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Laboratory note

Synthesis and benzodiazepine receptor binding of some imidazo-, pyrimido[2,1-*b*]benzoxazoles and pyrimido[1,2-*a*]benzimidazoles

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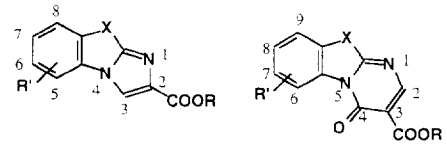
Summary — A series of imidazo[2,1-*b*]benzoxazoles **3a–c**, pyrimido[2,1-*b*]benzoxazoles **4–6**, and pyrimido[1,2-*a*]benzimidazoles **7–9** was synthesized and evaluated for affinity at the benzodiazepine receptor (BZR). These compounds generally possess BZR binding affinities lower than those observed for the corresponding benzothiazole analogues. However, imidazobenzoxazole **3d** possesses high binding affinity, showing an IC_{50} value of 77 nM. The pharmacological profile of **3d** was predicted by [^{35}S]TBPS binding as inverse agonist whereas antagonist or partial agonist activity was suggested by the GABA ratio value. Hence, a contrasting predictive capability of GABA ratio and [^{35}S]TBPS binding was observed. Compound **3d** should possess partial inverse agonist activity at BZR, because its [^{35}S]TBPS binding data is comparable to those of FG-7142.

imidazo[2,1-*b*]benzoxazole / pyrimido[2,1-*b*]benzoxazole / pyrimido[1,2-*a*]benzimidazole / central benzodiazepine receptor / peripheral benzodiazepine receptor / [^{35}S]TBPS binding / GABA ratio

Introduction

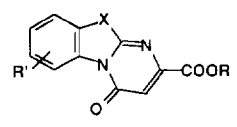
Benzodiazepine receptor (BZR) ligands act through the γ-aminobutyric acid A ($GABA_A$) receptor complex, eliciting a wide variety of pharmacological effects. Thus benzodiazepine receptor ligands cover a full continuum of activities from maximal positive allosteric modulation (agonist) to maximal negative allosteric modulation (inverse agonist); the effects of both classes of ligands are blocked by antagonists which have high affinity for BZR and which exhibit no pharmacological effects [1–3]. Even though the detailed structure of the benzodiazepine/GABA receptor complex remains obscure, recent results from cloning experiments suggest that multiple subpopulations of BZR exist in several regions of the mammalian brain, presumably serving different biological functions [4]. Thus, over the past few years many efforts have been focused on discovering new BZR ligands with more selectivity for the benzodiazepine/$GABA_A$ receptor subtypes and ligands with low intrinsic efficacy (partial agonists) in the hope of obtaining drugs with fewer unwanted side effects compared to full agonists.

Recently, we reported that some imidazo[2,1-*b*]-benzothiazoles **1** and 4*H*-pyrimido[2,1-*b*]benzothiazol-4-ones **2** bind to the BZR but show opposite pharmacological profiles [5]. In particular, it was found that while imidazobenzothiazoles **1a** (R = C_2H_5, R' = 5-OCH_3) and **1b** (R = OCH_3, R' = 5-Cl) possess inverse agonist profile, the pyrimidobenzothiazoles **2a** (R = C_2H_5, R' = 6-OCH_3) and **2b** (R = C_2H_5, R' = 6-Cl) show partial agonist properties.

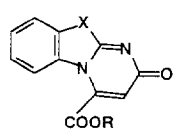


1 X = S
3 X = O

2 X = S
4 X = O
7 X = N-R



5 X = O
8 X = N-R



6 X = O
9 X = N-R

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As part of our research program on new BZR ligands, we now report on the synthesis and BZR binding affinity of some imidazo[2,1-*b*]benzoxazoles **3**, pyrimido[2,1-*b*]benzoxazoles **4–6** and pyrimido-[1,2-*a*]benzimidazole **7–9**. These compounds were designed considering that changes in binding affinity and potency might be produced by substitution of one heteroatom (sulfur for oxygen or nitrogen).

