Synthesis and Pharmacological Evaluation of Novel $GABA_A$ Subtype Receptor Ligands with Potential Anxiolytic-like and Anti-hyperalgesic Effect

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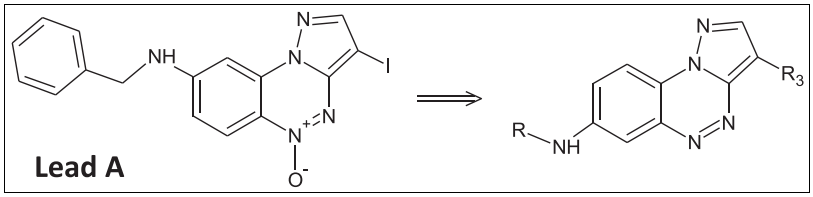

The identification of selective benzodiazepine site ligands, endowed with anxiolytic and anti-hyperalgesic action, is a relevant opportunity for the treatment of pain syndromes. Previously, we selected a compound with a promising anti-hyperalgesic profile, the 3-iodo-8-benzylaminopyrazolo [5,1-c][1,2,4]benzotriazine 5-oxide. Aimed to verify the structure–activity relationship, the corresponding 7-arylakylamino derivatives were synthesized. Compounds were tested for their affinity at $GABA_A$-receptor subtype; the compound **12** was further investigated in animal models of anxiety and persistent pain.

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INTRODUCTION

Chronic pain, a complex disorder associated with various pathological processes, is defined as pain that persists longer than the temporal course of natural healing; it has a profound impact on many aspects of daily life with quality-of-life connection and socioeconomic implications. In fact, a higher frequency of mood and anxiety disorders and the occurrence of suicidal ideation are not inconsequential in this pathological process. It is categorized as inflammatory or neuropathic pain and treated with opioids and non-steroidal anti-inflammatory drugs both inadequate for long-term therapy because of the well-known adverse events. The need to better understand chronic pain mechanisms and to create novel and effective multimodal treatment options strongly encourages the research in this field.

Among the neurotransmitters involved in pain signaling, the γ-aminobutyric acid (GABA) plays an important role. The block of the spinal GABAergic neurotransmission by intrathecal injection of GABA receptor antagonist produces hyper-sensitivity to innocuous stimuli, while compounds activating the $GABA_A$ receptors ($GABA_A$-Rs), such as agonists or positive allosteric modulators, have been proposed as potent analgesics in various models of inflammatory and neuropathic pain [1].

Briefly, $GABA_A$-Rs belong to the family of heteropentameric ion channel and present a typical composition containing 2α, 2β, 1γ subunits; inside the pentamer, the benzodiazepines site (Bdz site, also known as $GABA_A$-R subtype) lies between the α/γ subunits and the pharmacological effect of ligands depends on the type of α-subunit present (α1–6) [2,3]. It is well known that the α1 subunit is involved in an anticonvulsant and sedative effect, the α2/α3 subunits are involved in an anxiolytic-like effect, and the α5 subunit in memory [4]. After preliminary studies [5] indicating the relationships among analgesia, $GABA_A$ receptors, and benzodiazepine ligands, Knabl [6] and Ralvenius [7] presented innovative pharmacological data using several lines of mice with genetically modified $GABA_A$ receptors ($GABA_A$-Rs point-mutated mice) and demonstrated that the spinal anti-hyperalgesic effect was mediated by the α2- or α3-$GABA_A$R subtypes. Among the examples reported in Figure 1, compound L-838,417, a α2/α3-selective $GABA_A$-R subtype ligand, exerts pronounced anti-hyperalgesic action without producing sedation, motor impairment, or tolerance development [8]. Other partial allosteric modulators of α2-, α3-, and/or α5-containing $GABA_A$-Rs, NS11934, SL-651498, and TPA023 showed analgesic properties in models of inflammatory and neuropathic pain [5,9–11]. Moreover, recent literature reports HZ166 [12], with selective efficacy at α2 and α3 subunit-containing $GABA_A$-Rs, endowed with anti-hyperalgesic effect (Fig. 1). Such compounds might be useful for the treatment of chronic pain syndromes that have become unresponsive to classical analgesic drugs [13].

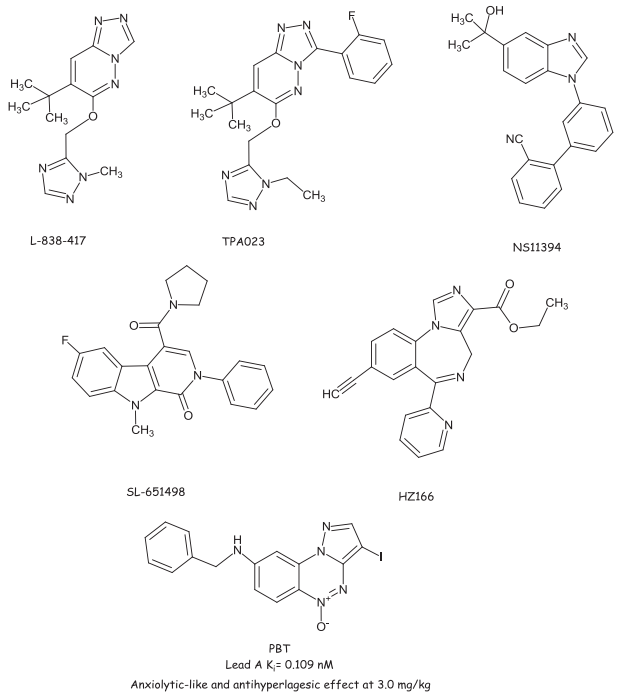


Figure 1. Chemical structures of reference compounds.

Our research group has been extensively involved in the design and synthesis of pyrazolo[5,1-c][1,2,4] benzotriazine (PBT) derivatives, with the aim to identify selective $GABA_A$-R subtype (Bdz site) ligands. Our previous studies let us to individuate the 3-iodo-8-benzylamino PBT (lead **A**) [14] reported in Figure 1, a promising compound with K_i = 0.109 ± 0.008 nM and provided with selective anxiolytic and anti-hyperalgesic effect, thus acting as a α2-subtype agonist. Its pain relieving efficacy was preclinically shown against trauma-induced peripheral nerve pain neuropathy and diabetic neuropathy, taking effect at 3 mg kg^{-1} per os.

Because the importance of the 8-NH group for the anxiolytic and anti-hyperalgesic effect was highlighted in the series of 3,8-disubstituted PBT [14], we aimed to verify if the shift of the NH from 8- to 7-position still allows the pharmacological activity. Thus, we synthesized 7-arylalkylaminopyrazolo [5,1-c][1,2,4benzotriazines 3-substituted (I, CH_3, triazole) to optimize lead **A** [14]. Moreover, some 8-arylalkylaminoderivatives were synthesized *ad hoc* to better compare the two series. All synthesized compounds were tested for their affinity at $GABA_A$-R subtype, and in vivo pharmacological tests were performed.

RESULTS AND DISCUSSION

Chemistry. All compounds described here are listed in Table 1. A first attempt to obtain the 3-iodo-7-arylalkylaminopyrazolo[5,1-c][1,2,4]benzotriazine derivatives was made starting from compound **1**, the 7-aminopyrazolo[5,1-c][1,2,4]benzotriazine [15] that was undergone to reductive N-alkylation by using the suitable aromatic aldehyde and sodium cianoborohydride in the presence of Lewis acid as $ZnCl_2$ [16]. The first term of this series, the 7-benzylamino derivative **3**, was easily obtained through the intermediate Schiff base (**2**) that was isolated and reduced. Unfortunately, the following iodination gave a mixture of 3,6- and 3,8-diiodio-7-benzylaminopyrazolo[5,1-c][1,2,4]benzotriazines, not easily separable (compounds **a** and **b**), Scheme 1. Thus, because the final iodination represented the critical step to obtain the desired compounds, we followed a different synthetic strategy using the 3-iodio-7-aminopyrazolo[5,1-c][1,2,4] pyrazolobenzotriazine, **6a**, as key intermediates (Scheme 2). This compound was indifferently obtained either by acid hydrolysis of **5** or by reduction with $SnCl_2$ [17] of **8** synthesized from the corresponding 3-unsubstituted compounds **4** and **7**, respectively [15,18], by treatment with iodine monochloride. Instead, to obtain the 3-methyl-7-aminopyrazolo[5,1-c][1,2,4] pyrazolobenzotriazine, **6b**, the reduction of **9** (3-carboxylic derivative) with borane dimethyl sulfide complex (Me_2SBH_3) was performed (Scheme 2).

