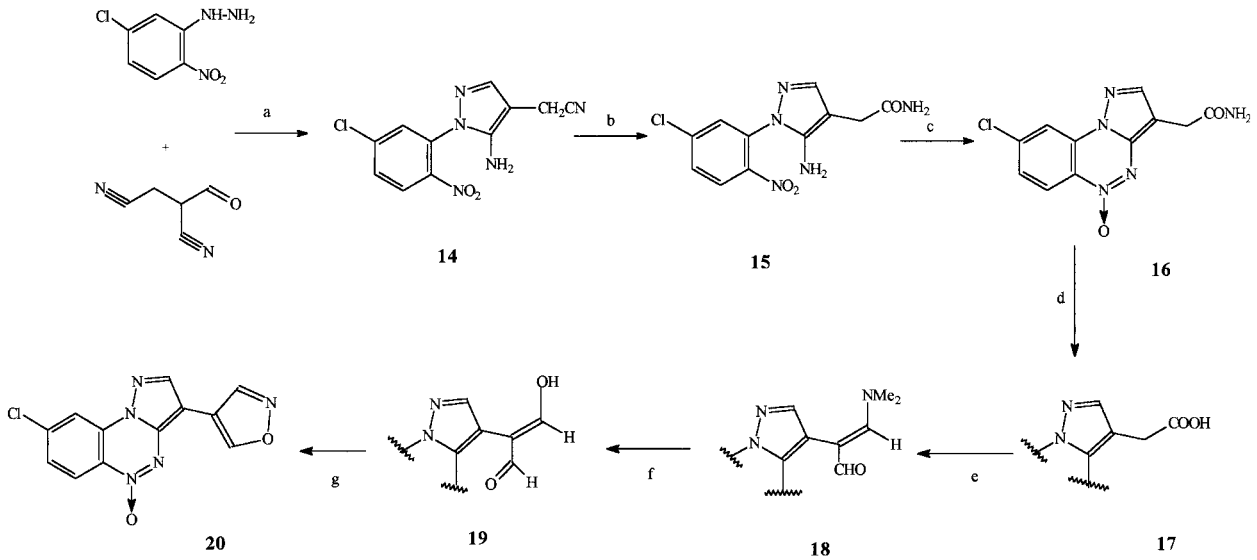
Scheme 2 depicts the route used to prepare the 3-(pyrrol-1-yl) derivative **11**. Treatment of 3-carboxy-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide[1] (**8**) with diphenyl phosphorazidate (DPPA)/triethylamine/*tert*-

Scheme 2[a,b]



[a] (a) Et_3N/DPPA; (b) acetic acid/hydrochloric acid; (c) dimethoxytetrahydrofuran. [b] (a) EtOH/hydrochloric acid as catalyst; (b) 10% aq NaOH.

Scheme 3[a]



[a] (a) EtOH; (b) sulfuric acid; (c) 10% aq NaOH; (d) H_2SO_4/$NaNO_2$; (e) $POCl_3$/Me_2NCHO; (f) 20% aq NaOH; (g) NH_2OH·HCl.

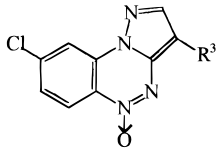
butyl alcohol[8] gave the 3-*tert*-butoxycarbamoylamino derivative **9**. The attempt to obtain **9** by another route with lead tetraacetate/*tert*-butyl alcohol[9] failed. Compound **9** was easily hydrolyzed with hot 12 M HCl and AcOH to the 3-amino derivative **10**. The 3-amino-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (**10**) was converted to the 3-(pyrrol-1-yl) derivative **11** by treatment with 2,5-dimethoxytetrahydrofuran in acetic acid.

The 2-nitro-5-chlorophenylhydrazine[1] was reacted with 2-(thien-2-yl)- and 2-(thien-3-yl)-3-oxapropanenitrile[10,11] (**c**,**d**) to obtain the aminopyrazoles bearing, at the 4-position, a 2-thienyl or 3-thienyl ring: **12c** and **12d**, respectively. These aminopyrazoles (**12c** and **12d**), in turn, were cyclized to the benzotriazine system in alkaline medium,[1−4] achieving the desired compounds pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxides **13c** and **13d** (see Scheme 2). Compound **13c**, 3-(thien-2-yl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide, was deoxidated to compound **13c′**, 3-(thien-2-yl)-8-chloropyrazolo-[5,1-*c*][1,2,4]benzotriazine, by treatment with triethyl phosphite(TEP)/toluene[3] to give us further information for the SAR.

A laborious synthetic path was used to obtain compound **20** bearing at the 3-position an isoxazole ring (see Scheme 3). As a precursor of the isoxazole ring, the malondialdehyde moiety was created at the 3-position of the pyrazolo[5,1-*c*][1,2,4]benzotriazine system. In fact this function is known to be very versatile and can cyclize to produce a large variety of aromatic as well as nonaromatic systems.[12−14]

The starting material to achieve the key intermediate, 3-(1-oxo-2-hydroxyvinyl)-8-chloropyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-oxide (**19**), was the 3-(carboxymethyl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (**17**) whose synthesis was rather laborious. The 2-nitro-5-chlorophenylhydrazine was reacted with 2-oxosuccinonitrile (see Experimental Section) to obtain the 1-(2-nitro-5-chlorophenyl)-5-aminopyrazole-4-acetonitrile (**14**). The acidic hydrolysis of this latter compound yielded the corresponding 4-acetamide derivative **15**, which was cyclized to benzotriazine system **16** in alkali medium. Treatment with concentrated H_2SO_4/$NaNO_2$ gave the desired 3-carboxymethyl derivative **17** which was, through the use of Vilsmeier−Haack reagent, transformed into 3-enamine **18** and after hydrolysis into 3-malondialdehyde derivative **19**. This latter compound exhibits an interesting ^1H NMR spectra run either in $CDCl_3$ or in DMSO-d_6. In $CDCl_3$, two signals as a broad singlet at 7.20 ppm for OH proton and at 9.20 ppm for