Chemistry

The general synthetic procedure employed to prepare compounds **3a–d** (table I) involves the reaction of the appropriate 2-aminobenzoxazoles **10** with ethyl bromopyruvate in refluxing DMF (scheme 1). Reaction of 2-aminobenzoxazoles **10** (R' = 5-Cl) with diethyl (ethoxymethylene)malonate (EMME) in ethanol gave the corresponding [[(2-benzothiazolyl)-amino]methylene]malonate **11** (10% yield). Successive cyclization of this last compound led to the desired pyrimidobenzoxazole **4** (R' = 5-Cl).

Compound **5** (R' = 7-Cl) was prepared in 24% yield by heating a mixture of 5-chloro-2-aminobenzoxazole **10** (R' = 5-Cl) and dimethyl 2-aminofumarate (DMAF) at 130 °C. Reaction of 5-chloro-2-aminobenzoxazole **10** (R' = 5-Cl) with dimethyl (DMAD) or diethyl acetylenedicarboxylate (DEAD) gave compounds **6a** and **6b**, in 26 and 40% yields, respectively. Reaction of 2-aminobenzimidazole **12** (R = H) with ethyl bromopyruvate in THF afforded an intermediate hydrobromide which was subsequently cyclized in refluxing EtOH to give compound **13a** (9% yield). The structural assignment for this last compound is based on its ^{1}H-NMR spectrum which shows a broad signal at δ 9.2 attributable to the NH proton deshielded by the ester carbonyl. Hence, the presence of the tautomer **13b** in $CDCl_3$ (scheme 2) can be ruled out.

Unfortunately, as far as the imidazobenzimidazole series is concerned, our attempts to prepare compounds substituted at the benzene ring by the above-mentioned procedure or by a variation of the reaction conditions (ie, longer reaction times or higher temperatures) failed.

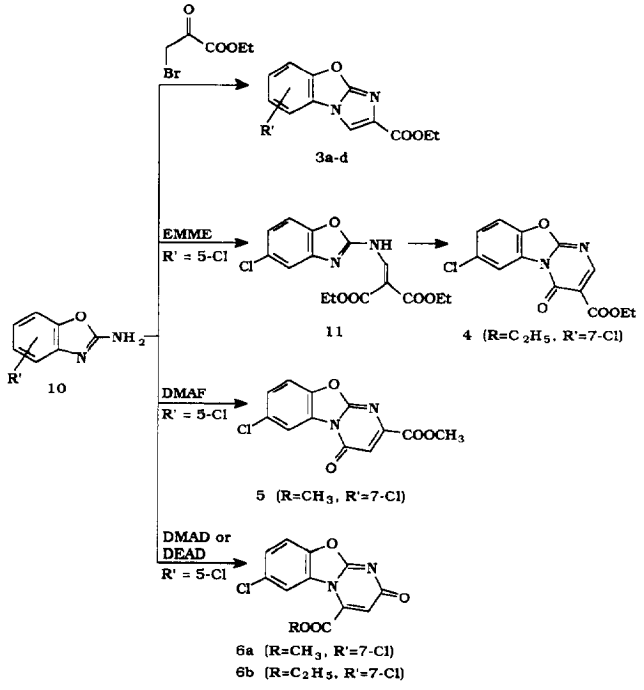
The pyrimidobenzimidazole compounds **7b** and **9b** and the known compound **8** (R = CH_3) [6] (table I) were synthesized as shown in scheme 3. These procedures essentially follow methods previously reported [6] for known compounds **7a** and **9a**.

Structural assignments for compounds **3–9** are based on IR, ^{1}H-NMR and mass spectral data. In particular, the ^{1}H-NMR spectra of **3a–d** are characterized by a singlet at δ 8.00 attributable to the proton linked to the C(3) carbon. Similarly, the ^{1}H-NMR spectra of **6a** and **6b** show a singlet at δ 7.00 attributable to the proton at C(3) carbon.

