Benzodiazepine Receptor Ligands. 7. Synthesis and Pharmacological Evaluation of New 3-Esters of the 8-Chloropyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide. 3-(2-Thienylmethoxycarbonyl) Derivative: An Anxioselective Agent in Rodents

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The synthesis and binding study of new 8-chloropyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide 3-ester compounds are reported. A pharmacological evaluation of the high-affinity ligands **1**−**4** belonging to the 3-heteroarylester series is made. The 3-(2-thienylmethoxycarbonyl) derivative **4** stands out from the other heteroarylesters and is found, using nine different behavioral methods, to be a functionally selective ligand in vivo: it shows anxiolytic-like activity in the conflict models (light-dark box and plus maze test) similarly to diazepam, without any sedative and amnesic properties or interference from alcohol.

Introduction

The $GABA_A$-receptor complex, which is directly associated with a chloride channel, represents the major inhibitory system in the mammalian brain. This receptor complex consists of five subunits, arranged in the membrane in cylindrical fashion to form the central ion channel. At present, a total of 21 subunits (6α, 4β, 4γ, 1δ, 1ϵ, 1π, 1θ, and 3ρ) have been cloned and sequenced. Although there is evidence for the existence of several different receptor types, most $GABA_A$ receptors are composed of α-, β-, and γ- subunits, and the sensitivity to benzodiazepines (BZs) is conferred by the γ subunit (γ_2 genetic variant) and adjacent α subunit (α_1, α_2, α_3, α_5 genetic variants).

The classical BZs bind all BZs-sensitive $GABA_A$ receptor subtypes. The heterogeneity of the receptor associated with a regionally distinct distribution in the central nervous system (CNS) has been indicated as the main factor responsible for the therapeutic actions displayed by classical BZs (in anxiety disorders, sleep disturbance, muscle spasm, and epilepsy) but also for their undesirable side effects such as memory impairment, cognition and motor disturbances, the potentiation of the alcohol effect, tolerance, and physical dependence. On the other hand, recent studies strongly suggest that a particular behavioral response might be associated with an action at a different $GABA_A$ receptor subtype.[1−4]

In particular, it is known from the literature that the α subunit may influence the ligands efficacy and their selective pharmacological actions. In fact, from independent research studies, it has been evidenced that sedative and anticonvulsant actions are mediated through α_1-containing $GABA_A$ receptors; α_2-containing $GABA_A$ receptors may be involved in anxiolytic-like activity, since these have a preferential brain distribution in circuits mediating emotional behavior. Moreover, α_2-, α_3-, or α_5-containing $GABA_A$ receptors may mediate the muscle relaxant effect.[5−15] These findings emphasize the need to find selective ligands to clarify the pharmacological role of subtype receptors and to obtain drugs that are devoid of side effects.

As an extension of our studies on benzodiazepine receptor (BZR) ligands, we recently reported two new series of 8-chloropyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide 3-ester and 3-heteroaryl derivatives, respectively, which showed a high affinity for BZR and a selective in vivo activity.[16−20]

Interaction with receptors has been proposed for these new ligands,[21] and the pharmacophoric descriptors (L_1/L_2/L_3/L_{Di}; H_1/H_2; S_1/S_2/S_3) are in accordance with those of the pharmacophore/receptor models formulated by other research groups.[3−4]

The pyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide system may bind the BZR through N1 and N4 atoms by means of a hydrogen bond involving H_2 and H_1 donor sites in the receptor protein. In particular, it has been focused that lipophilic, steric, and electronic features of the 3-substituent influence binding affinity; therefore, its interaction in a lipophilic pocket of limited size in the lipophilic region (L_1/L_2) can be supposed. Moreover, since the size of this lipophilic pocket may differ in various subtype receptors,[4] the particular fitting of the 3-substituent may be responsible for the in vivo selectivity observed.

In the 3-ester derivatives,[16−18] the substitution of the alkyl chain of the 3-ethoxycarbonyl group (**I**,[16] K_i = 35 nM in Chart 1) with an unsaturated chain (as in 3-(2-propinyl) ester[17] K_i = 21 nM) or a benzyl group (as in 3-benzyl ester[17] K_i = 11 nM and 3-(2-methoxy)benzyl

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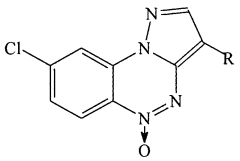
Chart 1

I R= COOEt

II R=$COOCH_2$-2-OMePh

III R= 3-thienyl

IV R= 2-furyl



ester[18] K_i = 1.0 nM, **II** in Chart 1) significantly enhanced the binding affinity. In the ester series, the lone pair orientation of the carbonyl oxygen electron reinforces the binding with receptor protein by means of a three-centered hydrogen bond (N4/H_1/CO). Moreover, a $\pi-\pi$ stacking interaction between the receptor and the 3-π-electron donor substituent (propinyl- or phenyl ring)[17,18] occurs and improves ligand affinity. The auxiliary role of the 5-oxide group to reinforce binding seems to be negligible in the 3-benzyl ester derivative, probably because the $\pi-\pi$ interaction works together in a more significant manner at anchorage than does the 5-oxide group that is indeed necessary in the 3-alkyl ester derivatives.

The in vivo tests showed that the 3-ester derivatives were endowed with a prevalently anxiolytic effect. In particular, the 3-(2-methoxybenzyloxycarbonyl) derivative **II** was a significantly selective anxiolytic at a dose of 30 mg/kg.[18]

In the 3-heteroarylpyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide derivatives,[19−20] the presence of five-member electron-rich rings, such as pyrrole, tiophene, and furan, gave compounds with high affinity with respect to derivatives bearing a five-member electron-poor ring (isoxazole, triazole, oxadiazole) at the same 3-position. The binding affinity of these ligands appears clearly influenced by electronic parameters of the 3-heterocyclic substituent, capable per se of providing $\pi-\pi$ interaction with the BZ receptor. Moreover, for these compounds the important role of the 5-oxide group reinforcing the hydrogen bond (N4/H_1 receptor) was also confirmed.[19,20] In animal models, the 3-heteroaryl derivatives are endowed with prevalently anticonvulsant activity, with the exception of the 3-(2-furyl)-8-chloropyrazolo[5,1-c]-[1,2,4]benzotriazine 5-oxide, which manifested a selectively anti-anxiety effect in a wide range of doses. For this latter compound, it is reasonable to believe that the lone-pair orientation of the α-oxygen atom furan ring can form a weak three-centered hydrogen bond which closely correlates the 3-(2-furyl) derivative to the 3-ester ligands, which showed predominantly anxiolytic-like effects.[20] From these results, it is clearly shown that chemical modifications at the 3-position of the pyrazolo-[5,1-c][1,2,4]benzotriazine 5-oxide system can influence the binding affinity and in vivo selectivity.

Within this context, to improve our knowledge of the main structural requirements needed for a high affinity and, possibly, a well-defined intrinsic activity, we synthesized a new series of 3-(heteroarylmethoxycarbonyl)-8-chloropyrazolo[5,1-c][1,2,4]benzotriazines and their 5-oxides. These new compounds, bearing an ester group and an electron-rich ring in the 3-position, possess all the favorable features of both the previously studied series.[16,20] In this new series of compounds, the importance of $\pi-\pi$ stacking interaction has been evidenced by introducing electron-rich, electron-poor, and saturated rings. Moreover, the effective role of the 5-oxide group has been further studied by synthesizing some 5-deoxyderivatives.

Moreover, to evaluate the size of supposed lipophilic pocket into which 3-substituent fits, compounds **7** and **8** were synthesized. The (±)1-phenylethoxycarbonyl derivative **7** and isopropylcarbonyl derivative **8** show a more steric hindrance than reference compounds, benzyl ester[17] and ethyl ester **I**,[16] respectively.