Table 2. BZR Ligand Affinity of Pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxides



compd	R^3	% inibition[a]	K_i (nM)[b]	GR[c]
2a	−CON=CHNMe$_2$	48.9 ± 1.32		
2b	−CON=C(NMe$_2$)Me	58.5 ± 2.53		
3a	−1,2,4-triazol-3-yl	44.4 ± 3.59		
3b	−1,2,4-triazol-5-Me-3-yl	15.2 ± 0.98		
4a	−1,2,4-triazol-1(2)(4)-Me-3-yl	0		
4b	−1,2,4-triazol-1(2)(4),5-diMe-3-yl	0		
5b	−1,2,4-oxadiazol-3-Me-5-yl	63.5 ± 4.57		
6	−CONHCHO	85.5 ± 6.89	844.2	2.07
7	−CON=CHNHOH	0		
9	−NHCOOtBu	0		
10	−NH$_2$	33.9 ± 1.50		
11	−pyrrol-1-yl	87.5 ± 6.30	61.5 ± 3.50	1.24
13c	−2-thienyl	82 ± 6.20	10.3 ± 0.81	1.54
13c′ [d]	−2-thienyl	66 ± 4.50		
13d	−3-thienyl	100 ± 6.70	36.3 ± 2.11	1.23
20	−4-isoxazolyl	66.0 ± 2.1		
I[e]	−COOEt	99.8 ± 5.0	35.0 ± 0.2[e]	1.91[e]
diazepam			10 ± 1.1	1.5

[a] Percent inhibition values of specific [^{3}H]Ro15-1788 binding at 10 μM concentration are means ± SEM of five determinations. [b] K_i values are means ± SEM of five determinations. [c] GR (GABA ratio) = IC_{50} compound + 10 μM GABA, performed in five independent experiments. [d] 5-Deoxy derivative. [e] See ref 2.

olefinic proton were seen; the aldehydic proton was evidenced only after D_2O treatment at 9.4 ppm, while the singlet at 7.20 ppm exchanges with the same treatment. In DMSO-d_6 the aldehydic and olefinic protons were seen as a broad singlet (8.7 ppm).

Finally, treatment of **19** with hydroxylamine hydrochloride in ethanol gave the desired compound 3-(isoxazol-4-yl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide (**20**). The same compound **19** could be the starting material to obtain the 3-(furan-3-yl) derivative, if treated with ethyl bromopropionate in alkali medium,[12] but in our case the synthesis failed.

Results and Discussion

Biological Results. The BZR binding affinity of pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxides was evaluated by their ability to displace [^{3}H]Ro15-1788 from its specific binding in bovine brain membranes. Binding data for all new compounds for lead compound **I** are reported in Table 2. As can be observed from these results, the reaction intermediates **2a**, **2b**, **6**, **7**, **9**, and **10**, which have at the 3-position hydrophilic or bulkier groups than the ethoxycarbonyl moiety in lead compound **I**, display no or very weak affinity to BZR. The 3-triazolyl, 3-oxadiazolyl, and 3-isoxazolyl derivatives **3a, 3b**, **4a**, **4b, 5b**, and **20** lack BZR affinity, excluding their bioisosterism with the 3-ethoxycarbonyl group.

Interestingly, in derivatives **11**, **13c**, and **13d** the replacement of the ester group of lead compound **I** by electron-rich five-membered rings, such as pyrrole or 2- or 3-thiophene, retains significant affinity to BZR. In fact the K_i values (61.5 nM for **11**, 36.3 nM for **13d**, and 10.3 nM for **13c**) indicate a lower, comparable to, and higher binding affinity respectively than that lead compound **I** (K_i 35.0 nM). Significantly the 3-(thien-2-yl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine (**13c′**), deoxy analogue derivative of **13c**, is devoid of affinity.

These new ligands (**11**, **13c**, and **13d**) bind to BZR with N-1 and N-4 by means of a hydrogen bond involving H_2 and H_1 donor receptor site as lead compound **I**.[4] In particular for these 3-heteroaryl derivatives, a $\pi-\pi$ stacking interaction between the receptor and the substituent at the 3-position, thiophene, and pyrrole, which are π-excessive rings, can be hypothesized. These rings, acting as a π-electron donor, might take place in a limited size lipophilic pocket in the lipophilic region L_1/L_2.[15−17] From literature it is well-recognized that the sulfur atom of a thiophene ring is substantially weak to form a hydrogen bond with an active proton.[16] Consequently the sulfur atom of compounds **13c** and **13d** cannot form a three-centered hydrogen bond as can the carbonyl group of the lead compound **I**. The enhanced affinity of ligand **13c** with respect to **13d** might be explained by a better accommodation of the thienyl ring of the 2-isomer than the 3-isomer into the lipophilic pocket reinforcing the $\pi-\pi$ stacking interaction. The important role of the 5-oxide group reinforcing the hydrogen bond (N-4/H_1 receptor) is confirmed by 5-deoxy derivative **13c′**, which is completely inactive (compare **13c** and **13c′**).

Compounds **3a**, **3b**, **4a**, **4b**, **5b**, and **20**, bearing at the 3-position a rigid isoster moiety of ester function, probably do not correctly accommodate the 3-substituent into the lipophilic pocket and consequently cannot contribute to form the three-centered hydrogen bonding with nitrogen or oxygen. In addition, oxadiazole, triazole, and isoxazole rings, which are more electron-poor than pyrrolo and thiophene, are unable to favor a $\pi-\pi$ stacking interaction.