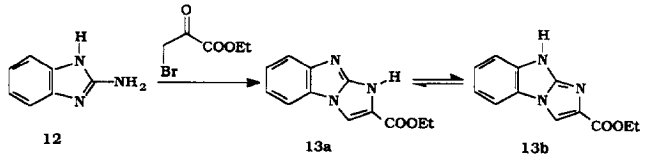
Results and discussion

The ability of compounds **3–9** to interact with BZR was investigated by a binding assay using [^{3}H]flunitrazepam as radioligand and membranes from brain tissues as receptor source. The percentage of inhibition of specific [^{3}H]flunitrazepam binding was determined by using a 40 μM concentration of the tested compounds followed by the determination of IC_{50} only for the most active ones (percentages of inhibition greater than 75%). The binding affinities measured are shown in table II.

The analysis of affinity showed some interesting differences between the series examined. Thus, in the imidazobenzoxazole series, the parent compound **3a**,



Scheme 1.



Scheme 2.

Table I. Physical and spectral data of 2-alkoxycarbonylimidazobenzoxazoles **3a–d**, pyrimidobenzoxazoles **4–6**, and pyrimidobenzimidazoles **7–9**.

Compound	*R*	*R'*	*Yield (%)*	*Mp (°C) (recrystallization solvent)*[a]	*Spectral data*
3a	C_2H_5	H	10	152–154 (A)	IR ν_{max} 1700 cm^{-1} ^{1}H-NMR ($CDCl_3$) δ: 1.43 (t, 3H, CH_3), 4.39 (q, 2H, CH_2), 7.3–7.6 (m, 4H, ArH), 8.00 (s, 1H, CH(3)) MS, *m/z*: 230 (36, M^+), 158 (base)
3b	C_2H_5	6-CH_3	40	156–158 (B)	IR ν_{max} 1690, 1630 cm^{-1} ^{1}H-NMR ($CDCl_3$) δ: 1.43 (t, 3H, CH_3), 2.50 (s, 3H,), 4.40 (q, 2H, CH_2), 7.1–7.5 (m, 3H, ArH), 7.93 (s, 1H, CH(3)) MS, *m/z*: 244 (32, M^+), 172 (base)
3c	C_2H_5	7-CH_3	40	169–170 (B)	IR ν_{max} 1710, 1640 cm^{-1} ^{1}H-NMR ($CDCl_3$) δ: 1.43 (t, 3H, CH_3), 2.16 (s, 3H, CH_3), 4.43 (q, 2H, CH_2), 7.1–7.6 (m, 3H, ArH), 7.96 (s, 1H, CH(3)) MS, *m/z*: 244 (32, M^+), 172 (base)
3d	C_2H_5	6-Cl	28	195–197 (A)	IR ν_{max} 1700, 1630 cm^{-1} ^{1}H-NMR ($CDCl_3$) δ: 1.43 (t, 3H, CH_3), 2.50 (s, 3H, CH_3), 4.43 (q, 2H, CH_2), 7.3–7.7 (m, 3H, ArH), 8.00 (s, 1H, CH(3)) MS, *m/z*: 264 (36, M^+), 158 (base)
3e	*i*-C_3H_7	6-Cl	24	210 (dec)	IR ν_{max} 1690, 1640 cm^{-1} ^{1}H-NMR (DMSO-d_6) δ: 1.33 (d, 6H, CH_3), 5.10 (m, 1H, CH), 7.0–8.2 (m, 3H, ArH), 8.43 (s, 1H, CH(3)) MS, *m/z*: 278 (30, M^+), 192 (base)
4	C_2H_5	7-Cl	10	150–152 (C)	IR ν_{max} 1750 cm^{-1} ^{1}H-NMR ($CDCl_3$) δ: 1.40 (t, 3H, CH_3), 4.40 (q, 2H, CH_2), 7.53 (m, 2H, ArH), 8.53 (s, 1H, ArH), 8.83 (s, 1H, CH(2)) MS, *m/z*: 292 (32, M^+), 220 (base)
5	CH_3	7-Cl	24	212–214 (D)	IR ν_{max} 1700, 1630 cm^{-1} ^{1}H-NMR ($CDCl_3$) δ: 4.00 (s, 3H, OCH_3), 7.20 (s, 1H, CH(3)), 7.53 (d, 2H, ArH), 8.50 (s, 1H, ArH) MS, *m/z*: 278 (M^+, base)
6a	CH_3	7-Cl	26	208–209 (E)	IR ν_{max} 1740, 1640 cm^{-1} ^{1}H-NMR ($CDCl_3$) δ: 4.10 (s, 3H, OCH_3), 7.00 (s, 1H, CH(3)), 7.4–7.5 (m, 2H, ArH), 8.0–8.1 (m, 1H, ArH) MS, *m/z*: 278 (92, M^+), 220 (base)
6b	C_2H_5	7-Cl	40	222–224 (C)	IR ν_{max} 1740, 1630 cm^{-1} ^{1}H-NMR ($CDCl_3$) δ: 1.50 (t, 3H, CH_3), 4.56 (q, 2H, CH_2), 7.00 (s, 1H, CH(3)), 7.4–7.5 (m, 2H, ArH), 8.0–8.1 (m, 1H, ArH) MS, *m/z*: 292 (99, M^+), 220 (base)
7b	C_2H_5		71	144–146 (E)	IR ν_{max} 1740, 1665 cm^{-1} ^{1}H-NMR ($CDCl_3$) δ: 1.2–1.7 (m, 6H, CH_3), 4.40 (q, 4H, CH_2), 7.3–7.5 (m, 3H, ArH), 8.7–8.8 (m, 1H, ArH), 8.86 (s, 1H, CH(2)) MS, *m/z*: 285 (59, M^+), 240 (base)
9b	C_2H_5		59	184–186 (E)	IR ν_{max} 1750, 1630 cm^{-1} ^{1}H-NMR ($CDCl_3$) δ: 1.43 (t, 3H, CH_3), 4.10 (s, 3H, OCH_3), 4.33 (q, 2H, CH_2), 6.80 (s, 1H, CH(3)), 7.2–7.9 (m, 4H, ArH) MS, *m/z*: 271 (31, M^+), 243 (base)