With the aim of extending studies of the structure−activity relationship (SAR), we concentrated on the importance of the distance between the 3-ester group and the pyrazolobenzotriazine system. We then introduced a methylene spacer, obtaining 3-aryloxy- and alkyloxycarbonylmethyl derivatives.

Chemistry

All pyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide (**1**−**8**, **11**−**19**) and the 5-deoxyderivatives (**9**−**10**) described here are listed in Table 1.

The starting materials for the synthesis of both types of 3-ester derivatives (**1**−**8** and **11**−**19**) were 3-carboxy-8-chloropyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide (**A**) and 3-carboxymethyl-8-chloropyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide (**B**), respectively, the syntheses of which have been previously described.[19,22] These carboxylic acids were treated with thionyl chloride and the suitable alcohol in 2-methyl-3-butene stabilized chloroform was added (Scheme 1). Compounds **9** and **10** obtained from 3-(2-methoxybenzyloxycarbonyl)pyrazolo[5,1-c][1,2,4]-benzotriazine 5-oxide[18] and from compound **4**, respectively, by treatment with triethyl phosphite/toluene,[16] were considered useful tools for SAR study.

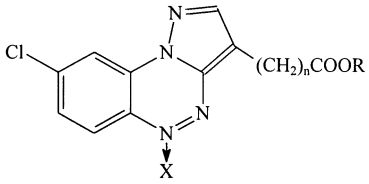
Results and Discussion

Biological Results. The BZR binding affinity of new derivatives **1**−**19** was evaluated by their ability to displace [^{3}H]-flumazenil ([^{3}H]Ro15-1788) from its specific binding in bovine brain membranes. Binding data and the GABA ratio for new compounds **1**−**19** and for reference compound 3-(2-methoxybenzylcarbonyl)-8-chloropyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide[18] **II** (see Chart 1), diazepam (Daz), and flumazenil (Flu), all useful for the following discussion of SAR, are reported in Table 2.

As can be observed, the co-presence in the 3-position of the ester function and of the π-excessive ring in the 5-oxide derivatives **1**−**4** yielded ligands with high affinity (K_i range 6.80−24.4 nM) that were comparable to those of the 3-heteroarylderivatives[19,20] (K_i range 10.3−36 nM), even if they were lower than that of lead compound 3-(2-methoxybenzylcarbonyl) derivative **II** (K_i = 1.0 nM). Intrinsic activity evaluated by means of the GABA ratio was in the partial agonist−agonist range (GR 1.72−2.07).

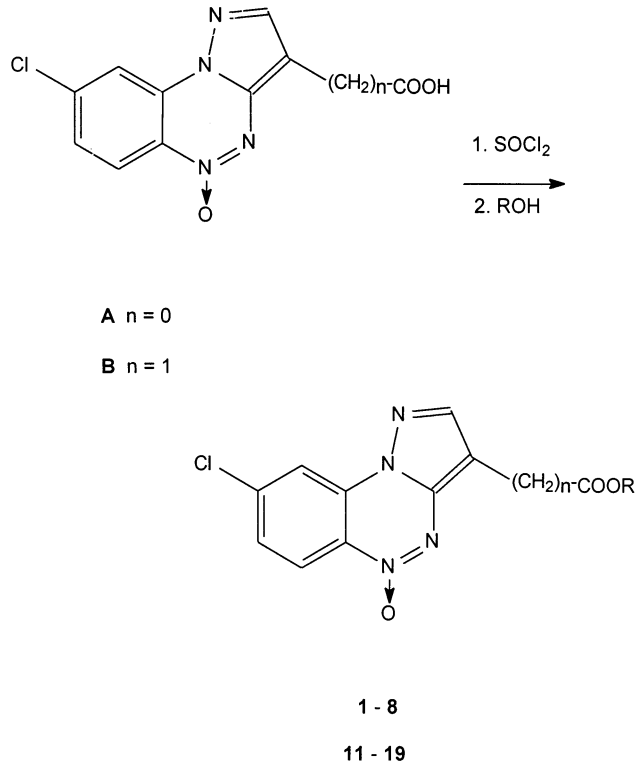
Replacement of the phenyl ring of lead compound with the 4-pyridine, electron-poor six-member heteroaryl ring, in compound **5**, was unfavorable to BZR binding (**5**: K_i = 44.5 nM vs **II**: K_i = 1.0 nM). Significantly, the 3-(4-pyridylmethoxycarbonyl)-8-chloropyrazolo[5,1-c]-[1,2,4]benzotriazine 5-oxide is endowed with an affinity value comparable to that of the 3-(4-nitrobenzyloxycarbonyl) derivative (K_i 41.8 nM).[18]

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



comp	n	R	X	MW (MF)	yield (%)	mp °C (recryst solvent)
1	0	−CH_2–3-furyl	O	344.72 ($C_{15}H_9N_4O_4Cl$)	35	164−5° (ethanol)
2	0	−CH_2–2-furyl	O	344.72 ($C_{15}H_9N_4O_4Cl$)	53	161−3° (ethanol)
3	0	−CH_2–3-thienyl	O	360.8 ($C_{15}H_9N_4O_3ClS$)	88	205−7° (ethanol)
4	0	−CH_2–2-thienyl	O	360.8 ($C_{15}H_9N_4O_3ClS$)	40	173−4° (ethanol)
5	0	−CH_2–4-Py	O	355.75 ($C_{16}H_{10}N_5O_3Cl$)	35	203−4° (ethanol)
6	0	−CH_2–3-tetrahydrofuryl	O	348.74 ($C_{15}H_{13}N_4O_4Cl$)	45	151−2° (ethanol)
7	0	(±)−$CH(CH_3)$Ph	O	368.78 ($C_{18}H_{13}N_4O_3Cl$)	43	132−4° (ethanol 80%)
8	0	−$CH(CH_3)_2$	O	306.59 ($C_{13}H_{11}N_4O_3Cl$)	45	160−1° (2-propanol)
9	0	−CH_2–2-MeOPh		368.78 (C_{18} $H_{13}N_4O_3Cl$)	35	189−0° (2-propanol)
10	0	−CH_2–2-thienyl		344.8 ($C_{15}H_9N_4O_2ClS$)	42	164−5° (2-propanol)
11	1	−CH_2Ph	O	368.78 (C_{18} $H_{13}N_4O_3Cl$)	60	156−7° (ethanol)
12	1	−CH_2–2-MeOPh	O	398.8 ($C_{19}H_{15}N_4O_4Cl$)	42	177−8° (ethanol)
13	1	−CH_2–3-thienyl	O	374.8 ($C_{16}H_{11}N_4O_3ClS$)	33	112−3° (ethanol)
14	1	−CH_2–2-thienyl	O	374.8 ($C_{16}H_{11}N_4O_3ClS$)	25	148−9° (ethanol)
15	1	−CH_2–CH_3	O	306.59 ($C_{13}H_{11}N_4O_3Cl$)	36	156−7° (ethanol)
16	1	−Ph	O	354.76 ($C_{17}H_{11}N_4O_3Cl$)	30	131−3° (ethanol/water)
17	1	−2-ClPh	O	389.2 ($C_{17}H_{10}N_4O_3Cl_2$)	40	126−7° (ethanol 80%)
18	1	−2-MeOPh	O	384.78 ($C_{18}H_{13}N_4O_4Cl$)	40	127−8° (ethanol)
19	1	−2-MePh	O	368.78 (C_{18} $H_{13}N_4O_3Cl$)	40	124−5° (ethanol 80%)

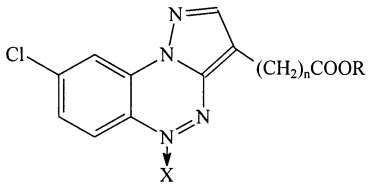
Scheme 1



Interestingly, when the 3-furyl ring of ester derivative **1** was replaced with the 3-tetrahydrofuryl ring, as in compound **6**, the lack of $\pi-\pi$ system and the more steric hindrance of the saturated ring were detrimental to binding (Ki 11.4 vs Ki 680 nM, respectively). Compound **7** (K_i 138 nM), having a chiral carbon, was tested in racemic mixture and displayed very moderate affinity. These results are probably related to higher steric hindrance of the 3-substituent, which cannot fit into the lipophilic pocket, rather than to stereochemical implications. The limited size of lipophilic pocket is further confirmed by the minor affinity of compound **8** with respect to **I** (**8**: K_i = 272 nM vs **I**:[16] K_i = 35 nM).