Pharmacological Results. Compounds **13c** and **13d** were chosen for in vivo testing. Muscle relaxant, anticonvulsant, and anxiolytic activities of these substances were evaluated in comparison to those of vehicle, lead compound **I**, and diazepam (used as positive control) (see

Table 3. Muscle Relaxant, Anticonvulsant, and Anxiolytic Effects of **13c**, **13d**, and **I** in Comparison with Diazepam

		muscle relaxant effect		anticonvulsant activity						
		rota-rod test		against PTZ-induced attacks	against MES-induced hindlimb extension[b]			antianxiety activity		
								light/dark box		
treatment[a]	mg/kg	*n*	*n* of falls from rotating rod	%	*n*	%	% lethality	*n*	*n* of crosses	% time in light
CMC, 1%	0.1 mL	113	0.24 ± 0.05	11.5	36	5.5	27.8	66	16.4 ± 0.9	100 ± 4.2
diazepam	0.1	21	0.23 ± 0.09	47.6***						
	0.3	16	0.56 ± 0.18	81.2***				8	22.0 ± 2.7*	95.5 ± 11.0
	1	15	0.73 ± 0.28*	100***	10	90***	10	13	28.5 ± 3.87***	144.9 ± 9.2***
	3	10	1.4 ± 0.4***	100***	10	100***	0*	10	16.5 ± 3.6	211.1 ± 29.9***
flumazenil	100	15	0.4 ± 0.16	0				10	15.2 ± 1.7	114.8 ± 8.8
flumazenil + diazepam	100	14	0.46 ± 0.2	14.2^^						
	0.3									
flumazenil + diazepam	100							10	18.6 ± 1.3^	104.1 ± 11.7^
	1									
13c	3							10	15.0 ± 2.1	88.9 ± 6.8
	10							12	20.0 ± 1.7	122.2 ± 6.4*
	30	10	0 ± 0	10	11	0	18.2	13	17.6 ± 2.5	115.5 ± 11.2
	100	12	0.08 ± 0.08	16.6	10	0	20	13	18.4 ± 2.2	114.5 ± 7.8
	300	8	0.2 ± 0.2	12.5	12	0	8.3*			
13c + diazepam	100	10	0.5 ± 0.31	50						
	0.3									
13c + diazepam	100	12	0.17 ± 0.1^	100				13	26.6 ± 3.2	130.9 ± 7.3
	1									
13d	10	11	0.43 ± 0.3	18.2	14	14.3	28.6	12	16.3 ± 2.0	94.2 ± 11.8
	30	13	0.08 ± 0.08	61.5***	18	33.3*	22.2	12	19.0 ± 2.9	104.3 ± 11.3
	100	13	0.15 ± 0.1	46.1***	11	27.3	18.2	13	15.3 ± 2.0	107.4 ± 7.0
13d + flumazenil	30	11	0.64 ± 0.24	36.4^						
	100									
I	30	20		15	9	0	22.2	10	19.1 ± 2.7	106.2 ± 10.7
	100	32	0.5 ± 0.15	28.1*	12	0	0**	11	15.2 ± 1.8	124.6 ± 10.8*
	300	20	0.33 ± 0.18	25						

[a] Treatment with compounds **13c**, **13d**, **21**, and diazepam was performed 30 min and flumazenil 40 min before the test. [b] Maximal electroshock (MES) = 40 mA, 0.2 s, 50 Hz. Tonic hindlimb extension was considered as end point. *P < 0.05, **P < 0.01, ***P < 0.001 versus control mice; ^P < 0.05, ^^P < 0.001 versus **13d**- and diazepam-treated mice, respectively.

Table 3). During the 30-min postdrug period, prior to test, the animals were carefully observed for gross behavior in their home cages. No behavior alterations were noticed following the administration of each of the substances under study.

None of the newly synthesized molecules, including the lead compound, which were tested in a wide range of doses, caused a statistically significant impairment in rota rod performance, thus indicating a total absence of any muscle relaxant effect, while diazepam, from 1 mg/kg po up, significantly diminished the mice's motor coordination.

Anticonvulsant activity was studied using two different kinds of convulsant stimuli: pentylenetetrazole (PTZ) for chemically induced convulsion and maximal electroshock (MES) for electrically induced ones. Diazepam was able to prevent both kinds of convulsion, although to a lesser extent those of MES. The lead molecule showed slight anticonvulsant activity only in the PTZ-induced attacks at the dose of 100 mg/kg po and significantly decreased, like diazepam, the MES-induced lethality.

On the other hand **13d**, even if it has a lower GABA ratio (1.23) with respect to **I** (1.91) and **13c** (1.54), prevented PTZ-induced convulsion in a statistically highly significant manner at doses of 30 and 100 mg/kg po. Flumazenil, an antagonist on the BZR, at a dose of 100 mg/kg ip was able to significantly antagonize the protective effects of both **13d** (30 mg/kg) and diazepam (0.3 mg/kg). Similarly to diazepam, **13d** yielded anticonvulsant activity also in the MES test. The minor efficacy of both the molecules is probably due to very strong attacks with a high degree of mortality observed in the MES test.

As for anxiolytic effects, diazepam was again used as reference molecule under our conditions. It demonstrated a dose-related effect at doses of 1 and 3 mg/kg po. Among the tested compounds, only the lead molecule **I** showed any anxiolytic effect at the same dose (100 mg/kg po) at which it demonstrated anticonvulsant effect. Compound **13c** provided neither muscle relaxant nor anticonvulsant activity, and its anxiolytic activity was very weak, but it was able (at a dose of 100 mg/kg po) to antagonize diazepam (1 mg/kg po)-induced falls from rotating rod, thus demonstrating its partial agonist profile.