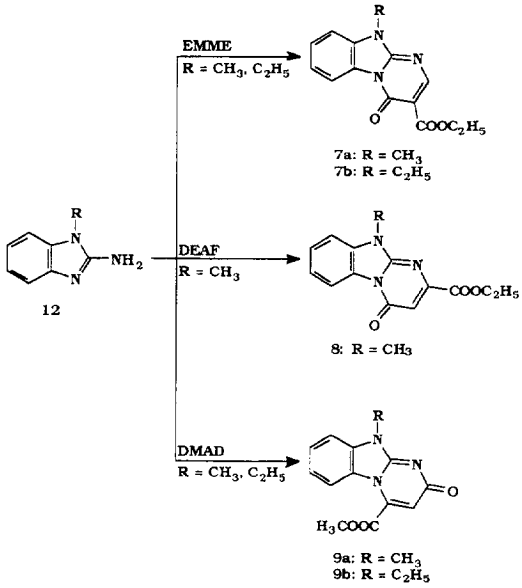
[a](A) Petroleum ether/AcOEt; (B) AcOEt; (C) EtOH; (D) MeOH/AcOEt; (E) MeOH. For data for **7a**, **8** and **9a** (R = CH_3) see reference [6].

compared to its benzothiazole analogue (compound **1** (R = C_2H_5, R' = H, IC_{50} = 120 nM)) [5], showed a ~ 40-fold decrease in binding affinity. It is interesting to note that the corresponding imidazobenzimidazole compound **13a** was found to inhibit the specific binding with an IC_{50} value of 440 nM. Furthermore, as previously observed for other substituted benzothiazole analogues (compounds **1**) [5], in the series **3a–d** the substitution at the fused benzene ring also leads to notable changes in BZR binding affinity. In fact, substitution with a methyl group and particularly with a chlorine at 6-position of the imidazobenzoxazole nucleus, led to a notable increase in binding affinity. However, by changing the alkyl group of the ester functionality of **3d** from ethyl to isopropyl, eg, **3e**, a decrease in affinity was observed (compare the affinity of **3d** with **3e**). Furthermore, substitution with a methyl group at 7-position of the imidazobenzoxazole nucleus again led to a decrease in binding potency.