New results as reported above were consistent with our hypothesis of the importance to binding of a $\pi-\pi$ stacking interaction between a receptor limited-size

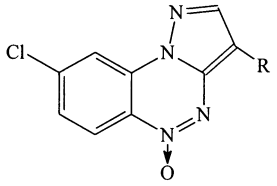
Table 2. BZR Ligand Affinity of Pyrazolo[5,1-c][1,2,4]benzotriazine Derivatives



comp	n	R	X	*I* %[a]	K_i (nM)[b]	GR[c]
1	0	−CH_2–3-furyl	O	96 ± 3	11.4 ± 1.1	2.07
2	0	−CH_2–2-furyl	O	100 ± 8	24.4 ± 2.0	1.94
3	0	−CH_2–3-thienyl	O	97 ± 6	13.3 ± 1.1	1.72
4	0	−CH_2–2-thienyl	O	93 ± 7	6.80 ± 0.40	1.88
5	0	−CH_2–4-Py	O	100 ± 8	44.5 ± 2.9	1.62
6	0	−CH_2–3-tetrahydrofuryl	O	85 ± 3	680 ± 20	
7	0	(±)−$CH(CH_3)$Ph	O	95	138 ± 10	1.50
8	0	$CH-(CH_3)_2$	O	97 ± 4	272 ± 26	
9	0	−CH_2–2-MeOPh		97 ± 3	1.70 ± 0.20	1.29
10	0	−CH_2–2-thienyl		96 ± 3	6.26 ± 0.50	1.91
11	1	−CH_2Ph	O	32 ± 2		
12	1	−CH_2–2-MeOPh	O	50 ± 4		
13	1	−CH_2–3-thienyl	O	45 ± 3		
14	1	−CH_2–2-thienyl	O	35 ± 6		
15	1	−CH_2–CH_3	O	53 ± 5		
16	1	−Ph	O	79 ± 7		
17	1	−2-ClPh	O	87 ± 1	489 ± 48	1.27
18	1	−2-MeOPh	O	88 ± 2	171 ± 10	1.33
19	1	−2-MePh	O	70 ± 6		
II[d]	0	−CH_2–2-MeOPh	O	100 ± 8	1.00 ± 0.10	1.33
Daz[e]					10.0 ± 1.1	1.50
Flu[e]					0.9 ± 0.05	0.90

[a] Percent of inhibition of specific [^{3}H]Ro15-1788 binding at 10 μM concentration are means ±SEM of five determinations. [b] K_i values are means ±SEM of five determinations. [c] GR (GABA ratio) = IC_{50} compounds/IC_{50} compounds + 10 μM GABA. [d] See ref 18. [e] See ref 20.

Table 3. Affinity Value at Recombinant $\alpha_x\beta_2\gamma_2$ and $\alpha_5\beta_3\gamma_2$ $GABA_A$/BZ Subtypes



		K_i[a] nM			
comp	R	α_1	α_2	α_3	α_5
4	$-COO-CH_2$–2-thienyl	3.00 ± 0.40	26.0 ± 3.0	12.0 ± 2.0	8.60 ± 0.70
III[b]	−3-thienyl	4.10 ± 0.40	12.0 ± 1.0	33.0 ± 4.0	18.0 ± 2.0
diazepam[c]		14.0 ± 2.1	7.80 ± 1.1	13.9 ± 0.5	9.80 ± 1.1
zolpidem[d]		26.7	156	383	>10000

[a] K_i values represent the means ±SEM derived from three independent experiments, conducted in triplicate. [b] See ref 19. [c] Data obtained from ref 9 for comparison purposes. [d] Data obtained from ref 3 for comparison purposes.

pocket (π-electron acceptor) and the 3-substituent (π-electron donor).

Deoxiderivatives **9** and **10** displayed high affinity, comparable to the 5-oxide counterparts **4** and lead compound **II**.[18] This result also showed that the carbonyl group of ester function in the 3-arylester derivatives plays a crucial role in binding, forming a stable three-centered hydrogen bond (N4/CO/H1), while the co-presence of the 5-oxide is negligible, unlike the derivatives of the 3-heteroaryl series.

A general loss of BZR recognition was evidenced for compounds **11**−**19**, indicating that the methylene spacer between the pyrazolobenzotriazine system and the ester function is always detrimental to binding. With the exception of compounds **17** and **18**, which showed low affinity values (**17**: $K_i = 489$ nM and **18**: $K_i = 171$ nM), the increased distance of the carbonyl group from the tricyclic system in all compounds did not permit the molecule to fit into the lipophilic pocket.

Binding of Selected Compounds at $\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_2\gamma_2$, $\alpha_3\beta_2\gamma_2$, and $\alpha_5\beta_3\gamma_2$ $GABA_A$/BZ Receptor Subtypes

Compounds **4,** which showed a notable selective anxiolytic activity (see pharmacological section), and **III** (see Chart 1), which represent the prototype ligand of the 3-heteroaryl series with good affinity in the cerebral cortex (K_i = 36.3 nM) and selective anticonvulsant activity,[19] were tested for their ability to displace [^{3}H]-Ro15-1788 from recombinant rat $\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_2\gamma_2$, $\alpha_3\beta_2\gamma_2$, and $\alpha_5\beta_3\gamma_2$ $GABA_A$/BZ receptor subtypes, which are stably expressed in human embryonic kidney cells (HEK293), in comparison with diazepam (full agonist) and zolpidem (α_1-selective agonist). As can be observed in Table 3, compounds **4** and **III** recognized all $GABA_A$/BZ receptor subtype studied and do not display pronounced preferential subtype selectivity. Both compounds show higher affinity for α_1-subtype ($K_i = 3.0$ nM and 4.1 nM, respectively) than α_2-, α_3-, and α_5-receptor combinations.

Pharmacological Results

The effects of newly synthesized molecules **1**−**4** were studied using different behavioral methods. The muscle relaxant, anticonvulsant, and anxiolytic activities of these substances were evaluated and compared with those of the diazepam used as positive control. Compound **4**, which showed a notable selective anxiolytic activity, without any anticonvulsant properties and motor impairment, was selected for further profiling: effects on mouse learning and on short- and long-term memory and on ethanol potentiation.

a. Effects on Motor Coordination. The effects of compounds **1**−**4** on animal motor coordination were investigated, using the mouse rota rod test as a screening method, to discover any ataxic effect as compared to diazepam (Table 4). In a wide range of doses, from 1 to 100 mg/kg po, the number of falls from the rotating rod (24 rpm) caused by **1**, **3**, and **4** never exceeded those of the control group. No significant effect was observed with compound **2** at doses of 10 and 30 mg/kg po: some excitation in mice was observed only at the highest dosage used (100 mg/kg). On the contrary, diazepam, dose-dependently from the dosage of 1 mg/kg po up, significantly impaired mouse motor coordination, scoring 0.78 ± 0.09 falls from the rotating rod in 30 s, as compared to the 0.25 ± 0.06 falls of the vehicle-treated group. Compound **4** was able to significantly prevent diazepam-induced falls from the rotating rod, while the other substances (**1**−**3**) were unable to do so.

b. Study on Spontaneous Motility. To make a detailed study of the effects on motility, additional experiments were carried out to investigate whether compound **4** was able to change the animals' spontaneous motility and curiosity (Figure 1). The hole board test was used for this; diazepam and flumazenil were used as reference molecules. As reported in Figure 1, statistically speaking, only diazepam at the dosage of 3 mg/kg po significantly decreased the cumulative scores for both holes and plane, while the effects caused by **4** (1, 3, and 10 mg/kg po) and flumazenil (100 mg/kg ip) did not differ from those of the controls.