Summary

In conclusion, the introduction in the 3-position of a pyrazolobenzotriazine system of an electron-rich ring, as 3-thienyl, yielded compound **13d** which is endowed with a good affinity (K_i 36.3 nM) and in vivo selective pharmacological profile. This derivative seems to be the most interesting because it manifested a selective anticonvulsant activity without affecting the animals' motor coordination. This effect is probably due to its agonist/antagonist characteristic according to its GABA ratio value (1.23), so that a specific $GABA_A$/BZ receptor subtype might be selectively activated.[18,19]

Unexpectedly the 2-thienyl isomer **13c**, having the best affinity (K_i 10.3 nM) in vitro, shows very weak intrinsic efficacy in vivo. The lack of activity in vivo of **13c** versus **13d** could be explained by different metabolic oxidation pathways of the thiophene ring in the

two isomers,[20] also considering that the conjugation of the heteroaryl ring with pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide system is different in the 3-thienyl than the 2-thienyl ring.

The results of this work have further focused on the critical role of the 3-substituent of the pyrazolo[5,1-*c*]-[1,2,4]benzotriazine 5-oxide ligands: it could fit in a lipophilic pocket into the lipophilic region of the receptor protein, which probably has a different size in the $GABA_A$/BZ subtype, affecting both ligand potency and intrinsic activity (efficacy), according to reported literature data.[18,19]

Experimental Section

The structures of all compounds were supported by their IR spectra (KBr pellets in Nujol mulls, Perkin-Elmer 681 spectrophotometer) and ^{1}H NMR data (measured with a Varian Gemini at 200 MHz, chemical shifts expressed in δ (ppm) using DMSO-d_6 or $CDCl_3$ as solvent). Melting points were determined with a Gallenkamp apparatus and were uncorrected. Elemental analyses were performed by the laboratories of Dipartimento Farmaco-Chimico-Tecnologico of Università di Siena, Italy, with a Perkin-Elmer, model 240C, elemental analyzer, and their results are within ±0.4% of theoretical values. The purity of samples was determined by means of TLC, which was performed using Machery-Nagel Duren, Alugram silica gel plates. The MS spectra were obtained with a QMD-1000 apparatus (Carlo Erba, Milano, Italy) after GC−MS analysis. GC experimental conditions: column SBP-5, i.d. 0.25 mm, l 30 m, gradient temperature elution at 50 °C, 2 min, 15 °C/min up to 280 °C. MS analysis: EI, 70 eV.

2-Oxosuccinonitrile. A suspension of 50% NaH in mineral oil (200 mmol, 4.8 g) was added to anhydrous toluene (300 mL) in a 1-L round-bottomed flask. After addition of *tert*-amyl alcohol (2 mL) the mixture was heated at 70 °C, and a solution of succinonitrile (100 mmol, 8 g) and ethyl formate (100 mmol, 8.5 mL) in anhydrous toluene (50 mL) was added dropwise in 1 h. After 6 h of stirring and heating at 70−80 °C, the resulting solid was allowed to stand overnight. The mixture was treated with ice−water (400 mL), and the aqueous phase was separated and washed with diethyl ether. Acidification with 12 M HCl causes the separation of an oil which was extracted with diethyl ether. The extracts were dried on anhydrous Na_2SO_4 and evaporated to dryness, and a red oil was obtained. The final product was identified by GC−MS spectra *m*/*z* (%): 79 (25.68), 53 (82.88), 40 (100).

General Procedure for Synthesis of 2a and 2b. To a suspension of compound **1**[1] (1.9 mmol, 500 mg) in anhydrous toluene (10 mL) and anhydrous DMF (1 mL) was added dimethylformamide−dimethyl acetal (DMF−DMA) or dimethylacetamide−dimethyl acetal (DMA−DMA) (6.8 mmol) to obtain the intermediate **2a** or **2b**, respectively. The reaction was kept at 100−110 °C and was monitored by TLC (toluene/EtOAc/AcOH, 8:2:1 v/v/v, as eluent). The obtained residue was filtered and washed by diethyl ether.

3-(*N*-(Dimethylaminomethylene)carbamoyl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide, 2a. From **1** with DMF−DMA; yellow crystals. IR ν cm^{-1}: 1640, 1560. ^{1}H NMR (DMSO-d_6) δ: 8.68 (s, 1H, CH); 8.64 (s, 1H, H-2); 8.46 (m, 2H, H-6, H-9); 7.82 (dd, 1H, H-7); 3.21 (s, 3H, N−CH_3); 3.19 (s, 3H, NCH_3). Anal. ($C_{13}H_{11}N_6O_2Cl$) C, H, N.

3-(*N*-(1-Dimethylaminoethylidene)carbamoyl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide, 2b. From **1** with DMA−DMA; yellow crystals. IR ν cm^{-1}: 1610, 1550. ^{1}H NMR ($CDCl_3$) δ: 8.56 (s, 1H, H-2); 8.48 (d, 1H, H-6); 8.42 (d, 1H, H-9); 7.58 (dd, 1H, H-7); 3.29 (s, 3H, N−CH_3); 3.18 (s, 3H, NCH_3); 2.41 (s, 3H, CCH_3). Anal. ($C_{14}H_{13}N_6O_2Cl$) C, H, N.

General Procedure for Synthesis of 3a and 3b. To a suspension of intermediate **2a** or **2b** (0.13 mmol) in AcOH (3 mL) was added hydrazine hydrate (0.30 mmol, 0.05 mL), and the reaction was kept at 90−100 °C for 2 h. The residue was filtered and recrystallized from a suitable solvent.

3-(1,2,4-Triazol-3-yl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide, 3a. From **2a**; orange crystals. IR ν cm^{-1}: 3000−2500, 1600, 1510. ^{1}H NMR (DMSO-d_6) δ: 8.69 (s, 1H, H-2); 8.56 (bs, 1H, CH triazole); 8.46 (d, 1H, H-6); 8.42 (d, 1H, H-9); 7.80 (dd, 1H, H-7). Anal. ($C_{11}H_6N_7OCl$) C, H, N.