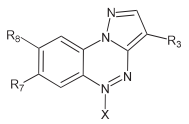
The key intermediate **6a** then treated with benzoylchloride to give the corresponding *N*-(3-iodopyrazolo[5,1-*c*][1,2,4]benzotriazin-7-yl)benzamide **10**; the reductive N-alkylation of **6a** with suitable (hetero) arylaldehyde and sodium cianoborohydride/$ZnCl_2$ afforded the corresponding 3-iodio-7-arylalkylaminoderivatives **11a–d**. Compound **11a** was, in turn, oxidized with m-chloroperbenzoic acid to the related N5-oxide, **12** (Scheme 3). Compound **6b** was transformed into the final products **13a–c** (Scheme 4) following the same method of reductive N-alkylation.

To achieve compounds 3-(1,2,4-triazol-5-yl)pyrazolo-[5,1-c][1,2,4]benzotriazine 7- and 8-N-arylalkyl substituted **18a–d** and **21b–d**, the first step was the building of the triazole ring at 3-position starting from the amide function. Following a procedure already described [19], the starting material, 3-carbamoyl- derivative **14** [18], was treated with dimethylformamide-dimethylacetal (DMF-DMA)/toluene to give the 3-(N-(dimethylaminomethylene) carbamoyl derivative **15**, and then with hydrazine hydrate to afford the 3-(1,2,4-triazol-5-yl) derivative **16**. The nitro group at position 7 of this latter was reduced with Sn/HCl to obtain the 7-amine, N5-deoxide compound **17**, which was, finally, treated as usual with the suitable aromatic aldehyde and sodium cianoborohydride to give compounds **18a–d** (Scheme 5).

The synthetic procedure followed to obtain PBTs bearing the arylalkylamino chain at position 8 is depicted in Scheme 6.

Table 1

Chemical data for PBT derivatives.



	R_3	R_7	R_8	X	Formula (MW)	mp°C (recryst. solvent)
1[a]	H	NH_2		—		
2	H	N=CHPh		—	$C_{16}H_{11}N_5$ (273.29)	280–281°C
3	H	$NHCH_2Ph$		—	$C_{16}H_{13}N_5$ (275.30)	292–293°C
4[a]	H	$NHCOCH_3$		—		
5	I	$NHCOCH_3$		—	$C_{11}H_8N_5OI$ (353.11)	248–249°C
6a	I	NH_2		—	$C_9H_6N_5I$ (311.14)	208–210°C
6b	CH_3	NH_2		—	$C_{10}H_9N_5$ (199.18)	298–299°C
7[b]	H	NO_2		O		
8	I	NO_2		O	$C_9H_4N_5O_3I$ (357.14)	270–272°C
9[b]	COOH	NO_2		O		
10	I	NHCOPh		—	$C_{16}H_{10}N_5OI$ (414.99)	>300°C
11a	I	$NHCH_2Ph$		—	$C_{16}H_{12}N_5I$ (401.4)	>300°C
11b	I	$NHCH_2$-4-Cl-Ph		—	$C_{16}H_{11}N_5ICl$ (435.53)	>300°C
11c	I	$NHCH_2$-2-Py		—	$C_{15}H_{11}N_6I$ (402.01)	>300°C
11d	I	$NHCH_2$-2-furyl		—	$C_{11}H_{10}N_5OI$ (391.16)	>300°C
12	I	$NHCH_2Ph$		O	$C_{16}H_{12}N_5OI$ (417.4)	>300°C
13a	CH_3	$NHCH_2Ph$		—	$C_{17}H_{15}N_5$ (289.31)	273–274°C
13b	CH_3	$NHCH_2$-4-Cl-Ph		—	$C_{17}H_{14}N_5Cl$ (323.75)	279–280°C
13c	CH_3	$NHCH_2$-2-Py		—	$C_{16}H_{14}N_6$ (290.28)	299–300°C
14[b]	$CONH_2$	NO_2		O		
15	$CON{=}CHN(CH_3)_2$	NO_2		O	$C_{13}H_{11}N_7O_4$ (329.27)	>300°C
16	1,2,4-triazol-5-yl	NO_2		O	$C_{11}H_6N_8O_3$ (298.22)	>300°C
17	1,2,4-triazol-5-yl	NH_2		—	$C_{11}H_8N_8$ (252.22)	>300°C
18a	1,2,4-triazol-5-yl	$NHCH_2Ph$		—	$C_{18}H_{14}N_8$ (342.28)	>300°C
18b	1,2,4-triazol-5-yl	$NHCH_2$-4-Cl-Ph		—	$C_{18}H_{13}N_8Cl$ (376.81)	>300°C
18c	1,2,4-triazol-5-yl	$NHCH_2$-2-Py		—	$C_{17}H_{13}N_9$ (343.35)	>300°C
18d	1,2,4-triazol-5-yl	$NHCH_2$-2-furyl		—	$C_{16}H_{12}N_8O$ (332.33)	>300°C
19[b]	$CONH_2$		Cl	O		
20[c]	1,2,4-triazol-5-yl		Cl	O		
21a	1,2,4-triazol-5-yl		$NHCH_2Ph$	O	$C_{18}H_{14}N_8O$ (358.28)	>300°C
21b	1,2,4-triazol-5-yl		$NHCH_2$-4-Cl-Ph	O	$C_{18}H_{13}N_8OCl$ (392.81)	>300°C
21c	1,2,4-triazol-5-yl		$NHCH_2$-2-Py	O	$C_{17}H_{13}N_9O$ (359.35)	>300°C
21d	1,2,4-triazol-5-yl		$NHCH_2$-2-furyl	O	$C_{16}H_{11}N_8O_2$ (348.33)	>300°C
22	$CONH_2$		$NHCH_2Ph$	O	$C_{17}H_{14}N_6O_2$ (334.12)	280°C dec.
23	$CON{=}CHN(CH_3)_2$		$NHCH_2Ph$	O	$C_{20}H_{19}N_7O_2$ (389.42)	>300°C

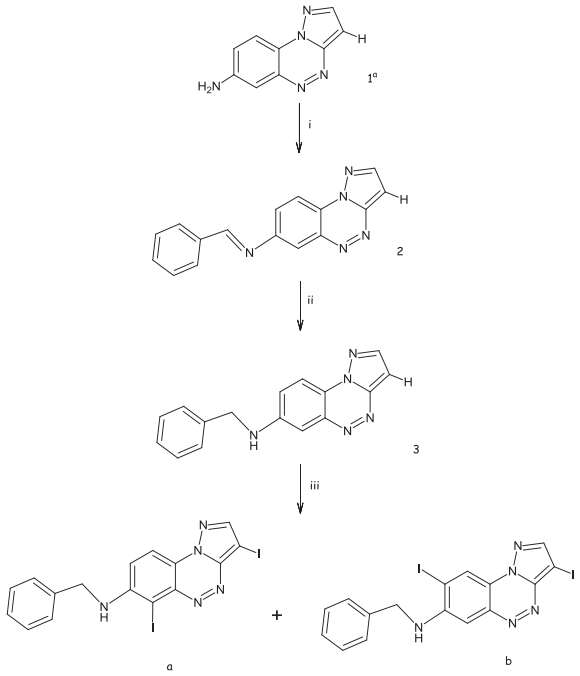
[a][15]
[b][18]
[c][19]

The 3-carbamoyl-8-chloropyrazolo[5,1-c][1,2,4]benzotr iazine 5-oxide **19** [18] was transformed into the 3-triazol-derivative **20** [19], which was then converted into the 8-N-arylalkylamino derivatives through the aromatic nucleophilic substitution of chlorine, in the presence of an excess of suitable aryl amine (final 5-N-oxide products **21b–d**). To obtain **21a** is more advantageous as yields pureness first to synthesize the 8-benzylamino derivative **22**, by the nucleophilic substitution of chlorine on the PBT **19** [18] with benzylamina, and then to perform the usual reaction with DMF-DMA (**23**), followed by the treatment with hydrazine hydrate (Scheme 6).

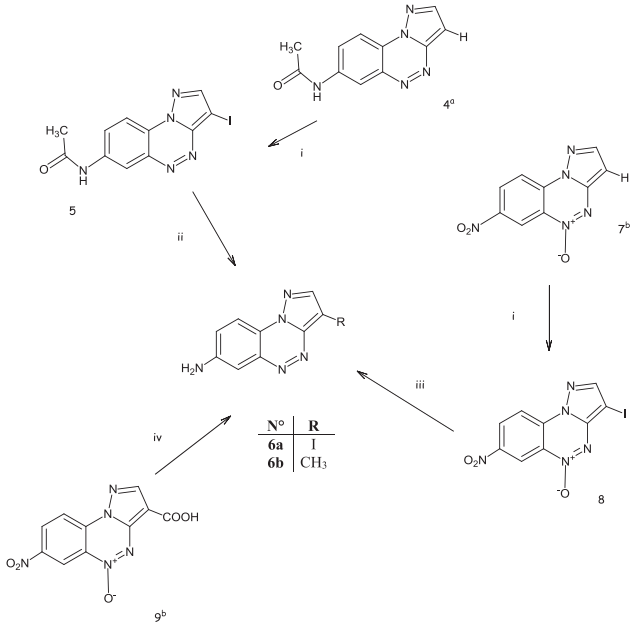
Biological results. ***In vitro binding.*** The Bz site/$GABA_A$-R binding affinity of newly synthesized compounds was evaluated for their ability to displace [^{3}H]flumazenil (Ro15–1788) from its specific binding in a bovine brain membrane.