As reported by Rudolph et al.[23] and McKernan et al.,[6] the sedative actions of benzodiazepine are due to the activation of α_1 subtype receptors. In light of these observations, it might be possible to exclude the $GABA_A$/BZ receptor α_1-subtype activation by our substances, because of the total absence of ataxic and sedative effects of all the compounds (**1**−**4**) in the rota rod test and, for compound **4**, also in the hole board test. Since compound **4** was also able to prevent the diazepam-induced falls from the rotating rod, its high affinity for the α_1-subtype receptor (K_i 3.0 nM, see Table 3) might reflect the observed antagonistic action on the same α_1-subtype receptor.

c. Effects against Chemically and Electrically Induced Convulsions. Anticonvulsant activity was

Table 4. Motor Coordination, Anticonvulsant, and Anxiolytic-Like Effects of Compounds **1**−**4** in Comparison with Diazepam

		motor coordination		anticonvulsant activity	anxiolytic-like activity		
	mg/kg	rota rod test		against PTZ-induced attacks	light-dark box		
treatment[a]	po	n	no. of falls in 30 s	%	n	no. of transfers	time (%) in light
CMC 1%	0.1 mL	64	0.25 ± 0.06	17.1	21	12.5 ± 1.08	100.0 ± 7.3
diazepam	0.3	18	0.39 ± 0.12	50*	8	22.0 ± 2.7*	114.6 ± 12.5
	1	32	0.78 ± 0.09***	93.8***	23	23.2 ± 2.87***	159.3 ± 13.2***
	3	8	1.6 ± 0.46***	100***	10	16.5 ± 3.6	243.2 ± 34.5***
1	3	7	0.14 ± 0.14	0			
	10	10	0.20 ± 0.20	20	14	16.57 ± 1.45*	118.95 ± 10.04
	30	10	0.10 ± 0.10	30	9	20.4 ± 2.4**	132.27 ± 10.65*
	100	11	0.18 ± 0.12	36.4	9	19.7 ± 2.3**	127.18 ± 17.12
1+ diazepam	10	9	0.44 ± 0.17	88.9			
	1						
2	10	11	0.27 ± 0.14	27.3	14	15.71 ± 1.44	96.8 ± 6.47
	30	13	0.08 ± 0.08	7.7	10	18.6 ± 2.5*	125.02 ± 10.38
	100	11	0.45 ± 0.20[b]	27.3	9	14.0 ± 1.7	96.16 ± 9.71
2+ diazepam	10	9	1.55 ± 0.5	88.9	10	27.1 ± 3.64	142.02 ± 16.53
	1						
3	3	10	0.2 ± 0.13	30	9	15.7 ± 1.7	110.3 ± 7.1
	10	11	0.36 ± 0.16	18.2	14	16.0 ± 1.92	91.84 ± 8.36
	30	11	0.27 ± 0.14	9.0			
3+ diazepam	10	9	0.55 ± 0.24	100	9	27.9 ± 3.2	149.16 ± 16.72
	1						
4	1	10	0.1 ± 0.1	20	10	21.1 ± 2.4	128.9 ± 9.44*
	3	20	0.1 ± 0.1	0	12	15.6 ± 2.9	170.8 ± 15.6***
	10	13	0.15 ± 0.1	15.38	10	16.6 ± 1.5	155.1 ± 8.83***
	30	10	0.1 ± 0.1	40	10	20.5 ± 2.1**	144.3 ± 11.18**
	100	10	0 ± 0	20	10	14.6 ± 1.5	157.1 ± 16.93**
4+ diazepam	3	10	0.3 ± 0.15∧	50∧∧			
	1						
flumazenil+ **4**	100				14	23.0 ± 2.1	124.08 ± 9.31∧
	3						
flumazenil	30 ip	18	0.33 ± 0.14	11.1	10	11.8 ± 2.4	90.36 ± 14.16
	100 ip	19	0.4 ± 0.16	5.3	10	15.2 ± 1.7	119.0 ± 9.17
flumazenil + diazepam	30				10	23.0 ± 4.0	154.8 ± 21.03
	1						
flumazenil + diazepam	100	5	0.4 ± 0.24	20∧∧	5	15.6 ± 1.4	107 ± 8.63∧
	1						

[a] Treatment with compounds **1**−**4** and diazepam (po) was performed 30 min and flumazenil (ip) 40 min before the test. [b] Excitation. *P < 0.05, **P < 0.01, ***P < 0.001 vs control mice; ∧P < 0.05, ∧∧P < 0.01 vs **4** and diazepam-treated mice, respectively. (χ^2 analysis for anticonvulsant activity; otherwise Student's t-test.)

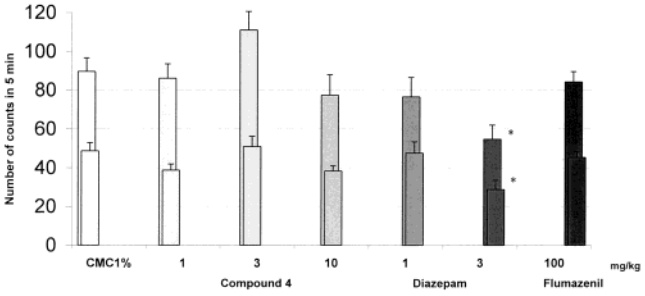


Figure 1. Effect of **4** on animal spontaneous motility, in comparison with diazepam and flumazenil, in the mouse hole board test. The test was performed 30, 20, and 40 min after administration of **4**, diazepam (po), and flumazenil (ip), respectively. Each column represents the mean ±SEM of 10−13 mice. The first plane columns represent cumulative scores of exploration of holes; the second plane columns, that of the plane. *p < 0.01 versus control mice (Student's t-test).

studied using two different kinds of convulsant stimuli: pentylenetetrazole (PTZ) for chemically induced convulsions (Table 4) and maximal electroshock seizure (MES) for electrically induced ones (only for **4**, Table 5). Diazepam (0.3, 1, 3 mg/kg po) dose-dependently and significantly protected mice from PTZ-induced convulsions (Table 4), while effects caused by **4** did not differ significantly from those of the controls. Likewise, diazepam (1, 3, 10 mg/kg po) protected the mice from MES-induced convulsions and from subsequent lethality. Compound **4**, at the dose of 30 mg/kg po, at which it had a little, although not significant, 40%-protective effect from PTZ-induced convulsions, in the MES-test behaved similarly to the vehicle, in regard to both the protection and lethality of mice. On the other hand, **4** was able to prevent the protective effect of diazepam on PTZ-induced convulsions (Table 4). None of the other newly synthesized molecules (**1**−**3**) demonstrated any anticonvulsant action; nor were they able to prevent the protective effect of diazepam on PTZ-

Table 5. Effect of **4** on Maximal Electroshock-Induced (MES) Convulsions and Lethality in Mice

		anticonvulsant activity		
	mg/kg	against MES-induced hind limb extension[a]		
treatment[a]	po	N[b]	%[d]	% lethality[c]
CMC 1%	0.1 mL	16	6.2	43.75
diazepam	1	10	90**	10
	3	10	100**	0*
	10	6	100**	0*
4	30	10	0	40

[a] Maximal electroshock (MES) = 40 mA, 0.2 s, 50 Hz. Tonic hind limb extension was considered as end point. Treatment was performed 30 min before MES. [b] N = number of mice. [c] *P < 0.05. [d] **P < 0.001 vs carboxymethylcellulose (CMC)-treated mice (χ^2 analysis).

induced convulsions. It has been reported[8,23] that the anticonvulsant effects are principally due to activation of the α_1 $GABA_A$/BZ receptor. Subsequent antagonism exerted by **4** against diazepam-induced protection was probably due, also in this case, to an antagonism on the α_1 subtype of this receptor.

d. Study of Anxiety in Mice. Effects on mouse anxiety of newly synthesized molecules and diazepam were studied using a light/dark apparatus. In our experiments, **2** and **3** had neither a significant agonist effect, nor were they able to prevent the anxiolytic-like effect of diazepam. Compound **1** was a weak anxiolytic at the dose of 30 mg/kg. On the contrary, **4** had a good anxiolytic-like effect in a large range of doses (Table 4). This was an effect that was significantly antagonized by flumazenil at the dose of 100 mg/kg ip, dose at which flumazenil was also able to antagonize the anxiolytic-like effect of diazepam.[19]

The potency of the anxiolytic-like activity of **4** was very similar to that of diazepam. From a dosage of 3 mg/kg po up to one of 100 mg/kg po, **4** prolonged the time in the light without any motor impairment as observed above.