3-((1,2,4-Triazol-5-methyl)-3-yl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide, 3b. From **2b**; orange crystals. IR ν cm^{-1}: 3280, 1600, 1540. ^{1}H NMR (DMSO-d_6) δ: 8.63 (s, 1H, H-2); 8.44 (d, 1H, H-6); 8.38 (d, 1H, H-9); 7.78 (dd, 1H, H-7); 2.42 (s, 3H, CH_3). Anal. ($C_{12}H_8N_7OCl$) C, H, N.

General Procedure for Synthesis of 4a and 4b. A suspension of compound **3a** or **3b** (0.35 mmol) in anhydrous DMF (15 mL) was added to a suspension of 50% NaH (0.76 mmol, 18.2 mg) and an excess of CH_3I (0.05 mL) and was kept at 50−60 °C for 18 h. The final solution was evaporated and the residue purified by recrystallization with 50% AcOH.

3-((1,2,4-Triazol-1(2)(4)-methyl)-3-yl)-8-chloropyrazolo-[5,1-*c*][1,2,4]benzotriazine 5-Oxide, 4a. From **3a**; orange crystals. IR ν cm^{-1}: 1600, 1520. ^{1}H NMR ($CDCl_3$) δ: 8.62 (s, 1H, H-2); 8.48 (m, 2H, H-6, H-9); 8.14 (s, 1H, CH triazole); 7.58 (dd, 1H, H-7); 4.03 (s, 3H, NCH_3). Anal. ($C_{12}H_8N_7OCl$) C, H, N.

3-((1,2,4-Triazol-1(2)(4),5-dimethyl)-3-yl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide, 4b. From **3b**; orange crystals. IR ν cm^{-1}: 1600, 1520. ^{1}H NMR ($CDCl_3$) δ: 8.58 (s, 1H, H-2); 8.50 (d, 1H, H-6); 8.46 (d, 1H, H-9); 8.57 (dd, 1H, H-7); 3.91 (s, 3H, NCH_3); 2.54 (s, 3H, CCH_3). Anal. ($C_{13}H_{10}N_7OCl$) C, H, N.

3-((1,2,4-Oxadiazol-3-methyl)-5-yl)-8-chloropyrazolo-[5,1-*c*][1,2,4]benzotriazine 5-Oxide, 5b. A suspension of **2b** (0.45 mmol, 150 mg) and NH_2OH hydrochloride (0.66 mmol, 50 mg) in dioxane (2.0 mL) was added to AcOH (2.0 mL) and a 10% aqueous solution of NaOH (0.4 mL). The suspension was kept at 100 °C for 5 h and monitored by TLC (CH_2Cl_2/MeOH, 10:0.5 v/v, as eluent). The final precipitate was filtered and purified by recrystallization: yellow crystals. IR ν cm^{-1}: 1600, 1550. ^{1}H NMR ($CDCl_3$) δ: 8.65 (s, 1H, H-2); 8.50 (d, 1H, H-6); 8.48 (d, 1H, H-9); 7.70 (dd, 1H, H-7); 2.50 (s, 3H, CH_3). Anal. ($C_{12}H_7N_6O_2Cl$) C, H, N.

3-(*N*-Formylcarbamoyl)-8-chloropyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-Oxide, 6. From compound **2a** (0.47 mmol, 150 mg) by treatment with H_2O/AcOH at 70−80 °C. On cooling the precipitate of the desired compound was formed: yellow crystals. IR ν cm^{-1}: 3220, 1730, 1680, 1560. ^{1}H NMR (DMSO-d_6) δ: 11.24 (bs, 1H, NH, exchang.); 9.25 (s, 1H, CHO); 8.92 (s, 1H, H-2); 8.50 (m, 2H, H-6, H-9); 7.88 (dd, 1H, H-7). Anal. ($C_{11}H_6N_5O_3Cl$) C, H, N.

3-(*N*-(Hydroxylaminomethylene)carbamoyl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide, 7. Compound **2a** (0.33 mmol, 100 mg) and NH_2OH hydrochloride (0.46 mmol, 30 mg) in dioxane (1.0 mL) were added to AcOH (1.0 mL) and a 10% aqueous solution of NaOH (0.2 mL). The suspension was kept at room temperature for 2 h, and the final precipitate was filtered and recrystallized: yellow crystals. IR ν cm^{-1}: 3320−3140, 1670, 1610, 1560. ^{1}H NMR (DMSO-d_6) δ: 11.04 (s, 1H, OH, exchang.); 10.00 (d, 1H, NH, exchang.); 8.82 (s, 1H, H-2); 8.52 (m, 2H, H-6, H-9); 7.91 (dd, 1H, H-7); 7.78 (d, 1H, CH, exchang.). Anal. ($C_{11}H_7N_6O_3Cl$) C, H, N.

3-((*tert*-Butoxycarbamoyl)amino)-8-chloropyrazolo-[5,1-*c*][1,2,4]benzotriazine 5-Oxide, 9. Compound **8**[1] (0.19 mmol, 50 mg) was suspended with NEt_3 and diphenyl phosphorazidate (DPPA), in equimolar amount, in *tert*-butyl alcohol (5 mL). The reaction was kept at refluxing temperature for 8 h, monitored by TLC (toluene/EtOAc/AcOH, 8:2:1 v/v/v, as eluent), and the final solution was evaporated to dryness obtaining a residue that was purified by recrystallization: red crystals. IR ν cm^{-1}: 3360, 1690, 1520. ^{1}H NMR ($CDCl_3$) δ: 8.56 (s, 1H, H-2); 8.43 (d, 1H, H-6); 8.30 (d, 1H, H-9); 7.51 (dd, 1H, H-7); 6.85 (bs, 1H, NH, exchang.); 1.50 (s, 9H, $(CH_3)_3$). Anal. ($C_{14}H_{14}N_5O_3Cl$) C, H, N.