As for the pyrimidobenzoxazole and pyrimidobenzimidazole series, once again very poor affinity was observed. These data, compared with those of benzothiazole analogues, clearly indicate that the nature of the heteroatom is an important structural determinant for BZR binding affinity. The differences in affinity between the sulfur- and nitrogen- or oxygen-containing heterocycles might be interpreted in terms of a possible combined negative lipophilic and electrostatic effect exerted by the nitrogen or oxygen heteroatoms.

An estimate of the efficacy of the compound having the highest affinity, eg, **3d**, was made by determining the GABA ratio (ie, IC_{50} without GABA/IC_{50} with GABA). Compound **3d** showed a GABA ratio value of 1.15 suggesting that this ligand may possess some partial agonist or antagonist properties. However, the usefulness of the GABA ratio as a predictor of antagonistic or inverse agonistic activity has been often been questioned [5]; in fact, only agonists have systematically displayed GABA ratios greater than unity.

Therefore, to further clarify the effect of compound **3d** at the level of the $GABA_A$ receptor complex, binding studies were carried out using [^{35}S]-*tert*-butylbicyclophosphorothionate ([^{35}S]TBPS) as radioligand in unwashed membranes from mouse cerebral cortex membranes. [^{35}S]TBPS is a specific ligand for a site located at the level of the chloride channel coupled to the $GABA_A$ receptor [7], and changes in its binding are correlated with parallel changes in the function of GABA-coupled chloride channel [8]. In particular, negative allosteric modulators of $GABA_A$ receptor function, such as the β-carboline derivative FG-7142 (*N*-methyl-β-carboline-3-carboxamide), bring about an increase in [^{35}S]TBPS binding. In contrast, positive allosteric modulators, such as benzodiazepines,



Scheme 3.

Table II. Effect of in vitro addition of the imidazobenzoxazoles **3a–d**, pyrimidobenzoxazoles **4–6** and pyrimidobenzimidazoles **7–9** on [^{3}H]flunitrazepam binding in rat cortical membranes.

Compound	*Inhibition % (40 μM)*	*IC_{50} (nM)*[a]
3a	89 ± 4	4560 ± 0.8
3b	96 ± 4	600 ± 0.02
3c	64 ± 1	
3d	98 ± 2	77 ± 2[b]
3e	94 ± 4	494 ± 13
4	50 ± 0.8	
5	91 ± 3	1080 ± 0.044
6a	49 ± 2	
6b	71 ± 1	
7a	50 ± 0.6	
7b	62 ± 0.8	
8	67 ± 1	
9a	16 ± 0.3	
9b	15 ± 0.6	

[a]Data from rat cortical membranes are means ± SD of two determinations. [b]GABA ratio = 1.15.

produce a decrease in [^{35}S]TBPS binding. Since **3d** increases [^{35}S]TBPS binding (see fig 1), **3d** should possess inverse agonist activity at BZR. Hence, a contrasting predictive capability of GABA ratio and [^{35}S]TBPS binding was observed as previously found [5] for some pyrimidobenzothiazoles. However, since it was also demonstrated [5] that for these compounds the in vivo effects are consistent with those predicted by [^{35}S]TBPS binding data, we tend to assign the meaning to the result of this assay rather than the GABA ratio value. As shown in figure 1, **3d** increases [^{35}S]TBPS binding in a concentration-dependent manner, an effect similar to that induced by the β-carboline derivatives DMCM (3-carbomethoxy-4-ethyl-6,7-dimethoxy-β-carboline) (full inverse agonist) and FG-7142 (partial inverse agonist), both negative modulators of the $GABA_A$ receptor function (fig 1). Hence, it is suggested that **3d** should possess partial inverse agonist activity at BZR because its [^{35}S]TBPS binding data are comparable to those of FG-7142.