Diazepam dose-dependently (0.3, 1, and 3 mg/kg po) increased both the time in the lighted area and the number of transfers from one compartment to the other. In the higher dosage of 3 mg/kg, diazepam no longer increased the number of transfers, reflecting its deleterious effects on motor coordination as evidenced with the rota rod test. Despite this, however, the mice chose to remain in the lighted compartment. On the other hand, even if **4** significantly prolonged the time in the light, compared to the vehicle-treated control group, its effect on the number of transfers made from one compartment to other did not change. Interestingly, on the basis of their observations, Young and Johnson[24] concluded that measurement of the time spent in the lighted area, but not the number of transfers, was the most consistent and useful parameter for assessing anxiolytic-like action. Furthermore, Lepicard et al.[25] reported that the time spent in the light was a stronger indication for the study of anxiety, whereas the number of transfers reflected both anxiety and exploration. These observations were in good agreement with our results.

e. Study of Anxiety in Rats. The elevated plus maze test was performed to verify whether the anxiolytic-like effect of **4** observed in mice could also be obtained in rats. This test is believed to produce unconditioned fear by a single exposure to open spaces and is considered a valid model for studying only substances that do not affect animal locomotor activity.[26] Consequently, the elevated plus maze is a very good model for studying **4**, that did not modify locomotor activity in either the rota rod or hole board tests. As can be observed in Figure 2, similarly to diazepam, **4** increased both the percentage entries into the open arms and, significantly, the percentage of time spent in the open arms, thus confirming its anxiolytic-like character also in rats.

As for the receptor subtypes responsible for anxiolytic-like effects, Löw et al.[11] and Möhler et al.[13] demonstrated in mice, with an α_2(H101R) knock-in point mutation, that diazepam no longer had an anxiolytic-like effect in either the light/dark box or in the elevated plus maze tests. However, its other effects, on locomotor activity and against PTZ-induced convulsions, remained unaltered. These authors therefore suggested that the anxiolytic-like effect of benzodiazepines is mediated by α_2 subtype $GABA_A$ receptors. McKernan et al.[6] do not exclude the participation also of the α_3 subtype in anxiolytic effects, since both these subtypes are highly located in two brain regions that mediate emotional behavior: in the amygdale and cortical regions. Moreover, Griebel et al.[9] also reported the anxiolylic-like effects of a new molecule selective for α_2- and α_3-containing $GABA_A$ receptors. The total absence of ataxic/sedative and anticonvulsant actions, the antagonism on α_1 subtype (see b and c sections) and the anxiolytic properties showed by **4**, make this compound a promising selective $\alpha_2/_3$ subtype agonist.

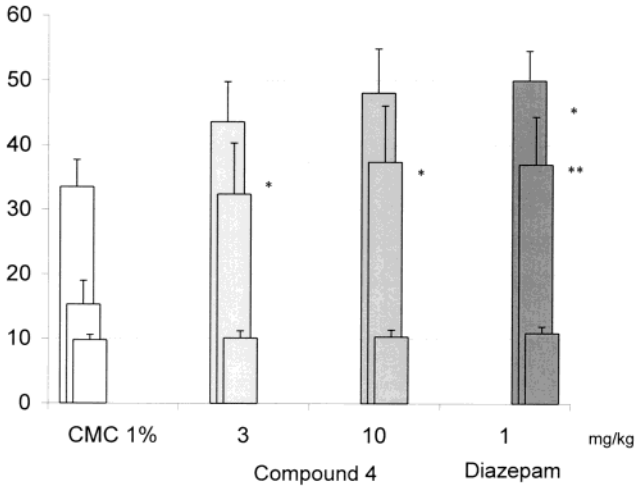


Figure 2. Anxiolytic-like effect of compound **4**, in comparison with diazepam, in the rat elevated plus maze test. Total number of arm entries made by rats during a 5-min observation period, first plane columns; percentage of time spent in open arms, second plane columns; percentage of open arms entries, third plane columns. Animals were treated (po) 30 min before the test. Each column represents the mean ±SEM of 9−15 rats. *$p < 0.05$, **$p < 0.01$ (Student's t-test).

f. Effects on Mouse Learning and on Short- and Long-Term Memory. The ability to learn and remember is often compromised by anxiolytics. The amnesic effect caused by benzodiazepine agonists has been well-known for a long time.[27] For this reason, we were interested in investigating the effects of **4** on mouse memory in a modified passive avoidance task, in which the punishment was not painful. Effects on both short-term and long-term memory were studied.

In the first set of experiments, the effect of **4**, at a dosage at which this substance has been found to be anxiolytic, was compared to that of diazepam and that of flumazenil, a nonselective benzodiazepine antagonist. Mice were treated before the training session; in this circumstance, the retention session was performed 24 h later. As reported in Figure 3, at all doses used (1, 3, and 10 mg/kg po), **4** never impaired mouse memory: indeed, at the dosage of 3 mg/kg, the Student's t-test revealed a statistically significant improvement in mouse memory performance. On the contrary, the effects of diazepam (1 and 3 mg/kg po) were definitely harmful.

As for the effects on long-term memory, treatment was performed immediately after the training session

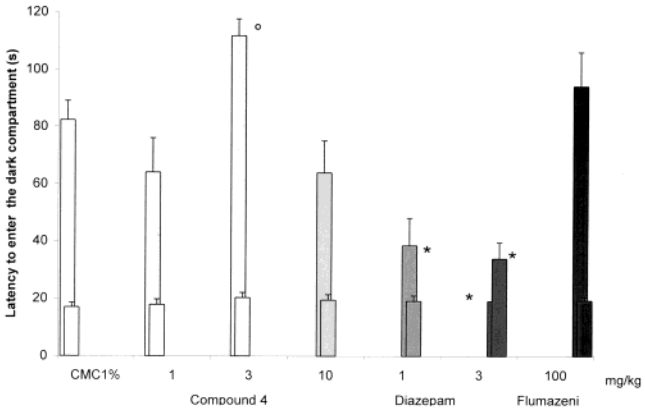


Figure 3. Lack of short memory impairment from compound **4** (po), in comparison with diazepam (po) and flumazenil (ip), in the mouse step-through passive avoidance test. Mice were treated 30 min before training test (first plane columns). The retention test was performed 24 h later (second plane columns). Each column represents the mean ±SEM of 11−36 mice. *$p < 0.01$; **$p < 0.001$ vs CMC-treated mice (ANOVA, followed by Scheffe's multiple comparison test) °$p < 0.01$ vs CMC-treated mice (Student's t-test).