3-Amino-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide, 10. Compound **9** (0.15 mmol, 50 mg) was hydrolyzed in a solution of 16 M HCl (1.0 mL) and concentrated AcOH (1.5 mL) for 1 h at refluxing temperature. The reaction was monitored by TLC (toluene/EtOAc/AcOH, 8:2:1 v/v/v, as eluent). The residue obtained by alkalinization with a 10% aqueous solution of NaOH was filtered and purified: red crystals. IR ν cm^{-1}: 3440−3360, 1570. ^{1}H NMR ($CDCl_3$) δ: 8.41 (d, 1H, H-6); 8.19 (d, 1H, H-9); 7.73 (s, 1H, H-2); 7.45 (dd, 1H, H-7); 3.66 (bs, 2H, NH_2, exchang.). Anal. ($C_9H_6N_5OCl$) C, H, N.

3-(Pyrrol-1-yl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide, 11. A suspension of compound **10** (0.42 mmol, 100 mg) in dimethoxytetrahydrofurane (0.42 mmol, 0.05 mL) and AcOH (6.0 mL) was refluxed for 2 h, following a described procedure.[21] The obtained solution was then evaporated to dryness and the residue purified: red crystals. IR ν cm^{-1}: 1570. ^{1}H NMR (DMSO-d_6) δ: 8.72 (s, 1H, H-2); 8.42 (d, 1H, H-6); 8.35 (d, 1H, H-9); 7.76 (dd, 1H, H-7); 7.45 (m, 2H, H-3′, H-4′ pyrr.); 6.34 (m, 2H, H-2′, H-5′ pyrr.). Anal. ($C_{13}H_8N_5OCl$) C, H, N.

General Procedure for Synthesis of 12c and 12d. The suitable 3-oxopropanenitrile (**c**,**d**) was reacted with 5-chloro-2-nitrophenylhydrazine following a reported method.[4]

1-(2-Nitro-5-chlorophenyl)-4-(thien-2-yl)-5-aminopyrazole, 12c. From oxopropanenitrile **c**; reaction run under nitrogen; orange crystals, yield 35%; mp 126−127 °C after recrystallization from EtOH/H_2O. IR ν cm^{-1}: 3420−3280. ^{1}H NMR ($CDCl_3$) δ: 7.98 (d, 1H, H-3′); 7.70 (m, 2H, H-6′, H-3); 7.58 (dd, 1H, H-4′); 7.26 (m, 1H, H-4″ 4-thienyl); 7.08 (m, 2H, H-2″, H-3″ 4-thienyl); 3.90 (bs, 2H, NH_2, exchang.). Anal. ($C_{13}H_9N_4O_2SCl$) C, H, N.

1-(2-Nitro-5-chlorophenyl)-4-(thien-3-yl)-5-aminopyrazole, 12d. From oxopropanenitrile **d**; orange crystals, yield 40%; mp 133−135 °C after recrystallization from ethanol/water. IR ν cm^{-1}: 3460−3180. ^{1}H NMR ($CDCl_3$) δ: 7.95 (d, 1H, H-3′); 7.70 (d, 1H, H-6′); 7.65 (s, 1H, H-3); 7.55 (dd, 1H, H-4′); 7.43 (dd, 1H, H-4″ 4-thienyl); 7.22 (m, 2H, H-2″, H-5″ 4-thienyl); 3.80 (bs, 2H, NH_2, exchang.). Anal. ($C_{13}H_9N_4O_2SCl$) C, H, N.

General Procedure for Synthesis of 13c and 13d. The cyclization to pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide system was obtained from the suitable 5-aminopyrazoles **12c** and **12d** in alkaline medium, according to a previously reported method.[1−4]

3-(Thien-2-yl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide, 13c. From **12c**; red crystals. IR ν cm^{-1}: 1570. ^{1}H NMR ($CDCl_3$) δ: 8.48 (d, 1H, H-6); 8.36 (d, 1H, H-9); 8.28 (s, 1H, H-2); 7.62 (dd, 1H, H-4′ 3-thienyl); 7.56 (dd, 1H, H-7); 7.34 (dd, 1H, H-2′ 3-thienyl); 7.12 (dd, 1H, H-3′ 3-thienyl). Anal. ($C_{13}H_7N_4OClS$) C, H, N.

3-(Thien-3-yl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide, 13d. From **12d**; red crystals. IR ν cm^{-1}: 1570; ^{1}H NMR ($CDCl_3$) δ: 8.48 (d, 1H, H-6); 8.38 (d, 1H, H-9); 8.32 (s, 1H, H-2); 7.88 (dd, 1H, H-2′ 3-thienyl); 7.62 (dd, 1H, H-5′ 3-thienyl); 7.54 (dd, 1H, H-7); 7.42 (dd, 1H, H-4′ 3-thienyl). Anal. ($C_{13}H_7N_4OClS$) C, H, N.

3-(Thien-2-yl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine, 13c′. From **13c** by treatment with triethyl phosphite according a previously cited method;[3] light-red crystals. IR ν cm^{-1}: 1610. ^{1}H NMR ($CDCl_3$) δ: 8.56 (d, 1H, H-6); 8.44 (m, 2H, H-2 and H-9); 7.92 (dd, 1H, H-4′ 3-thienyl); 7.70 (dd, 1H, H-7); 7.44 (dd, 1H, H-2′ 3-thienyl); 7.22 (dd, 1H, H-3′ 3-thienyl). Anal. ($C_{13}H_7N_4OClS$) C, H, N.