Finally, with the aim of exploring whether compound **3d** interacts selectively at the central BZR, binding studies were carried out on this compound using RO 5-4864 as specific radioligand for peripheral BZR sites and membranes from renal cells as the mitochondrial BZR source. Since, in this experiment no binding affinity was observed (data not shown), it was deduced that **3d** acts specifically at a central level.

In conclusion, the results obtained in the present study indicate that imidazobenzoxazoles **3a–c**, pyrimidobenzoxazoles **4–6**, and pyrimidobenzoimidazoles **7–9** generally possess BZR binding affinities lower than those observed for the corresponding benzothiazole analogues **1** and **2** [5]. These data constitute further evidence of the important role played by the heteroatom considered. However, compound **3d** possesses high binding affinity (IC_{50} = 77 nM) and, based on its [^{35}S]TBPS binding data, should possess partial inverse agonist activity at BZR.

Experimental protocols

Chemistry

Melting points were determined in open capillary tubes with a Büchi apparatus and are uncorrected. IR spectra were obtained on a Perkin-Elmer 283 spectrophotometer (KBr pellets). ^{1}H-NMR spectra were determined on a Varian 390 or Bruker instruments operating at 90 and 300 MHz, respectively. Chemical shifts are given in δ values downfield from Me_4Si as internal standard. Mass spectra were recorded on a Hewlett-Packard 5995c GC-MS low resolution spectrometer. All compounds showed appropriate IR, ^{1}H-NMR and mass spectra. Elemental analyses were carried out with a Carlo Erba model 1106 analyzer and results were within ±0.40% of the theoretical values. Silica gel 60 (Merck 70-230 mesh) was used for column chromatography. Yields and physical data of new compounds are reported in table I unless otherwise stated.

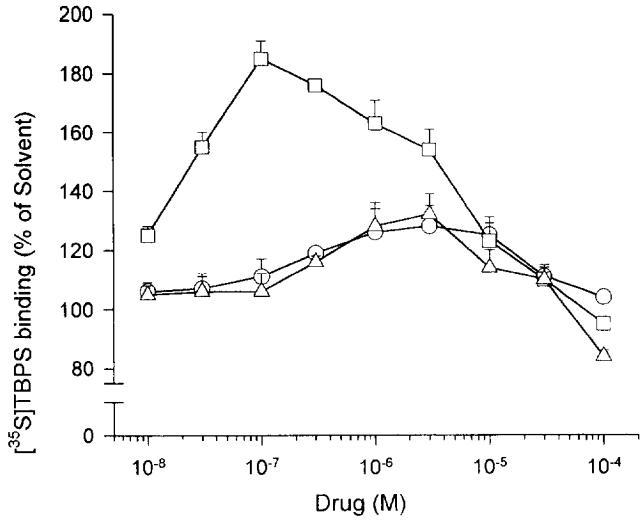


Fig 1. Effect of **3d** (○), DMCM (□) and FG 7142 (△) on [^{35}S]TBPS (2 nM) binding measured in fresh, unwashed membranes from mouse cerebral cortex. Data are expressed as percentage increase in binding from control values and are the means ± SEM from four separate experiments.

*General procedure for the preparation of ethyl imidazo[2,1-b]-benzoxazol-2-carboxylates **3a–d***

Ethyl bromopyruvate (20 mmol) was added dropwise to a stirred solution of the appropriate 2-aminobenzoxazole (15 mmol) in DMF (15 mL) and the mixture was refluxed for 6 h. Evaporation of the solvent under reduced pressure gave a residue which was purified by column chromatography (chloroform/ethanol 98:2 v/v as eluent).