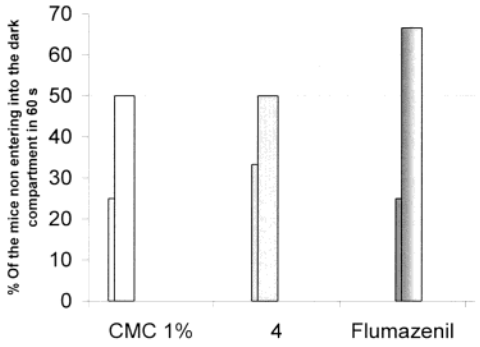


Figure 4. Effect of compound **4** (3 mg/kg po) on mice memory extinction, in comparison with flumazenil (3 mg/kg ip) and zolpidem (3 mg/kg po), in the passive avoidance test. The punishment consisted of a fall into cold water. Treatment was performed immediately after the training session. Columns represent the percentage of mice not entering the dark compartment in 60 s. First plane columns: retention test performed 7 days after the training test. Second plane columns: retention test performed 14 days after the training test. Each column represents the mean of 11−35 mice.

with **4** and flumazenil (nonselective antagonist) using the same dosage of 3 mg/kg po for both drugs. As reported in Figure 4, compound **4** caused, like flumazenil, no effect on forgetting rates.

According to studies[23,28] about the contribution of α_1 subtype in the amnesic activity of BZ agonists, and if we take into account the fact that compound **4** prevents the effects of diazepam in the rota rod- and PTZ-induced convulsion-tests, it would seem likely that also its effects on mouse short- and long-term memory are not ascribable to the activation, but to some antagonism in receptors containing the α_1 subtype (Figures 3 and 4).

g. Effect on Ethanol-Induced Sleeping Time. The effects of ethanol on GABA-ergic neurotransmission are well-known,[29−30] as alcohol intake may compromise the daytime use of anxiolytics. For this reason, our next approach to the study of **4** was to verify whether **4** might be capable of influencing ethanol-induced sleep time, as was to be expected on the basis of the observations of Rudolph et al.,[23] who demonstrated that ethanol potentiation is due to the activation of the α_2, α_3, or α_5 subtypes of $GABA_A$/BZ receptors. The experiment was conducted on mice, using both **4** and diazepam at doses which had induced anxiolytic-like effects in previous tests. Diazepam potentiated the sleep time induced by ethanol in highly significant manner, while surprisingly, the effect with **4** did not differ from that of the controls (Figure 5). In other words, **4** caused no further central depression.

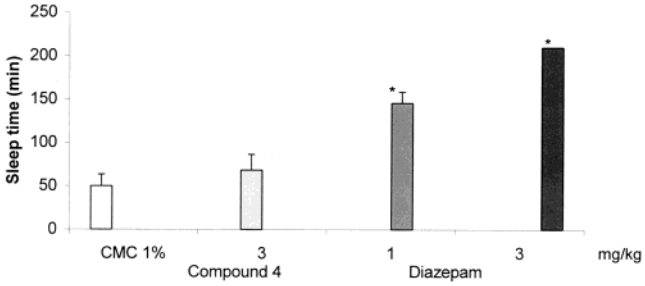


Figure 5. Effect of compound **4** (3 mg/kg po) on ethanol-induced sleep time, in comparison with diazepam (po). Each column represents the mean ±SEM of 9−18 mice. Substances were administered 30 min before ethanol (4 g/kg ip). $p < 0.001$ (Student's t-test).

If it is assumed that the observed anxiolytic-like effects of **4** are due to the activation of α_2 or α_3 subtype receptors, the consequent lack of ethanol potentiation must have some other explanation. Recently, June et al.[31] reported that the α_5 subtype containing receptors in the hippocampus play an important role in regulating alcohol-seeking behavior in the alcohol-preferring rat. Furthermore, Ro 15-4513, a partial inverse-agonist, the most α_5-selective compound reported to date,[32] significantly attenuated ethanol-induced motor incoordination.[33] Compound **4** has a good K_i value in α_5 subtype receptors (8.6 ± 0.7 nM). Therefore, in the light of these final observations, it can be hypothesized that, concerning the ethanol test, **4** is endowed, in the α_5 subtype-containing receptors, with an antagonist property or agonistic activity with low efficacy.

Summary

The combination in the 3-position of the pyrazolobenzotriazine moiety of an ester function and an electron-rich heteroaryl ring yielded a new series of 3-heteroaryl ester ligands **1**−**4** with high affinity to the BZ receptor. In the in vivo tests, compound **4** stands out clearly and in accordance with literature reports about compounds with mixed agonist/antagonists profile;[3,6,13,15,34] we might consider **4** as a functionally selective ligand, although it does not display a pronounced preferential subtype selectivity. The differential profile in vivo of **4** may be related to its different intrinsic efficacy for certain $GABA_A$ receptor subtype. In fact, eliciting in vivo anxyolitic-like properties, it probably acts as agonist at α_2- and α_3-subtypes. The lack of sedative and amnesic properties per se and the ability of **4** to prevent the effects of diazepam on PTZ-induced convulsion and on rota rod, might be due to its antagonist profile at α_1-subtypes. The lack of ethanol potentiation could be explained because **4** probably acts as antagonist or low efficacy agonist at α_5-subtypes. Moreover, the in vivo results are in accordance with our previous findings[18]

relative to a prevalent anxiolytic effect shown by the pyrazolo[5,1-c][1,2,4]benzotriazine 3-ester series.

Similarly, the selective anticonvulsant agonist profile showed by compound **III** may be related to its preferential efficacy on α_1-subtype receptor.

On the basis of these findings, further chemical modifications of the 3-substituent features appear to be a valid strategy for obtaining new benzodiazepine receptor ligands with greater affinity and pharmacological selectivity.

Experimental Section

Melting points were determined with a Gallenkamp apparatus and were uncorrected. 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 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.

General Procedure for the Synthesis of 3-Ester Derivatives, 1−8, 11−19. From 3-carboxy-8-chloropyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide[22] (**A**) (100 mg, 0.37 mmol) and 3-carboxymethyl-8-chloropyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide (**B**) (100 mg, 0.36 mmol),[19] the corresponding 3-carbonyl chloride was obtained by means of treatment with 2.0 mL of thionyl chloride. The final solution was evaporated to dryness, the residue was suspended in 2-methyl-3-butene stabilized chloroform (3 mL), and the suitable alcohol was added. In some cases, two drops of pyridine (for compounds **16**, **17**, **19**) or triethylamine (for compounds **1**, **2**) were added. The reaction was monitored by TLC and stopped when the starting material disappeared (10−12 h). The final solution was washed with sodium hydrogen carbonate solution, and after the normal work up, the residue was treated with isopropyl ether or ethyl ether, filtered, and recrystallized by a suitable solvent.

3-(3-Furylmethoxycarbonyl)-8-chloropyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide (1). Yellow crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1710, 1560; ^{1}H NMR ($CDCl_3$) δ 8.52 (s, 1H, H-2); 8.50 (d, 1H, H-6); 8.45 (d, 1H, H-9); 7.64 (dd, 1H, H-7); 7.62 (m, 1H, H-2′ 3-furyl); 7.43 (m, 1H, H-5′ 3-furyl); 6.57 (m, 1H, H-4′ 3-furyl); 5.31 (s, 2H, CH_2). Anal. C, H, N.

3-(2-Furylmethoxycarbonyl)-8-chloropyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide (2). Yellow crystals; TLC eluent: toluene/ethyl acetate/8:3 v/v; IR ν^{-1} 1720, 1570; ^{1}H NMR ($CDCl_3$) δ 8.51 (s, 1H, H-2); 8.48 (d, 1H, H-6); 8.43 (d, 1H, H-9); 7.64 (dd, 1H, H-7); 7.45 (m, 1H, H-5′ 2-furyl); 6.53 (m, 1H, H-3′ 2-furyl); 6.38 (m, 1H, H-4′ 2-furyl); 5.37 (s, 2H, CH_2). Anal. C, H, N.