1-(2-Nitro-5-chlorophenyl)-5-aminopyrazole-4-acetonitrile, 14. The 2-nitro-5-chlorophenylhydrazine[1] (5.34 mmol, 1 g) was reacted with 2-oxosuccinonitrile (8.01 mmol, 865 mg) in EtOH at refluxing temperature. The reaction was monitored by TLC (toluene/EtOAc, 8:2 v/v, as eluent) until the starting material disappeared.The final solution was evaporated to dryness, and the final residue was purified by recrystallization with an 80% aqueous solution of EtOH: yellow crystals, yield 52%; mp 183−184 °C. IR ν cm^{-1}: 3400−3340, 2240. ^{1}H NMR (DMSO-d_6) δ: 8.10 (d, 1H, H-3′); 7.85 (d, 1H, H-6′); 7.75 (dd, 1H, H-4′); 7.40 (s, 1H, H-3); 5.80 (bs, 2H, NH_2 exch.); 3.70 (s, 2H, CH_2). Anal. ($C_{11}H_8N_5O_2Cl$) C, H, N.

1-(2-Nitro-5-chlorophenyl)-5-aminopyrazole-4-acetamide, 15. From **14** by treatment with concentrated H_2SO_4 at 60 °C. The end of the reaction was monitored by TLC ($CHCl_3$/MeOH, 10:1 v/v, as eluent). The final solution was added to ice−H_2O and made alkaline with aqueous NH_3. The obtained precipitate was filtered and recrystallized with H_2O: yellow crystals, yield 60%; mp 181−182 °C. IR ν cm^{-1}: 3400−3200. ^{1}H NMR (DMSO-d_6) δ: 8.05 (d, 1H, H-3′); 7.80 (d, 1H, H-6′); 7.70 (dd, 1H, H-4′); 7.25 (s, 1H, H-3); 7.20 (bs, 1H, NH exch.); 6.90 (bs, 1H, NH exch.); 5.40 (s, 2H, NH_2 exch.); 3.10 (s, 2H, CH_2). Anal. ($C_{11}H_{10}N_5O_3Cl$) C, H, N.

3-(Carbamoylmethyl)-8-chloropyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-Oxide, 16. From **15** by treatment with a 10% aqueous solution of NaOH and few milliliters of diethylene glycol dimethyl ether (diglyme) at room temperature. The final precipitate was filtered and purified: light-yellow crystals. IR ν cm^{-1}: 3400−3120, 1570. ^{1}H NMR (DMSO-d_6) δ: 8.45 (d, 1H, H-6); 8.35 (d, 1H, H-9); 8.25 (s, 1H, H-2); 7.75 (dd, 1H, H-7); 7.45 (bs, 1H, NH exch.); 7.05 (bs, 1H, NH exch.); 3.55 (s, 2H, CH_2). Anal. ($C_{11}H_8N_5O_2Cl$) C, H, N.

3-Carbomethyl-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide, 17. From **16** by treatment with concentrated H_2SO_4/$NaNO_2$ as previously described:[3] dark-yellow crystals. IR ν cm^{-1}: 3300−2800, 1570. ^{1}H NMR (DMSO-d_6) δ: 12.6 (bs, 1H, OH exch.); 8.42 (d, 1H, H-6); 8.34 (d, 1H, H-9); 8.26 (s, 1H, H-2); 7.74 (dd, 1H, H-7); 3.80 (s, 2H, CH_2). Anal. ($C_{11}H_7N_4O_3$-Cl) C, H, N.

3-(1-Oxo-2-(dimethylamino)vinyl)-8-chloropyrazolo-[5,1-*c*][1,2,4]benzotriazine 5-Oxide, 18. Following the method described in the literature,[13] anhydrous DMF (3.88 mmol, 0.301 mL) was added dropwise, with vigorous stirring, to $POCl_3$ (3.24 mmol, 0.3 mL), mantaining the temperature at maximum 30 °C. The mixture was stirring for 5 min; then compound **17** (1.08 mmol, 300 mg) solubilized in anhydrous DMF was added. The resulting solution was stirred at 70 °C for 2 h and then was poured on ice and neutralized by the addition of anhydrous K_2CO_3. The final suspension was made strongly alkaline with a 40% aqueous solution of NaOH, mantaining the temperature at 50 °C to completely eliminate the $NHMe_2$ which is formed during the reaction.The final precipitate was filtered and washed well with H_2O. The obtained product was enough pure for the next step: dark-red crystals. IR ν cm^{-1}: 1650, 1570. ^{1}H NMR ($CDCl_3$) δ: 9.14 (s, 1H, CHO); 8.43 (d, 1H, H-6); 8.35 (d, 1H, H-9); 8.10 (s, 1H, H-2); 7.54 (dd, 1H, H-7); 7.05 (s, 1H, $CHNMe_2$); 2.98 (s, 6H, NMe_2). Anal. ($C_{13}H_{12}N_5O_2Cl$) C, H, N.

3-(1-Oxo-2-hydroxyvinyl)-8-chloropyrazolo[5,1-*c*][1,2,4]-benzotriazine 5-Oxide, 19. Compound **18** (1.1 mmol, 350 mg) was suspended in 2.5 mL of diglyme and 2.0 mL of a 20% aqueous solution of NaOH, at 50 °C. After 2 h the starting product was completely hydrolyzed. The suspension was cooled and acified with 6 M HCl obtaining a red precipitate that was purified by recrystallization: red crystals. IR ν cm^{-1}: 1570. ^{1}H NMR ($CDCl_3$) δ: 9.20 (bs, 1H, C*H*OH); 8.88 (bs, 1H, H-2); 8.42 (m, 2H, H-6 and H-9); 7.58 (dd, 1H, H-7); 7.20 (d, 1H, CHO*H*); after exchange with D_2O the spectra appeared; 9.40 (s, 1H, CHO); 9.20 (s, 1H, C*H*OH); 8.88 (s, 1H, H-2); 8.42 (m, 2H, H-6 and H-9); 7.58 (dd, 1H, H-7). ^{1}H NMR (DMSO-d_6) δ: 8.70 (bs, 2H, CHO, C*H*OH); 8.42 (d, 1H, H-6); 8.36 (d, 1H, H-9); 8.26 (bs, H, H-2); 7.76 (dd, 1H, H-7). Anal. ($C_{13}H_7N_4O_2Cl$) C, H, N.