*Isopropyl 6-chloro-imidazo[2,1-b]benzoxazol-2-carboxylate **3e***

A solution of **3d** (0.5 g, 1.9 mmol) in 1 N NaOH (2.4 mL) and EtOH (30 mL) was refluxed for 20 h. Evaporation of the solvent under reduced pressure gave a residue which was treated with HCl (pH 3). The resulting precipitate was the corresponding carboxylic acid (0.4 g). This last compound was dissolved in 2-propanol (30 mL) and boron trifluoride etherate (0.5 mL) was added. Such a mixture was refluxed for 7 h. The solvent was then removed and the residue was dissolved in $CHCl_3$ (30 mL). The organic phase washed with 10% $NaHCO_3$, dried (Na_2SO_4) and evaporated. The resulting residue was the pure compound **3e**.

*Diethyl[[2-(4-chlorobenzoxazolyl)amino]methylene]malonate **11***

A solution of **10** (R' = 5-Cl) (5 g, 29.8 mmol) and EMME (6.27 g, 29 mmol) in ethanol (50 mL) was stirred at room temperature overnight. Evaporation of the solvent under reduced pressure gave a residue which was crystallized from ethanol to give the pure compound **11** (1.0 g, 10% yield). Mp 124–125 °C. IR ν_{max} 1720, 1670, 1620 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 1.36 (t, 3H, CH_3), 1.40 (t, 3H, CH_3), 4.33 (m, 4H, CH_2), 7.1–7.5 (m, 3H, ArH), 8.66 (d, 1H, CH=), 11.33 (d, 1H, NH). MS *m/z*: 220 (base).

Ethyl 7-chloro-4-oxo-4H-pyrimido[2,1-b]benzoxazol-3-carboxylate **4** *(R' = 7-Cl)*
Compound **11** (1.0 g, 3 mmol) was heated under vacuum at 183 °C for 0.5 h. After cooling, crystallization of the crude mixture from ethanol gave the compound **4.**

Methyl 7-chloro-4-oxo-4H-pyrimido[2,1-b]benzoxazol-2-carboxylate **5** *(R' = 7-Cl)*
A solution of **10** (R' = 5-Cl) (1.5 g, 8 mmol) and 2-aminofumarate (3 g, 19 mmol) was heated at 130 °C for 1 h. After cooling at room temperature, evaporation of the solvent gave a residue which was purified by column chromatography (chloroform/methanol 7:3 v/v as eluent) to give the pure compound **5** (R' = Cl).

Methyl 7-chloro-2-oxo-2H-pyrimido[2,1-b]benzoxazol-2-carboxylate ***6a***
A solution of **10** (R' = 5-Cl) (2 g, 12 mmol) and dimethyl acetylenedicarboxylate (1.69 g, 12 mmol) in methanol (50 mL) was refluxed for 7 h. After cooling at room temperature, evaporation of the solvent gave a residue which was purified on column chromatography (chloroform as eluent) to give the pure compound **6a**.

In the same manner, compound **6b** was prepared by using diethyl acetylenedicarboxylate.

Ethyl 1H-imidazo[1,2-a]benzimidazol-2-carboxylate ***13a***
Ethyl bromopyruvate (2.3 g, 15 mmol) was added dropwise to a stirred solution of the 2-aminobenzimidazole **12** (R = H) (2.0 g, 15 mmol) in dry THF (20 mL). The mixture was stirred for 4 h, then EtOH (10 mL) was added and the resulting solution was refluxed for 3 h. Solvent was evaporated under reduced pressure and the residue was purified by column chromatography (chloroform/ethanol 95:5, v/v as eluent) to give the pure **13a** (0.08 g, 9% yield). Mp 206–208 °C, IR ν_{max} 1720, 1640 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 1.437 (t, 3H, CH_3), 4.39 (q, 2H, CH_2), 7.1–7.7 (m, 4H, ArH), 7.89 (s, 1H, CH(3)), 9.2 (br s, 1H, NH). MS, *m/z*: 229 (87, M^+), 157 (base).

Ethyl 10-ethyl-4-oxo-4H-pyrimido[1,2-a]benzimidazol-3-carboxylate ***7b***
This compound was prepared following the procedure reported in reference [6] starting from 1-ethyl-2-aminobenzimidazole **12** (R = C_2H_5).