3-(3-Thienylmethoxycarbonyl)-8-chloropyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide (3). Yellow crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1720, 1560; ^{1}H NMR ($CDCl_3$) δ 8.52 (s, 1H, H-2); 8.47 (d, 1H, H-6); 8.44 (d, 1H, H-9); 7.64 (dd, 1H, H-7); 7.46 (m, 1H, H-2′ 3-thienyl); 7.34 (m, 1H, H-5′ 3-thienyl); 7.24 (m, 1H, H-4′ 3-thienyl); 5.43 (s, 2H, CH_2). Anal. C, H, N.

3-(2-Thienylmethoxycarbonyl)-8-chloropyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide (4). Yellow crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1720, 1560; ^{1}H NMR ($CDCl_3$) δ 8.52 (s, 1H, H-2); 8.48 (d, 1H, H-6); 8.44 (d, 1H, H-9); 7.65 (dd, 1H, H-7); 7.33 (m, 1H, H-5′ 2-thienyl); 7.23 (m, 1H, H-3′ 2-thienyl); 7.01 (m, 1H, H-4′ 2-thienyl); 5.57 (s, 2H, CH_2). Anal. C, H, N.

3-(4-Pyridylmethoxycarbonyl)-8-chloropyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide (5). Yellow crystals; TLC eluent: ethyl acetate/ciclohexane 2:1 v/v; IR ν^{-1} 1720, 1570; ^{1}H NMR ($CDCl_3$) δ 8.66 (d, 2H, Py); 8.57 (s, 1H, H-2); 8.54 (d, 1H, H-6); 8.47 (d, 1H, H-9); 7.67 (dd, 1H, H-7); 7.48 (d, 2H, Py); 5.48 (s, 2H, CH_2). Anal. C, H, N.

3-(Tetrahydro-3-furylmethoxycarbonyl)-8-chloropyrazolo[5,1-c][1,2,4]benz otriazine 5-oxide (6). Yellow crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1720, 1570; ^{1}H NMR ($CDCl_3$) δ 8.55 (s, 1H, H-2); 8.50 (d, 1H, H-6); 8.43 (d, 1H, H-9); 7.65 (dd, 1H, H-7); 4.38 (m, 3H, CH_2− and H-3 tetrahydrofuryl); 3.93 (m, 2H, CH_2-tetrahydrofuryl); 1.94 (m, 4H, $(CH_2)_2$-tetrahydrofuryl). Anal. C, H, N.

(±)3-(1-Phenylethoxycarbonyl)-8-chloropyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide (7). Yellow crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1710, 1570; ^{1}H NMR ($CDCl_3$) δ 8.55 (s, 1H, H-2); 8.50 (d, 1H, H-6); 8.45 (d, 1H, H-9); 7.65 (dd, 1H, H-7); 7.55 (m, 2H, Ph); 7.38 (m, 3H, Ph); 6.20 (q, 1H, −CH−); 1.75 (d, 3H, CH_3). Anal.C, H, N.

3-Isopropylcarbonyl-8-chloropyrazolo[5,1-c][1,2,4]-benzotriazine 5-oxide (8). Yellow crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1720, 1570; ^{1}H NMR ($CDCl_3$) δ 8.55 (m, 2H, H-2, H-9); 8.45 (d, 1H, H-6); 7.65 (dd, 1H, H-7); 5.25 (m, 1H, −CH−); 1.40 (m, 6H, $(CH_3)_2$). Anal. C, H, N.

3-Benzyloxycarbonylmethyl-8-chloropyrazolo[5,1-c]-[1,2,4]benzotriazine 5-oxide (11). Yellow crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1740, 1570; ^{1}H NMR ($CDCl_3$) δ 8.48 (s, 1H, H-6); 8.38 (d, 1H, H-9); 8.15 (d, 1H, H-2); 7.55 (dd, 1H, H-7); 7.36 (m, 5H, Ph); 5.20 (s, 2H, $-CH_2-$Ph); 3.90 (s, 2H, $-CH_2-$CO−). Anal. C, H, N.

3-(2-Methoxybenzyloxycarbonylmethyl)-8-chloropyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide (12). Yellow crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1740, 1570; ^{1}H NMR ($CDCl_3$) δ 8.42 (s, 1H, H-6); 8.35 (d, 1H, H-9); 8.28 (d, 1H, H-2); 7.75 (dd, 1H, H-7); 7.35 (m, 2H, Ph); 7.00 (m, 2H, Ph); 5.15 (s, 2H, $-CH_2-$Ph); 3.90 (s, 2H, $-CH_2-$CO−); 3.80 (s, 3H, OCH_3). Anal. C, H, N.

3-(3-Thienylmethoxycarbonylmethyl)-8-chloropyrazolo-[5,1-c][1,2,4]benzotriazine 5-oxide(13). Yellow crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1720, 1560; ^{1}H NMR ($CDCl_3$) δ 8.50 (s, 1H, H-6); 8.39 (d, 1H, H-9); 8.25 (d, 1H, H-2); 7.55 (dd, 1H, H-7); 7.28−7.24 (m, 2H, H-2′ and H-5′ 3-thienyl); 7.22 (m, 1H, H-4′ 3-thienyl); 5.20 (s, 2H, $-CH_2$-thienyl); 3.90 (s, 2H, $-CH_2$CO). Anal. C, H, N.

3-(2-Thienylmethoxycarbonylmethyl)-8-chloropyrazolo-[5,1-c][1,2,4]benzotriazine 5-oxide(14). Yellow crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1730, 1560; ^{1}H NMR ($CDCl_3$) δ 8.50 (s, 1H, H-6); 8.45 (d, 1H, H-9); 8.13 (d, 1H, H-2); 7.55 (dd, 1H, H-7); 7.35 (m, 1H, H-3′ 2-thienyl); 7.12 (m, 1H, H-5′ 2-thienyl); 7.00 (m, 1H, H-4′ 2-thienyl); 5.34 (s, 1H, CH_2-thienyl); 3.87 (s, 2H, $-CH_2$CO). Anal. C, H, N.

3-Ethoxycarbonylmethyl-8-chloropyrazolo[5,1-c][1,2,4]-benzotriazine 5-oxide (15). Yellow crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1720, 1560; ^{1}H NMR ($CDCl_3$) δ 8.47 (s, 1H, H-6); 8.36 (d, 1H, H-9); 8.14 (d, 1H, H-2); 7.54 (dd, 1H, H-7); 4.18 (q, 2H, CH_2); 1.30 (t, 3H, CH_3). Anal. C, H, N.

3-Phenoxycarbonylmethyl-8-chloropyrazolo[5,1-c][1,2,4]-benzotriazine 5-oxide (16). Yellow crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1710, 1570; ^{1}H NMR ($CDCl_3$) δ 8.49 (s, 1H, H-6); 8.39 (d, 1H, H-9); 8.22 (d, 1H, H-2); 7.57 (dd, 1H, H-7); 7.39 (m, 3H, Ph); 7.13 (m, 2H, Ph); 4.10 (q, 2H, CH_2). Anal. C, H, N.

3-(2-Chlorophenoxycarbonylmethyl)-8-chloropyrazolo-[5,1-c][1,2,4]benzotriazine 5-oxide (17). Yellow crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1720, 1570; ^{1}H NMR ($CDCl_3$) δ 8.50 (s, 1H, H-6); 8.41 (d, 1H, H-9); 8.22 (d, 1H, H-2); 7.50 (dd, 1H, H-7); 7.28−7.22 (m, 4H, Ph); 4.20 (q, 2H, CH_2). Anal. C, H, N.