Final products (**10–13, 18a–d, 21a–d**) were tested at fixed concentrations of 10 μM, and the K_i value was expressed only for those terms inhibiting radioligand binding by more than 80%. Looking at Table 2, it emerges that, for the most part of the new products, this value is less than 60% with only few exceptions. In the series of 3-iodo PBT, only the 7-benzylamino derivatives **11a** and **12**

Scheme 1. Reagents and conditions: (i) PhCHO/MeOH/AcOH; (ii) $MeOH/NaBH_4$; (iii) ICl/CH_2Cl_2. [a]See [15].



Scheme 2. Reagents and conditions: (i) ICl/CH_2Cl_2; (ii) HCl, NaOH; (iii) $SnCl_2$/EtOH; (iv) $THF/(CH_3)_2SBH_3$. [a]See [15]. [b]See [18].

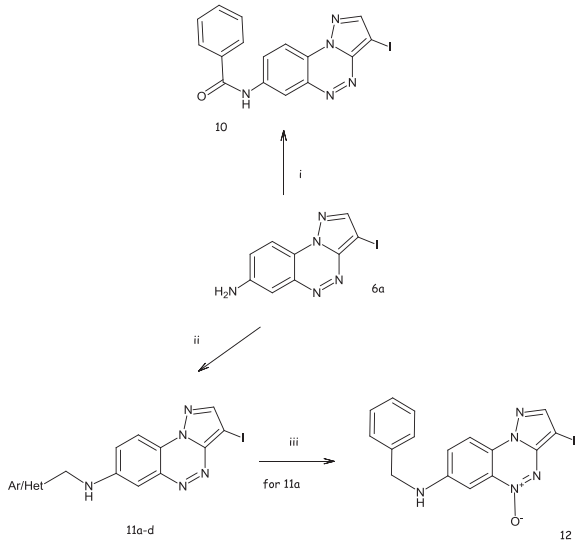


N°	R
6a	I
6b	CH_3

show an appreciable affinity: $K_i = 0.914 \pm 0.10$ μM and $K_i = 0.28 \pm 0.029$ μM, respectively.

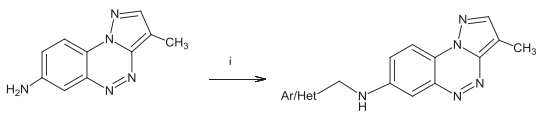
Also in the 3-methyl- and 3-(triazol-5-yl)- series (**13a–c** and **18a–d**), the only compounds endowed with a certain affinity are **13a** and **18a**, both showing the benzylamino fragment at position 7.

Scheme 3. Reagents and conditions: (i) NaOH, benzoylchloride; (ii) Ar/HetCHO (**a-d**, benzaldehyde **a**, 4-chlorobenzaldheyde **b**, 2-pyridincarboxyaldheyde **c** and 2-furylcarboxyaldheide **d**), $NaCNBH_3/ZnCl_2$/MeOH; (iii) $MCPBA/CH_2Cl_2$.



N°	Ar/Het
11a	Ph
11b	4-ClPh
11c	2-Py
11d	2-Fur

Scheme 4. Reagent and conditions: (i) Ar/HetCHO (**a-c**, benzaldehyde **a**, 4-chlorobenzaldheyde **b**, 2-pyridincarboxyaldheyde **c**), $NaCNBH_3/ZnCl_2$/MeOH.

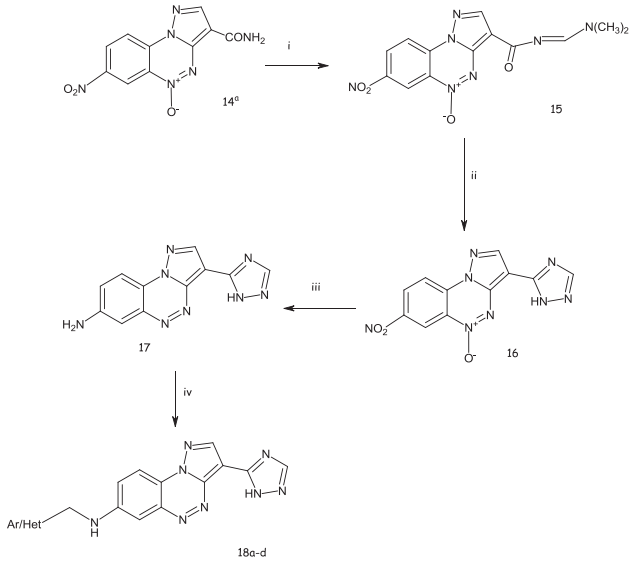


N°	Ar/Het
13a	Ph
13b	4-ClPh
13c	2-Py

Finally, the biological data of compounds **21a–d** (the isomers of **18a–d**), in which the (hetero)arylamino moiety was shifted from 7 to 8-position, further confirm the importance of the benzylamino fragment for binding affinity (**21a**, $K_i = 1.44 \pm 0.11$ μM).

In vivo activity. Compound **12** was selected for its best affinity, and we decided to investigate the pharmacological profile by evaluating the anxiolytic-like, anticonvulsant, and anti-hyperalgesic activity. Derivative **12** is the 7-isomer of our lead compound **A**, (3-iodo-8-benzylaminopyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide Fig. 1), characterized by a selective anxiolytic profile joined to anti-hyperalgesic effects in animal pain models, starting from the dose of 3 mg kg^{-1} per os [14]. Accordingly to the notion that the benzodiazepines are generally not analgesic *per se*, the $GABA_A$-subtype

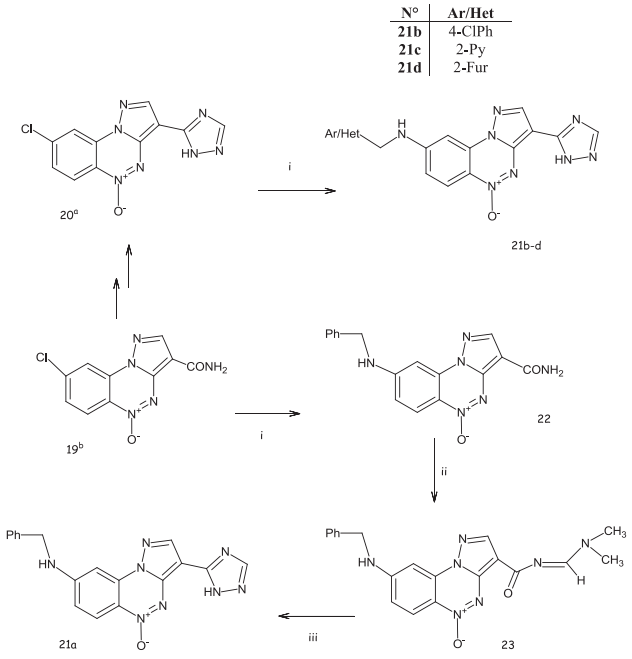
Scheme 5. Reagent and conditions: (i) DMF-DMA/Toluene; (ii) N_2H_4.H_2O/AcOH; (iii) Sn/HCl; (iv) Ar/HetCHO (**a-d**, benzaldehyde **a**, 4-chlorobenzaldheyde **b**, 2-pyridincarboxyaldheyde **c** and 2-furylcarboxyaldheyde **d**), $NaCNBH_3/ZnCl_2$/MeOH. [a]See [18].



N°	Ar/Het
18a	Ph
18b	4-ClPh
18c	2-Py
18d	2-Fur

Scheme 6. Reagents and conditions: (i) $PhCH_2NH_2$ for **22** and Ar/HetCH_2NH_2 for **21b–d**; (ii) DMF-DMA/Toluene; (iii) $N_2H_4.H_2O$/AcOH. [a]See [19]. [b]See [18].

N°	Ar/Het
21b	4-ClPh
21c	2-Py
21d	2-Fur



ligands can exert anti-hyperalgesic action overall when the pain sensitivity is pathologically increased (inflammation or neuropathic injury) [9,20].

The effects on mouse anxiety were studied using a light/dark box apparatus. Compound **12** was tested in the dose range 10–30 mg kg^{-1} p.o. showing good anxiolytic-like effect with an efficacy comparable to diazepam (standard reference). The anxiolytic-like effects were completely antagonized by flumazenil (100 mg kg^{-1} intraperitoneally), suggesting that the anxiolytic-like effect is exerted through the $GABA_A$-R subtypes (Bdz site), Table 3.