3-(Isoxazol-4-yl)-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-Oxide, 20. Compound **19** (0.180 mmol, 50 mg) was suspended in EtOH, and NH_2OH hydrochloride was added and maintained at refluxing temperature. The reaction was monitored by TLC ($CHCl_3$/MeOH, 10:1 v/v, as eluent), and after 3 h the final product was completely formed. The solvent was removed under reduced pressure, and the red residue was purified: red crystals. IR ν cm^{-1}: 1570. ^{1}H NMR ($CDCl_3$) δ: 9.20 (s, 1H, H-5 isox); 8.80 (s, 1H, H-3 isox); 8.50 (d, 1H, H-6); 8.38 (d, 1H, H-9); 8.26 (s, 1H, H-2); 7.60 (dd, 1H, H-7). Anal. ($C_{12}H_6N_5O_2Cl$) C, H, N.

Radioligand Binding Assay. [^{3}H]Ro15-1788 (s.a. 32.2 Ci/m mol^{-1}) was purchased from DuPont New England Nuclear (Boston, MA). All other reagents were obtained from commercial suppliers.

Bovine cerebral cortex membranes were prepared as previously described.[22] In brief, tissue was homogenized in10 volumes of ice-cold 0.32 M sucrose containing protease inhibitors. The homogenate was centrifuged for 5 min at 2000*g* at 4 °C, and the suspension was recentrifuged for 30 min at 30000*g* at 4 °C. The resulting pellet was resuspended in 50 mM Tris-citrate buffer at pH 7.4 (buffer T), homogenized, and centrifuged for 30 min at 30000*g* at 4 °C. The resulting pellet was subjected to washing procedures to remove endogenous GABA.[23] For this purpose the membranes were frozen, thawed, resuspended in 32 volumes of T buffer, containing 0.01% Triton X-100 and protease inhibitors, homogenized, incubated for 60 min at 37 °C, and centrifuged for 30 min at 30000*g* at 4 °C. The pellet was resuspended in buffer T, homogenized, and recentrifuged. The washing procedure was repeated three times. The washed membranes were incubated with [^{3}H]Ro15-1788 (~0.2 nM, K_d = 0.60 nM) for 90 min at 0 °C in 500 μL of buffer T. The incubation was terminated by dilution to 5 mL with ice-cold buffer T, followed immediately by rapid filtration through glass fiber Whatman GF/C filters. The filters were then washed (2 × 5 mL) with buffer T, and the amount of radioactivity retained on the filters was determinated by Packard 1600 TR liquid scintillation counter at 66% efficiency. Nonspecific binding was estimated in the presence of 10 μM diazepam. The compounds were dissolved in EtOH (or DMSO) and tested at a concentration of 10 μM. For the active compounds, the IC_{50} values were determined and K_i values were derived according to the equation of Cheng and Pursoff.[24] Protein concentration was assayed by the method of Lowry et al.[25] IC_{50} determination for GABA ratio values was carried out in the absence and presence of 10 μM GABA.

Pharmacological Methods. Male Swiss−Webster mice weighing 22−26 g (Harlan Nossan) were used. Twelve mice were housed per cage. The cages were taken into the experimental room 24 h before testing and left under a normal 12-h light/dark cycle (lights on 7:00 h). The animals were fed a standard laboratory diet and tap water ad libitum. All experiments were conducted between 9:00 and 17:00.

Drugs: The following commercial drugs were used diazepam (valium 10, Roche), flumazenil (Ro15-1788, Roche), and pentylenetetrazole (Sigma). Drug concentrations were prepared in such a way that the necessary dose could be injected in a 10 mL/kg volume of carboxymethylcellulose (CMC), 1%.

Rota-rod test:[26] The apparatus consisted of a base platform and a rotating rod of 3-cm diameter with a nonslippery surface. This rod was placed at a height of 15 cm from the base. The integrity of motor coordination was assessed at a rotating speed of 24 rpm, counting the number of falls from the rod in 30 s, 25 min after treatment. The statistical analysis was performed by means of the Student's *t*-test.

Anticonvulsant effects: The tests used for evaluation of anticonvulsant activity were the pentylenetetrazole (PTZ)-induced convulsions (quantal effects) and the maximal electroshock (MES) test. The first test was performed by using PTZ at a dose (75 mg/kg sc) which induced generalized tonic-clonic seizure in 88.4% of control mice observed during the following 30 min. MES-induced convulsions in mice were performed via transcorneal electrodes by means of a Hugo Sachs Elektronik stimulator (40 mA, 50 Hz, 0.2 s). The chosen parameters were able to cause tonic hindlimb extension in 92.3% of control mice. All the animals were treated 30 min before the PTZ- or MES convulsant stimulus. The incidence of nonconvulsant mice was evaluated using χ^2-statistical analysis.

Assessment of anxiolytic activity: The light−dark box (length 500 mm, width 205 mm, height 190 mm) was constructed from two equal compartments, one white (lighted) and the other black (dark), which were separated by a driver with a 100 × 32 opening at floor level. A 60-W white bulb was positioned 400 mm above the white compartment floor. The experimental room was slightly illuminated. Mice were treated po with either drugs or CMC and 30 min later individually placed into the center of the lighted side of the box, facing away from the divider. Behavior was observed for 5 min, and time spent in the illuminated side of the box and the number of crosses from one side to the other were measured. A crossover was counted when all four paws were in the opposite side of the box. Statistical analysis was performed by means of Student's *t*-test.

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