3-(2-Methoxyphenoxycarbonylmethyl)-8-chloropyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide (18). Yellow crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1780, 1570; ^{1}H NMR ($CDCl_3$) δ 8.50 (s, 1H, H-6); 8.40 (d, 1H, H-9); 8.22 (d, 1H, H-2); 7.58 (dd, 1H, H-7); 7.15−6.95 (m, 4H, Ph); 4.19 (q, 2H, CH_2); 3.82 (s, 3H, CH_3). Anal. C, H, N.

3-(2-Methylphenoxycarbonylmethyl)-8-chloropyrazolo-[5,1-c][1,2,4]benzotriazine 5-oxide (19). Yellow crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1760, 1570; ^{1}H NMR ($CDCl_3$) δ 8.50 (s, 1H, H-6); 8.41 (d, 1H, H-9); 8.23 (d, 1H, H-2); 7.59 (dd, 1H, H-7); 7.22−7.18 (m, 4H, Ph); 4.18 (q, 2H, CH_2); 2.20 (s, 3H, CH_3). Anal. C, H, N.

General Procedure for Synthesis of 9−10. These compounds were obtained by following a previously described procedure of reduction with triethyl phosphite (3 mL) in toluene[16] (15 mL) at refluxing temperature for 6−8 h, starting from 3-(2-methoxybenzyloxycarbonyl)-8-chloropyrazolo[5,1-c]-[1,2,4]benzotriazine 5-oxide[18] (0.2 mmol) and compound **4** (0.2 mmol), respectively.

3-(2-Methoxybenzyloxycarbonyl)-8-chloro pyrazolo-[5,1-c][1,2,4]benzotriazine (9). Yellow crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1780; ^{1}H NMR ($CDCl_3$) δ 8.70 (s, 1H, H-6); 8.68 (d, 1H, H-2); 8.54 (d, 1H, H-9); 7.80 (dd, 1H, H-7); 7.63 (d, 1H, H-3′ Ph); 7.34 (m, 1H, H-5′ Ph); 6.98 (m, 2H, H-4′ and H-6′ Ph); 5.60 (s, 2H, CH_2); 3.90 (s, 3H, OCH_3). Anal. C, H, N.

3-(2-Thienylmethoxycarbonyl)-8-chloropyrazolo[5,1-c]-[1,2,4]benzotriazine (10). Yellow crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν^{-1} 1720; ^{1}H NMR ($CDCl_3$) δ 8.69 (m, 2H, H-2 and H-6); 8.53 (d, 1H, H-9); 7.80 (dd, 1H, H-7); 7.36 (m, 1H, H-5′ 2-thienyl); 7.28 (m, 1H, H-3′ 2-thienyl); 7.03 (m, 1H, H-4′ 2-thienyl); 5.69 (s, 2H, CH_2). Anal. C, H, N.

Radioligand Binding Assay

Binding Studies. [^{3}H]Ro15-1788 (specific activity 70.8 Ci/mmol) was obtained from NEN Life Sciences products. All the other chemicals, which were of reagent grade, were obtained from commercial suppliers.

Bovine cerebral cortex membranes were prepared as previously described.[35,36] 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.[37] [^{3}H]Ro15-1788 binding studies were performed as previously reported.[19] Clonal mammalian cell lines, expressing relatively high levels of $GABA_A$ receptor subtypes ($\alpha_1\beta_2\gamma_2$, $\alpha_1\beta_2\gamma_2$, $\alpha_3\beta_2\gamma_2$, $\alpha_5\beta_3\gamma_2$) were maintained as previously described[38] in Minimum Essential Medium Eagle with EBSS, supplemented with 10% fetal calf serum, l-glutamine (2 mM), penicillin (100 units/mL), and streptomycin (100 μg/mL) in a humidified atmosphere of 5% CO_2/95% air at 37 °C. After removal, the cells were harvested by centrifugation at 500 × g. The crude membranes were prepared after homogenization in 10 mM potassium phosphate, pH 7.4, and differential centrifugation at 48000 × g for 30 min at 4 °C. The pellets were washed twice in this manner before final resuspension in 10 mM potassium phosphate, pH 7.4, that contained 100 mM potassium chloride.[38] [^{3}H]Ro15-1788 binding assays to transfected cell membranes were carried out as previously described.[38] In brief, the cell line membranes were incubated in a volume of 500 μL, which contained [^{3}H]Ro15-1788 at a concentration of 1−2 nM and test compound in the 10^{-9}−10^{-5} M range. Nonspecific binding was defined by 10^{-5} M diazepam. Assays were incubated to equilibrium for 1 h at 4 °C. The compounds were dissolved in DMSO, the level of which did not exceed 1% and which was maintained constant in all tubes. At least six different concentrations of each compound were used. The data of $n = 5$ experiments carried out in triplicate were analyzed by means of an iterative curve-fitting procedure (program Prism, GraphPad, San Diego, CA), which provided IC_{50}, K_i, and SEM values for tested compounds, the Ki values being calculated from the Cheng and Prusoff equation.[39]

The potencies of the new synthesized compounds to inhibit [^{3}H]Ro15-1788 binding in the presence and absence of GABA were compared. Differences obtained were expressed as GABA shifts (namely, the ratios of the K_i values obtained in the absence of GABA over the K_i values obtained in the presence of GABA) have provided indications as to agonist, partial agonist, antagonist, and inverse agonist pharmacological activity.

Pharmacological Methods

The experiments were carried out in accordance with the Animal Protection Law of the Republic of Italy, DL No. 116/1992, on the basis of 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 numbers of falls from the rod in 30 s, 25 min after drug administration, were counted.

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

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.

Maximal Electroshock Seizure (MES) Test. Groups of 10−16 mice were observed for the occurrence of maximal seizures following application of an electroshock (40 mA, 0.2 s, 50 Hz) to their corneas 30 min after drug administration. The incidence of tonic extensive convulsions and lethality was determined.

Mouse Light/Dark Box Test. The apparatus (50 cm long, 20 cm wide, and 20 cm high) consisted of two equal acrylic compartments, one dark and one light, illuminated by a 60 W bulb lamp and separated by a divider with a 10 × 3-cm opening at floor level. Each mouse was tested by placing it in the center of the lighted area, facing away from the dark one, and allowing it to explore the novel environment for 5 min. The number of transfers from one compartment to the other and the time spent in the illuminated side were measured. This test exploited the conflict between the animal's tendency to explore a new environment and its fear of bright light.

Rat Elevated Plus Maze Test. The plus maze consisted of two open arms (50 × 10 cm) and two enclosed arms (50 × 10 × 40 cm) with an open roof,

arranged so that the two arms of each type were opposite each other. The floor was covered with black rubber. The maze was elevated to a height of 50 cm. The rats were placed in the center of the apparatus, facing one of the open arms, and were left to explore the maze for five min, 30 min after drug administration. An observer sitting in the same laboratory noted the number of entries and the time spent in both the open and the closed arms.

Step through Passive Avoidance Tests. The apparatus (50 cm long, 20 cm wide, 20 cm high) consisted of two equal compartments, one dark and one white, lighted with a 60W light bulb, and separated by a divider with a 10 × 3-cm opening at floor level. The dark compartment had a pitfall floor. Punishment consisted of a fall (40 cm) into cold water (10 °C),[40] instead of a painful electric foot shock.

Each mouse was placed in turn in the lighted compartment, facing away from the dark one. When the mouse entered the dark compartment, it fell into the water. Treatment was given 30 min before the training session. Twenty-four hours later, a retention trial was performed. The step-through latency for entering the dark compartment was again recorded.

In a second set of experiments, treatment was given soon after the training session; in this case, the retention trials were carried out one and two weeks later.

In the retention trials, if the mouse did not enter the dark compartment within 120 s, the test was interrupted and the step-through latency was recorded as 120 s.

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 the drugs 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 the po route, except for flumazenil which was administered ip. Drug concentrations were prepared in such a way that the necessary dose could be administered in a 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 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* values of less than 0.05 were considered significant.[41]

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