Moreover, the pain relieving profile of compound **12** (3–10-30 mg kg^{-1} p.o.) was evaluated in animals that underwent the chronic constriction injury of the sciatic nerve (according to the method described by Bennett and Xie [21]). Behavioral measurements were performed on day 14 after the surgery when hyperalgesia was established. The nociceptive threshold was determined by the paw pressure test measuring the sensitivity to a mechanical noxious stimulus [22]. The pain threshold of the ipsilateral paw was significantly reduced in comparison to the contralateral, uninjured, paw (Table 4). As shown in Table 4, 30 mg kg^{-1}, compound **12** p.o. induced a statistically significant anti-hyperalgesic activity in comparison with the vehicle-treated group (Table 4).

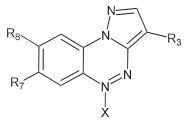
Compound **12** was also tested in the animal model of streptozotocin (STZ)-induced hyperalgesia, which reproduces pain related to diabetic neuropathy in laboratory animals. STZ is toxic for the insulin-producing beta cells of the pancreas in mammals, and it drastically increases the serum glucose level. In diabetic patients, hyperglycemia-induced oxidative stress has been considered the main cause of diabetic neuropathy [23]. STZ-induced hyperalgesia was measured by the hot plate test (Table 5); **12** (30 mg kg^{-1} p.o.) significantly increased the pain threshold 15, 30, 45, and 60 min after the administration (Table 5).

On the other hand, compound **12** (10–30 mg kg^{-1} p.o.) did not show antinociceptive activity in the Writhing test, a model of irritative pain induced by the injection i.p. of acetic acid. Finally, the protection from convulsions was evaluated in mice using pentylentetrazole (PTZ) as a chemical convulsant agent. Compound **12** (10–30 mg kg^{-1} p.o.) was devoid of any effect on PTZ-shock with respect to the reference drug (diazepam, 1 mg kg^{-1} i.p.).

Because it was reported that the anticonvulsant effects are principally because of the activation of the α1 $GABA_A$-R subtype [24,25], the lack of this effect could be related to the antagonist efficacy of **12** at this receptor subtype. This result suggests that the mechanism of action responsible for the anti-hyperalgesic effect of **12** is not associated with anticonvulsant activity [26–29].

Table 2

BZR ligand affinity of new synthesized compounds.



	R_3	R_7	R_8	X	I% or K_i (μM)[a]
10	I	NHCOPh		—	5%
11a	I	$NHCH_2Ph$		—	0.914 ± 0.10
11b	I	$NHCH_2$-4-Cl-Ph		—	25%
11c	I	$NHCH_2$-2-Py		—	57%
11d	I	$NHCH_2$-2-furyl		—	35%
12	I	$NHCH_2Ph$		O	0.28 ± 0.02
13a	CH_3	$NHCH_2Ph$		—	5.08 ± 0.3
13b	CH_3	$NHCH_2$-4-Cl-Ph		—	34.5%
13c	CH_3	$NHCH_2$-2-Py		—	1%
18a	1,2,4-triazol-5-yl	$NHCH_2Ph$		—	1.88 ± 0.23
18b	1,2,4-triazol-5-yl	$NHCH_2$-4-Cl-Ph		—	15.3%
18c	1,2,4-triazol-5-yl	$NHCH_2$-2-Py		—	48%
18d	1,2,4-triazol-5-yl	$NHCH_2$-2-furyl		—	28%
21a	1,2,4-triazol-5-yl		$NHCH_2Ph$	O	1.44 ± 0.11
21b	1,2,4-triazol-5-yl		$NHCH_2$-4-Cl-Ph	O	9%
21c	1,2,4-triazol-5-yl		$NHCH_2$-2-Py	O	33.4%
21d	1,2,4-triazol-5-yl		$NHCH_2$-2-furyl	O	43%
Lead **A**	I		$NHCH_2Ph$	O	0.109 ± 0.008 nM

[a]Percent of inhibition of specific [^{3}H]Ro 15-1788 binding at 10 μM concentration or K_i, are means ± SEM of five determinations.

Table 3

Anxiolytic-like effect of 12 in light–dark box test.

Treatment	mg/kg	Persistent time in light box (min)
CMC 1%		119.3 ± 9.1
flumazenil	100 mg kg^{-1} (i.p.)	120.6 ± 7.3
diazepam 1	10 mg kg^{-1}	165.6 ± 9.1*
12	10 mg kg^{-1}	129.4 ± 7.7
12	30 mg kg^{-1}	153.6 ± 7.2*
12 + flumazenil	30 mg kg^{-1} + 100 mg kg^{-1}	129.3 ± 8.9**

Compound (p.o.) and diazepam were administered 30 min and flumazenil (flu, i.p.) before the test. Each value represents at least eight mice.
*$P < 0.01$ versus saline-treated mice.
**$P < 0.01$ versus the flumazenil-treated mice.

Table 4

Anti-hyperalgesic effect of 12 (CCI, paw-pressure test).

Treatment mg kg^{-1} p.o		Paw	Paw pressure in rats (g)			
			Before treatment	After treatment		
				15 min	30 min	45 min
	CMC	contra	60.3 ± 3.5	63.5 ± 3.1	62.9 ± 3.1	59.6 ± 2.6
	CMC	ipsi	28.5 ± 3.1	25.7 ± 3.8	24.2 ± 3.9	28.2 ± 3.3
12	3	contra	58.7 ± 3.2	63.3 ± 3.2	59.4 ± 2.8	64.5 ± 3.6
	3	ipsi	25.3 ± 3.3	32.7 ± 3.1	35.7 ± 3.5	33.9 ± 2.4
	10	contra	62.6 ± 3.0	58.4 ± 3.6	64.2 ± 3.4	60.8 ± 2.5
	10	ipsi	27.8 ± 2.9	34.5 ± 3.8	34.8 ± 4.1	30.3 ± 3.4
	30	contra	58.5 ± 3.2	66.4 ± 4.0	57.3 ± 3.9	61.9 ± 3.5
	30	ipsi	28.9 ± 3.7	47.9 ± 3.9*	46.2 ± 2.8*	38.0 ± 3.2

The paw pressure test was performed on the ipsilater paw (ipsi) in comparison to the contralateral (contra) unaltered paw. There were at least six rats per group.
*$P < 0.01$.

Table 5

Anti-hyperalgesic effect of 12 (SZT, mouse hot-plate test).

Pre-treatment	Treatment i.p. mg kg^{-1}	Licking latency in mice (s)				
		Before treatment	after treatment			
			15 min	30 min	45 min	60 min
CMC	CMC	16.9 ± 0.9	18.4 ± 1.3	19.2 ± 1.2	18.5 ± 1.3	18.1 ± 1.4
STZ	CMC	9.6 ± 0.5	10.1 ± 1.2	10.2 ± 0.7	9.9 ± 1.1	9.2 ± 0.9
CMC	**12** 30	15.1 ± 1.1	17.0 ± 1.2	17.8 ± 1.0	15.3 ± 1.5	15.6 ± 1.2
STZ	**12** 30	9.3 ± 0.9	15.4 ± 1.0*	15.9 ± 0.7*	16.7 ± 1.0*	15.5 ± 1.3*

Streptozotocine (STZ) 200 mg kg^{-1} i.p. was dissolved in citrate buffer and injected 21 days before experiment. Each value represents the mean of at least 10 mice.
*$P < 0.01$ in comparison with STZ-treated mice.

On the basis of binding results, it emerges that only derivatives bearing the 7-benzylamino group (**11a**, **12**, and **13a**) show receptor recognition (K_i range 0.28–5.08 μM). Thus it is possible to hypothesize that, despite the simultaneous presence of a lipophilic group at position 3 (I and CH_3) and of a group that engages a hydrogen bond at 7-position (NH), [14] a different fit into receptor protein with respect the 8-isomers occurs (data not published). The same trend is verified also in the 3-triazole derivatives (**18a** and **21a**). All these molecules will be inserted in a next study of molecular dynamic to evaluate the occurrence of hydrogen bonds and Van der Walls interactions, between the ligand and the amino acids of receptor protein [30].

CONCLUSION

All new compounds 7-arylalkylamino derivatives, designed as isomers of the active 8-arylalkylaminopyrazolo[5,1-c][1,2,4]benzotriazines, surprisingly have very low affinity at $GABA_A$-R subtype. Within this series, only the 7-benzylamino derivatives **11a**, **12**, **13a**, and **18a** have affinity in micromolar range, showing that the phenyl of the benzylamino chain must be unsubstituted and it cannot be replaced with a heteroaryl ring. The most affine compound was **12**, and because it is the 7-isomer of the lead **A**, pharmacological in vivo activity was assessed. Compound **12** showed anxiolytic-like effect and anti-hyperalgesic profile in mono-neuropathic pain and in diabetic neuropathy at 10–30 mg kg^{-1}, while it was not able to revert the inflammatory pain. Thus, the shift of the NH moiety from 8- to 7-position (from lead **A** to compound **12**) was detrimental for the binding affinity, probably because the NH hydrogen bond donor is not in the suitable position to engage strong interaction with the receptor protein [14,31] responsible for a good affinity value. On the other hand, the retention of anxiolytic-like and anti-hyperalgesic activity seems to indicate that the evoked efficacy (intrinsic activity) by compound **12** is however that of a α2-agonist, analogously to the 8-arylalkylamino isomers, confirming that between affinity and efficacy, the efficacy parameter is more important.

EXPERIMENTAL

Chemistry. ***General.*** Melting points were determined with a Gallenkamp apparatus and were uncorrected. Silica gel plates (Merk F_{254}) and silica gel 60 (Merk 70–230 mesh) were used for analytical and column chromatography, respectively. The structures of all compounds were supported by their IR spectra (KBr pellets in nujol mulls, Perkin-Elmer 1420 spectrophotometer) that showed characteristic absorption band corresponding to N—H stretching, and ^{1}H-NMR data (measured with a Bruker 400 MHz). Chemical shifts were expressed in δ ppm, using DMSO-d_6 or $CDCl_3$ as solvent. The chemical and physical data of new compounds are shown in Table 1; microanalyses were performed with a Perkin-Elmer 260 analyzer for C, H, N; the molecular formula and formula weight are reported in Table 1.

7-Benzylaminopyrazolo[5,1-c][1,2,4]benzotriazine (3). A solution of 7-aminopyrazolo[5,1-c][1,2,4]benzotriazine (**1**) [15] (0.25 mmol) in methanol (10 mL) and five drops of acetic acid was maintained under anhydrous N_2 for 1 h. Benzaldehyde (1:1, 0.026 mL) was added, and the reaction was maintained under reflux until the starting material disappeared (TLC). The solvent was evaporated to dryness, and the formation of the intermediate Base of Schiff **2** was evaluated by ^{1}H-NMR on raw material. TLC eluent: dichloromethane/ethyl acetate 7:3 *v*/v; ^{1}H-NMR (DMSO-d_6) δ 8.92 (s, 1H, CH); 8.53 (m, 2H, H-6 and H-9); 8.47 (d, 1H, H-2); 8.10 (dd, 1H, H-8); 8.07 (dd, 2H, H-2′, and H-6′); 7.65 (d, 1H, H-3); 7.61 (m, 3H, H-3′, H-4′, and H-5′). *Anal.* Calcd C, H, N: C, 70.32%; H, 4.06%; N, 25.63%. Found: C, 70.25%; H, 4.08%; N, 25.59%.

The second step of the reaction was the reduction of Base of Schiff **2**, by the addition of sodium borohydride (0.5 mmol) in methanol at reflux temperature for 2 h. At the end of the reaction (monitored by TLC), a solution of 10% sodium hydroxide was added and the precipitate, compound **3**, was filtered and recrystallized by suitable ethanol. TLC eluent: dichloromethane/ethyl acetate 7:3 *v*/v; IR ν cm^{-1} 3331. ^{1}H-NMR ($CDCl_3$) δ 8.27 (d, 1H, H2); 8.24 (d, 1H, H-9); 7.58 (dd, 1H, H-8); 7.46 (d, 2H, H-2′, and H-6′); 7.43 (m, 2H, H-3, and H-6); 7.38 (t, 2H, H-3′, H-4′); 7.28 (t, 1H, H-4′); 7.20 (t, 1H, NH exch.); 4.48 (d, 1H, CH_2). *Anal.* Calcd C, H, N: C, 69.80%; H, 4.76%; N, 25.44%. Found: C, 69.82%; H, 4.73%; N, 25.49%.

General procedure for the synthesis of 5 and **8**. The starting materials, 7-acetamidopyrazolo[5,1-c][1,2,4]benzotriazine, **4** and 7-nitropyrazolo[5,1-c][1,2,4]pyrazolobenzotriazine 5-oxide, **7** [[15],[18]] (0.2 mmol), respectively, were dissolved in dichloromethane (5 mL), and a solution of iodine monochloride in dichloromenthane (0.4 mmol/2 mL) was added and maintained at room temperature. The reaction was monitored by TLC; after 5 h, the solution was evaporated to dryness and the residue was recovered by treatment with water, made slightly alkaline with a 5% sodium hydroxide solution.

3-Iodo-7-acetamidopyrazolo[5,1-c][1,2,4]benzotriazine (5). From **4** [15]; yellow crystals; yield 70% from ethanol; TLC eluent: toluene/ethyl acetate/methanol 8:2:1 *v*/v/v; IR ν cm^{-1} 3214, 1680; ^{1}H-NMR (DMSO-d_6) δ 10.56 (s, 1H, NH exch.); 9.01 (d, 1H, H-6); 8.52 (s, 1H, H-2); 8.42 (d, 1H, H-9); 8.15 (dd, 1H, H-8); 2.12 (s, 3H, CH_3). *Anal.* Calcd C, H, N: C, 37.41%; H, 2.28%; N, 19.83%. Found: C, 37.53%; H, 2.25%; N, 19.88%.

3-Iodo-7-nitropyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide (8). From **7** [18]; yellow crystals; yield 85% from ethanol; TLC eluent: toluene/ethyl acetate/methanol 8:2:1 *v*/v/v; IR ν cm^{-1} 1570, 1540, 1350; ^{1}H-NMR (DMSO-d_6) δ 9.02 (d, 1H, H-6); 8.80 (dd, 1H, H-8); 8.52 (s, 1H, H-2); 8.49 (d, 1H, H-9). *Anal.* Calcd C, H, N: C, 30.27%; H, 1.13%; N, 19.61%. Found: C, 30.41%; H, 1.15%; N, 19.68%.

3-Iodo-7-aminopyrazolo[5,1-c][1,2,4]benzotriazine (6a). This product was obtained from **5** or **8** in two different methods.

A suspension of **5** (0.2 mmol) in 15 mL of 6N hydrochloride acid was maintained at reflux temperature for 5 h. After cooling, the residue was treated with 10% sodium hydroxide solution and the precipitate was filtered and recrystallized. Yield 80%.

A solution of **8** (0.2 mmol) in methanol (5 mL) was added of $SnCl_2$ [17] in a ratio of 1:5. The reaction was maintained at reflux temperature for 10 h until the starting material disappeared; the final solution was made alkaline with water/sodium hydrogen carbonate and extracted with ethyl acetate. The evaporation of the organic layer gave a residue recrystallized with ethanol 80%. Yield 85%. Red crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 *v*/v/v; IR ν cm^{-1} 3460, 3360; ^{1}H-NMR (DMSO-d_6) δ 8.37 (s, 1H, H-2); 8.18 (d, 1H, H-9); 7.58 (d, 1H, H-6); 7.45 (dd, 1H, H-8); 6.05 (bs, 2H, NH_2 exch.). *Anal.* Calcd C, H, N: C, 34.75%; H, 1.94%; N, 22.51%. Found: C, 34.55%; H, 1.96%; N, 22.49%.

3-Methyl-7-aminopyrazolo[5,1-c][1,2,4]benzotriazine (6b). A suspension of 3-carboxy-7-nitropyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide **9** [18] (0.350 mmol) in THF anhydrous (10 mL) was maintained at 0°C for 30 min, and then 0.1 mL of borane-dimethylsulfide complex in THF was slowly added [32]. The solution was gentle heated to reflux until the starting material disappeared and again cooled in an ice bath, added with 10 mL of methanol and stirred for 1 h. After, the solution was acidified with hydrochloride acid and was again refluxed for 30 min. The addition of 10 mL of methanol and the following evaporation of the solvent gave a residue that was made alkaline with 10% sodium hydroxide solution. Finally, the suspension was extracted with ethyl ether obtaining, after evaporation, a desired crude product that was recrystallized by water. Red crystals; yield 45%; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 *v*/v/v; IR ν cm^{-1} 3460, 3360; ^{1}H-NMR (DMSO-d_6) δ 8.13 (d, 1H, H-9); 8.08 (s, 1H, H-2); 7.56 (d, 1H, H-6); 7.38 (dd, 1H, H-8); 5.91 (bs, 2H, NH_2 exch.); 2.56 (s, 3H, CH_3). *Anal.* Calcd C, H, N: C, 60.29%; H, 4.55%; N, 35.16%. Found: C, 60.35%; H, 4.57%; N, 35.21%.

3-Iodo-7-benzamidopyrazolo[5,1-c][1,2,4]benzotriazine (10). A suspension of **6a** (0.170 mmol) in a 10% sodium hydroxide solution (3 mL) was added of benzoyl chloride (0.5 mL), with attention, because the reaction is exothermic. The reaction was maintained at room temperature for 1 h, and then the precipitate was filtered and washed with water. Pale brown crystals; Yield 70% from ethanol; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 *v*/v/v; IR ν cm^{-1} 3200; ^{1}H-NMR (DMSO-d_6) δ 10.88 (s, 1H, NH exch.); 9.19 (d, 1H, H-6); 8.54 (s, 1H, H-2); 8.47 (m, 2H, H-9 and H-8); 8.05 (d, 2H, Ph); 7.58–7.66 (m, 3H, Ph). *Anal.* Calcd C, H, N: C, 46.29%; H, 2.43%; N, 16.87%. Found: C, 46.40%; H, 2.47%; N, 16.82%.

General procedure for the synthesis of 11a–d, **13a–c**, **18a–d**. A mixture of starting material **6a**, **6b**, and **17** (0.225 mmol) and the suitable aldehyde (benzaldehyde, 4-chlorobenzaldehyde, 2-pyridincarboxyaldehyde, 2-furaldehyde, **a-d,** 0.3 mmol) was solubilized in methanol (4 mL), and a solution of sodium cyanoborohydride (0.8 mmol) and zinc chloride (0.8 mmol) in methanol (2 mL) was added; the reaction was maintained at room temperature for 8 h and monitored by TLC. The final suspension was filtered, to eliminate the inorganic residue, and washed with methanol. The solvent was evaporated to dryness and alkalinized (pH 8) with a 10% sodium hydroxide solution. The precipitate was filtered and recrystallized by suitable solvent.

3-Iodo-7-benzylaminopyrazolo[5,1-c][1,2,4]benzotriazine (11a). From **6a**; yield 70% from ethanol; red crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 *v*/v/v; IR ν cm^{-1} 3328; ^{1}H-NMR (DMSO-d_6) δ 8.27 (d, 1H, H-6); 8.24 (d, 1H, H-9); 7.55 (dd, 1H, H-8); 7.50–7.42 (m, 6H, Ph and H-2); 7.27 (t, 1H, NH exch.); 4.50 (d, 2H, CH_2). *Anal.* Calcd C, H, N: C, 47.90%; H, 3.01%; N, 17.46%. Found: C, 48.28%; H, 3.03%; N, 17.42%.

3-Iodo-7-(4-chlorobenzylamino)pyrazolo[5,1-c][1,2,4] benzotriazine (11b). From **6a**; yield 63% from ethanol; orange crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 *v*/v/v; IR ν cm^{-1} 3324; ^{1}H-NMR (DMSO-d_6) δ 8.27 (d, 1H, H-6); 8.24 (d, 1H, H-9); 7.55 (dd, 1H, H-8); 7.50 (d, 2H, Ph); 7.42 (m, 3H, Ph and H-2); 7.24 (t, 1H, NH exch.); 4.49 (d, 2H, CH_2). *Anal.* Calcd C, H, N: C, 44.11%; H, 2.55%; N, 16.08%. Found: C, 44.28%; H, 2.54%; N, 16.06%.

3-Iodo-7-(2-pyridinylmethylamino)pyrazolo[5,1-c][1,2,4] benzotriazine (11c). From **6a**; yield 58% from 2-propanol; red crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 *v*/v/v; IR ν cm^{-1} 3331; ^{1}H-NMR (DMSO-d_6) δ 8.59 (d, 1H, Py); 8.37 (d, 1H, H-6); 8.23 (d, 1H, H-9); 7.78 (t, 1H, Py); 7.59 (dd, 1H, H-8); 7.48–7.42 (m, 2H, H-2 and Py); 7.34 (t, 1H, Py); 7.30 (t, 1H, NH exch.); 4.58 (d, 2H, CH_2). *Anal.* Calcd C, H, N: C, 44.79%; H, 2.76%; N, 20.90%. Found: C, 44.38%; H, 2.72%; N, 21.01%.

3-Iodo-7-(furan-2-methylamino)pyrazolo[5,1-c][1,2,4] benzotriazine (11d). From **6a**; yield 71% from ethanol; brown crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 *v*/v/v; IR ν cm^{-1} 3328; ^{1}H-NMR (DMSO-d_6) δ 9.02 (d, 1H, H-6); 8.81(dd, 1H, H-8); 8.52 (s, 1H, H-2); 8.24 (d, 1H, H-9); 7.60 (m, 2H, furane); 7.36 (m, 1H, furane); 6.89 (t, 1H, NH exch.); 4.50 (d, 2H, CH_2). *Anal.* Calcd C, H, N: C, 42.99%; H, 2.58%; N, 17.90%. Found: C, 43.10%; H, 2.52%; N, 17.85%.

3-Iodo-7-benzylaminopyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide (12). To a solution of compound **11a** (0.50 mmol) in

CH_2Cl_2, an excess of m-chloroperbenzoic acid was added. The reaction was kept at 60°C and monitored by TLC. The final precipitate was filtered and recrystallized. Yield 65% from ethanol; red crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 *v*/v/v; IR ν cm^{-1} 3315, 1580; ^{1}H-NMR (DMSO-d_6) δ 8.37 (s, 1H, H-2); 8.22 (d, 1H, H-9); 7.54 (dd, 1H, H-8); 7.48 (m, 2H, Ph); 7.42 (m, 3H, Ph and H-6); 7.30 (d, 1H, Ph); 7.27 (t, 1H, NH exch.); 4.50 (d, 2H, CH_2). *Anal.* Calcd C, H, N: C, 46.06%; H, 2.90%; N, 16.79%. Found: C, 40.35%; H, 2.88%; N, 16.85%.

3-Methyl-7-benzylaminopyrazolo[5,1-c][1,2,4]benzotriazine (13a). From **6b**; yield 68% from ethanol; orange crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 *v*/v/v; IR ν cm^{-1} 3339; ^{1}H-NMR (DMSO-d_6) δ 8.18 (d, 1H, H-9); 8.08 (s, 1H, H-2); 7.47 (dd, 1H, H-8); 7.41 (d, 2H, Ph); 7.38 (d, 1H, H-6); 7.36 (d, 2H, Ph); 7.29 (t, 1H, Ph); 7.12 (t, 1H, NH exch.); 4.48 (d, 2H, CH_2); 2.55 (s, 3H, CH_3). *Anal.* Calcd C, H, N: C, 70.57%; H, 5.23%; N, 24.21%. Found: C, 70.43%; H, 5.26%; N, 24.26%.

3-Methyl-7-(4-chlorobenzylamino)pyrazolo[5,1-c][1,2,4]benzotriazine (13b). From **6b**; yield 54% from 2-propanol; orange crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 *v*/v/v; IR ν cm^{-1} 3252; ^{1}H-NMR (DMSO-d_6) δ 9.34 (d, 1H, H-9); 8.81 (dd, 1H, H-8); 8.59 (d, 1H, H-6); 8.10 (s, 1H, H-2); 7.49 (d, 2H, Ph); 7.43 (m, 2H, Ph); 7.18 (t, 1H, NH exch.); 4.49 (d, 2H, CH_2); 2.55 (s, 3H, CH_3). *Anal.* Calcd C, H, N: C, 63.06%; H, 4.36%; N, 21.63%. Found: C, 63.47%; H, 4.32%; N, 21.59%.

3-Methyl-7-(2-pyridinylmethylamino)pyrazolo[5,1-c][1,2,4]benzotriazine (13c). From **6b**; yield 35% from 2-propanol; brown crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 *v*/v/v; IR ν cm^{-1} 3327; ^{1}H-NMR (DMSO-d_6) δ 9.39 (d, 1H, H-9); 8.81 (dd, 1H, H-8); 8.59 (d, 1H, Py); 8.47 (m, 2H, H-2 and H-6); 8.17 (m, 1H, Py); 7.48–7.42 (m, 2H, Py); 7.34 (t, 1H, Py); 7.25 (t, 1H, NH exch.); 4.55 (d, 2H, CH_2); 2.62 (s, 3H, CH_3). *Anal.* Calcd C, H, N: C, 66.19%; H, 4.86%; N, 28.95%. Found: C, 66.25%; H, 4.82%; N, 29.05%.

3-(1,2,4-Triazol-3-yl)-7-benzylaminopyrazolo[5,1-c][1,2,4]benzotriazine (18a). From **17**; yield 65.4% from methoxyethanol; red crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 *v*/v/v; IR ν cm^{-1} 3331, 3210; ^{1}H-NMR (DMSO-d_6) δ 9.71 (bs, 1H, NH triazole exch.), 8.54 (s, 1H, H-2); 8.38 (s, 1H, CH); 8.24 (d, 1H, H-9); 7.58 (dd, 1H, H-8); 7.46 (m, 3H, H-6 and Ph); 7.36 (t, 2H, Ph); 7.26 (t, 1H, Ph); 7.21 (t, 1H, NH exch.); 4.51 (d, 2H, CH_2). *Anal.* Calcd C, H, N: C, 63.15%; H, 4.12%; N, 32.73%. Found: C, 63.37%; H, 4.18%; N, 32.78%.

3-(1,2,4-Triazol-3-yl)-7-(4-chlorobenzylamino)pyrazolo[5,1-c][1,2,4]benzotriazine (18b). From **17**; yield 58% from methoxyethanol; red crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 *v*/v/v; IR ν cm^{-1} 3281, 3210; ^{1}H-NMR (DMSO-d_6) δ 10.1 (bs, 1H, NH exch.); 8.50 (s, 1H, H-2); 8.35 (s, 1H, CH); 8.24 (d, 1H, H-9); 7.60 (dd, 1H, H-8); 7.39 (m, 4H, Ph); 7.37 (d, 1H, H-6); 5.27 (t, 1H, NH exch.); 4.53 (d, 2H, CH_2). *Anal.* Calcd C, H, N: C, 53.38%; H, 3.48%; N, 29.74%. Found: C, 57.45%; H, 3.43%; N, 29.71%.

3-(1,2,4-Triazol-3-yl)-7-(2-pyridinylmethylamino)pyrazolo[5,1-c][1,2,4]benzotriazine (18c). From **17**; yield 35% from ethanol; red crystals; TLC eluent: toluene/ethyl acetate/methanol 8:2:1 *v*/v/v; IR ν cm^{-1} 3331, 3220; ^{1}H-NMR (DMSO-d_6) δ 10.1 (bs, 1H, NH exch.); 8.48 (d, 1H, Py); 8.26 (s, 1H, H-2); 8.17 (m, 2H, CH and H-9); 7.76 (m, 1H, Py); 7.54 (dd, 1H, H-8); 7.46 (m, 2H, H-6 and Py); 7.24 (m, 1H, Py); 5.38 (t, 1H, NH exch.); 4.56 (d, 2H, CH_2). *Anal.* Calcd C, H, N: C, 59.47%; H, 3.82%; N, 36.72%. Found: C, 59.51%; H, 3.85%; N, 36.77%.

3-(1,2,4-Triazol-3-yl)-7-(furan-2-methylamino)pyrazolo[5,1-c][1,2,4]benzotriazine (18d). From **17**; yield 60.5% from ethanol; light violet crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 *v*/v/v; IR ν cm^{-1} 3318, 3210; ^{1}H-NMR (DMSO-d_6) δ 9.20 (bs, 1H, NH exch.); 8.11 (m, 2H, H-2 and CH); 7.65 (d, 1H, H-9); 7.55 (m, 1H, furane); 6.60 (d, 1H, H-8); 6.43 (m, 2H, H-8 and furane); 6.26 (m, 1H, furane); 6.89 (t, 1H, NH exch.); 4.50 (d, 2H, CH_2). *Anal.* Calcd C, H, N: C, 57.83%; H, 3.64%; N, 33.72%. Found: C, 57.47%; H, 3.59%; N, 33.68%.

General procedure for the synthesis of 15 and 23. The starting material, 3-carbamoyl-7-nitropyrazolo[5,1-c][1,2,4] benzotriazine 5-oxide **14** [18] and **22** (0.182 mmol), was solubilized in a mixture of anhydrous toluene (5 mL) and anhydrous dimethylformamide (2 mL), then DMF-DMA (0.3 mL) was added. The reaction was maintained at reflux temperature and monitored by TLC until the starting material disappeared. The final suspension was filtered, and the product, washed with di-isopropyl ether, was pure enough and used in the next reaction.

3-(N-(Dimethylaminomethylene)carbamoyl)-7-nitropyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide (15). From **14** [18]; green-yellow crystals; yield 78%; TLC eluent: toluene/ethyl acetate/methanol 8:2:2 *v*/v/v; IR ν cm^{-1} 1640, 1570, 1540, 1340; ^{1}H-NMR (DMSO-d_6) δ 9.04 (d, 1H, H-6); 8.83 (dd, 1H, H-8); 8.74 (s, 1H, H-2); 8.64 (s, 1H, CH); 8.57 (d, 1H, H-9); 3.21 (s, 3H, CH_3); 3.19 (s, 3H, CH_3). *Anal.* Calcd C, H, N: C, 47.42%; H, 3.37%; N, 29.78%. Found: C, 47.30%; H, 3.32%; N, 29.75%.

3-(N-(Dimethylaminomethylene)carbamoyl)-8-benzylaminopyrazolo[5,1-c][1,2,4]benzo triazine 5-oxide (23). From **22**; green-yellow crystals; yield 74%; TLC eluent: toluene/ethyl acetate/methanol 8:2:2 *v*/v/v; IR ν cm^{-1} 3350, 1640, 1550; ^{1}H-NMR (DMSO-d_6) δ 8.58 (s, 1H, H-5); 8.45 (s, 1H, H-2); 8.38 (t, 1H, NH exch.); 8.14 (d, 1H, H-6); 7.40 (m, 4H, Ph and H-9); 7.30 (m, 2H, Ph); 7.10 (m, 1H, H-7); 4.56 (d, 2H, CH_2); 3.18 (s, 3H, CH_3); 3.14 (s, 3H, CH_3). *Anal.* Calcd C, H, N: C, 61.69%; H, 4.92%; N, 25.18%. Found: C, 61.41%; H, 4.89%; N, 25.22%.

General procedure for the synthesis of 16 and 21a. To a suspension of **15** or **23** (0.150 mmol) in glacial acetic acid (3 mL) was added hydrazine hydrate 55% (0.35 mmol, 0.017 mL). The reaction was kept at 90–100°C and monitored by TLC until the starting material disappeared. The residue was diluted with diethyl ether, filtered and recrystallized by suitable solvent.

3-(1,2,4-Triazol-3-yl)-7-nitropyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide (16). From **15**; orange crystals; yield 83% from ethanol; TLC eluent: toluene/ethyl acetate/methanol 8:2:2 *v*/v; IR ν cm^{-1} 3312, 1570, 1540, 1350; ^{1}H-NMR (DMSO-d_6) δ 11.7 (br, 1H, NH exch.); 9.05 (d, 1H, H-6); 8.83 (dd, 1H, H-8); 8.78 (s, 1H, H-2); 8.58 (s, 1H, CH); 8.56 (d, 1H, H-9). *Anal.* Calcd C, H, N: C, 44.30%; H, 2.03%; N, 37.57%. Found: C, 44.47%; H, 2.08%; N, 37.59%.

3-(1,2,4-Triazol-3-yl)-8-benzylaminopyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide (21a). From **23**; orange crystals; yield 83% from 2-propanol; TLC eluent: toluene/ethyl acetate/methanol 8:2:1.5 *v*/v/v; IR ν cm^{-1} 3321, 3231, 1550; ^{1}H–NMR (DMSO-d_6) δ 14.08 (br, 1H, NH exch.); 8.51 (s, 1H, H-2); 8.37 (s, 1H, H-5); 8.15 (d, 1H, H-6); 7.40 (m, 4H,

Ph and H-9); 7.30 (m, 2H, Ph); 7.10 (m, 2H, H-7 and NH exch.); 4.56 (d, 2H, CH_2). *Anal.* Calcd C, H, N: C, 60.33%; H, 3.94%; N, 31.27%. Found: C, 60.47%; H, 3.98%; N, 31.31%.

3-(1,2,4-Triazol-3-yl)-7-aminopyrazolo[5,1-c][1,2,4]benzotriazine (17). To a suspension of **16** (0.150 mmol) in conc. hydrochloride acid (3 mL) at 0°C was added, in portions, tin (150 mg). After 15 min, the reaction finished (by TLC) and the inorganic precipitate, $SnCl_4$, was solubilized by treatment with a 10% sodium hydroxide solution until pH 10.

The desired final product, instead, was recovered as red precipitate, filtered and recrystallized by ethanol. Yield 72%; TLC eluent: dichloromethane/methanol 10:0.5 *v*/v; IR ν cm^{-1} 3344, 3240, 3218; ^{1}H-NMR (DMSO-d_6) δ 14.2 (br, 1H, NH exch.); 8.72 (s, 1H, H-2); 8.27 (d, 1H, H-9); 8.12 (s, 1H, CH); 7.61 (d, 1H, H-6); 7.54 (dd, 1H, H-8); 6.05 (bs, 2H, NH_2 exch.); *Anal.* Calcd C, H, N: C, 52.38%; H, 3.20%; N, 44.42%. Found: C, 52.45%; H, 3.25%; N, 44.39%.

General procedure for the synthesis of 21b–d and 22. A solution of starting material 3-(1,2,4-triazol-3-yl)-8-chloropyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide **20** [19] or **19** [18] (0.150 mmol) in dimethylformamide (5 mL) was added of the suitable aromatic amine (2 mL, benzylamine 4-chlorobenzylamine, 2-aminomethylpyridine, furfurylamine) and vrefluxed monitoring the reaction by TLC. The final solution was cooled, and the addition of water gave a precipitate that was recrystallized or purified by chromatography column.

3-(1,2,4-Triazol-3-yl)-8-(4-chlorobenzylamino)pyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide (21b). From **20** [19] with 4-chlorobenzylamine, yield 48% from chromatography column (eluent toluene/ethyl acetate/acetic acid 8:2:1 *v*/v); orange crystals; IR ν cm^{-1} 3331, 3210, 1570; ^{1}H-NMR (DMSO-d_6) δ 14.1 (bs, 1H, NH exch.); 7.91 (m, 2H, H-2 and CH); 7.56 (d, 1H, H-6); 7.39–7.32 (m, 5H, Ph and NH exch.); 6.60 (dd, 1H, H-7); 6.43 (d, 1H, H-9); 4.46 (d, 2H, CH_2). *Anal.* Calcd C, H, N: C, 55.04%; H, 3.34%; N, 28.53%. Found: C, 55.31%; H, 3.37%; N, 28.50%.

3-(1,2,4-Triazol-3-yl)-8-(4-pyridinilmethylamino)pyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide (21c). From **20** [19] with 2-aminomethylpyridine; yield 55% from water; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 *v*/v; orange crystals; IR ν cm^{-1} 3319, 3213, 1570; ^{1}H-NMR (DMSO-d_6) δ 9.74 (bs, 1H, NH exch.); 8.63 (m, 2H, Py); 8.54 (s, 1H, H-2); 8.52 (s, 1H, CH); 8.06 (d, 1H, H-6); 7.83 (d, 1H, H-9); 7.53 (m, 2H, Py); 7.43 (dd, 1H, H-7); 7.21 (t, 1H, NH exch.); 4.52 (d, 2H, CH_2). *Anal.* Calcd C, H, N: C, 56.82%; H, 3.65%; N, 35.08%. Found: C, 56.59%; H, 3.62%; N, 35.18%.

3-(1,2,4-Triazol-3-yl)-8-(furan-2-methylamino)pyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide (21d). From **20** [19] with furfurylamine; yield 48.3% from water; brown crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 *v*/v/v; IR ν cm^{-1} 3331, 3220, 1550; ^{1}H-NMR (DMSO-d_6) δ 9.20 (bs, 1H, NH exch.); 7.90 (s, 1H, CH); 7.58 (m, 2H, H-6 and H-2); 7.55 (m, 1H, furane); 7.18 (d, 1H, H-9); 7.03 (dd, 1H, H-7); 6.43 (m, 2H, furane); 6.89 (t, 1H, NH exch.); 4.55 (d, 2H, CH_2). *Anal.* Calcd C, H, N: C, 55.17%; H, 3.47%; N, 32.17%. Found: C, 55.31%; H, 3.43%; N, 32.15%.

3-Carbamoyl-8-benzylaminopyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide (22). From **19** [18] with benzylamine, orange crystals; yield 86%; TLC eluent: toluene/ethyl acetate/ 8:2 *v*/v; IR ν cm^{-1} 3335, 3211 1640, 1550; ^{1}H-NMR (DMSO-d_6) δ 8.43 (t, 1H, NH exch.); 8.41 (s, 1H, H-2); 8.15 (d, 1H, H-6); 7.45 (bs, 1H, NH exch.); 7.40 (m, 4H, Ph, and H-9); 7.30 (m, 2H, Ph); 7.10 (m, 1H, H-7); 6.98 (bs, 1H, NH exch.); 4.56 (d, 1H, CH_2). *Anal.* Calcd C, H, N: C, 61.07%; H, 4.22%; N, 25.14%. Found: C, 61.32%; H, 4.25%; N, 25.20%.

Biological. ***Radioligand binding assay.*** [^{3}H]Ro 15-1788 (specific activity 78.8 Ci/mmol) was obtained from Perkin Elmer. All the other chemicals, which were of reagent grade, were obtained from commercial suppliers. Bovine cerebral cortex membranes were prepared as previously described [33,34]. 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.* [35]; [^{3}H]Ro 15-1788 binding studies were performed as previously reported [19]. At least six different concentrations of each compound were used. The data of $n = 5$ experiments carried out in triplicate were analyzed by means of an iterative curve-fitting procedure (program Prism, GraphPad, San Diego, CA), which provided IC_{50}, K_i, and SEM values for tested compounds, the K_i values being calculated from the Cheng and Prusoff equation [36].

Pharmacological methods.

Animals. Male Swiss albino mice (23–30 g) and male Sprague Dowley rats from the Harlan-(Varese) breeding farm were used. Fifteen mice or four rats were housed per cage. The cages were placed in the experimental room 24 h before the test for acclimatization. The animals were kept at 23 ± 1°C with a 12 h light/dark cycle, light at 7 AM, with food and water ad libitum. All experiments were carried out according to the guidelines of the European Community Council.

Pentylenetetrazole-induced seizure. Pentylentetrazole (90 mg/kg s.c.) 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.

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.

Hot plate test. The method adopted was described by O'Callaghan and Holzman [37]. Mice were placed inside a stainless steel container, thermostatically set at 52.5 ± 0.1°C in a precision water-bath from KW Mechanical Workshop, Siena, Italy. Reaction times (s), were measured with a stop-watch before i.c.v. injections and at regular intervals (15 min) up to a maximum of 60 min after treatment (cut-off) in order to prevent tissue damage. The endpoint used was the licking of the fore or hind paws. Antinociception was seen as increased latencies to the responses evaluated, while increased nociception was seen by shorter latencies. Those mice scoring below 12 and over 18 s in the pretest were rejected (30%). An arbitrary cut-off time of 45 s was adopted.

Abdominal constriction test. Mice were injected i.p. with a 0.6% solution of acetic acid (10 mL/kg), according to Koster [38]. The number of stretching movements was counted for 10 min, starting 5 min after acetic acid injection.

Paw pressure test. The nociceptive threshold in the rat was determined with an analgesimeter (Ugo Basile, Varese, Italy), according to the method described by Leighton [22]. The instrument exerts a force that is applied at a costant rate (32 g per second) with a cone-shaped pusher on the upper surface of the rat hind paw. The force is continuously monitored by a pointer moving along a linear scale. The pain threshold is given by the force that induces the first struggling from the rat. Pretested rats that scored below 40 g or over 75 g during the test before drug administration (25%) were rejected. An arbitrary cut off value of 250 g was adopted.

Chronic constriction injury. A peripheral mononeuropathy was produced in adult rats by placing loosely constrictive ligatures around the common sciatic nerve according to the method described by Bennett [21]. Rats were anesthetized with chloral hydrate. The common sciatic nerve was exposed at the level of the middle of the thigh by blunt dissection through biceps femoris. Proximal to sciatica's tri furcation, about 1 cm of the nerve was freed of adhering tissue and four ligatures (3/0 silk tread) were tied loosely around it with about 1 mm spacing. The length of the nerve thus affected was 4–5 mm long. Great care was taken to tie the ligatures such that the diameter of the nerve was seen to be just barely constricted when viewed with 40× magnification. In every animal, an identical dissection was performed on the opposite side except that the sciatic nerve was not ligated. The left paw was untouched.

Induction of diabetes (streptozotocin treatment). Streptozotocin (200 mg kg^{-1}) was solubilized in a saline solution (pH 4.5 in 0.1 N sodium citrate buffer). Mice were divided in two groups that received in the tail vein STZ or vehicle, respectively. STZ increases serum glucose level (above 300 mg/dl; measured by the glucose oxidase method) 7, 14, and 21 days after injection treatment. A significant thermal hyperalgesia is well established on day 21 [39]. Behavioral tests were performed on day 21, after a preliminary evaluation of the pain threshold (mice scoring over 20 s in the hot plate test were rejected). Measurements were performed over time (30, 60, 90, and 120 min) after the administration of test compound.

Drugs. Diazepam (Valium 10 – Roche), flumazenil (Roche), PTZ (Sigma), and zolpidem (Tocris) were used. All drugs except PTZ were suspended in 1% carboxymethylcellulose sodium salt and sonicated immediately before use. PTZ was dissolved in an isotonic (NaCl 0.9%) saline solution and injected s.c. All benzodiazepine receptor ligands were administered by p.o. route, except for flumazenil, which was administered i.p. Drug concentrations were prepared in such a way that the necessary dose could be administered in a volume of 10 mL/kg by the p.o., i.p., or s.c. routes.

Statistical analysis. All experimental results are given as the mean ± S.E.M. Analysis of variance, followed by Fisher's Protected Least Significant Difference procedure for post hoc comparison, was used to verify significance between two means. Data were analyzed with the StatView software for the Macintosh (1992). *P* values of less than 0.05 were considered significant.

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