Methyl 10-ethyl-2-oxo-2H-pyrimido[1,2-a]benzimidazol-3-carboxylate ***9b***
This compound was prepared following the procedure reported in reference [6] starting from 1-ethyl-2-aminobenzimidazole **12** (R = C_2H_5).

Biological methods. Radioligand binding assays

Ligand binding to rat brain
[^{3}H]Flunitrazepam (New England Nuclear, Boston, USA) had a specific activity of 84.3 Ci/mmol and a radiochemical purity > 99%. Male Wistar rats (180–200 g) (Charles River, Italy) were killed by decapitation and whole brains (excluding cerebellum and pons medulla) were quickly removed. The brains were homogenized in 20 volumes of ice-cold 0.32 M sucrose with a Potter. The homogenate was centrifuged for 5 min at 2000 x *g* at 4 °C and the supernatant was centrifuged for 10 min at 40 000 x *g* at 4 °C. The pellet was suspended in 30 mL of 50 mM cold Tris-HCl buffer, pH 7.4, and centrifuged for 30 min at 40 000 x *g* at 4 °C. This pellet was suspended in 8–10 mL of cold Tris-HCl buffer. BZR binding activity was determined as follows: 50 µL of membrane suspension was incubated in triplicate with 0.67 nM [^{3}H]flunitrazepam and with 40 µM of each tested compound for 90 min at 4 °C in 50 mM cold Tris-HCl buffer (500 µL final volume). After this incubation time, the samples were diluted with 5 mL of cold Tris-HCl buffer and immediately filtered under reduced pressure through glass-fiber filter disks (Wathman, GF/C), with a vacuum filtration manifold (Millipore, model 1225). The filters were washed with 5 mL of the same cold buffer and the retained radioactivity was counted in pico vials in 4 mL of Ready Protein Beckman liquid scintillation cocktail. Compounds **3–9** were dissolved in 50% ethanol/Tris-HCl buffer and added to the assay mixture. Non-specific binding was determined by incubating membranes and [^{3}H]flunitrazepam in the presence of 10 µM diazepam. Specific binding was obtained by subtracting non-specific binding from total binding and was approximately 90% of the total binding. Six to eight concentrations of the compounds in triplicate were added to samples to determine IC_{50} values in the absence and in the presence of 10 µM GABA in parallel experiments. Values shown for the GABA ratio are means of the ratio of IC_{50} without GABA to IC_{50} with 10 µM GABA in three independent experiments.

[N-Methyl-^{3}H]-Ro 5-4864 binding
[*N*-Methyl-^{3}H]-Ro 5-4864 (New England Nuclear, Boston, USA) had a specific activity of 84.7 Ci/mmol and a radiochemical purity > 99%. Male Wistar rats (180–200 g) (Charles River, Italy) were killed by decapitation. The kidneys were removed and homogenized with a Potter in 20 volumes of ice-cold 0.32 M sucrose. The homogenate was centrifuged at 2000 x *g* for 5 min and the supernatant was centrifuged at 40 000 x *g* for 10 min at 4 °C. The membranes were suspended and lysed in 30 mL of 50 mM cold Tris-HCl buffer (pH 7.4) and centrifuged at 4000 x *g* for 30 min at 4 °C [9]. The resulting pellet was suspended in 7–8 mL of 50 mM Tris-HCl cold buffer. Studies of [*N*-methyl-^{3}H]-Ro 5-4864 binding activity of kidney mitochondrial preparation were performed as follows: 50 µL of mitochondrial suspension (200–250 mg protein) were incubated in triplicate with 0.9 nM [*N*-methyl-^{3}H]-Ro 5-4864 and with 40 µM of each tested compound for 90 min at 4 °C in a total volume of 500 µL of 50 mM cold Tris-HCl buffer. After incubation, samples were diluted with 5 mL of cold Tris-HCl buffer and immediately filtered under reduced pressure through glass-fiber filter disks (Wathman, GF/C) with a vacuum filtration manifold (Millipore, model 1225). The filters were rinsed with 5 mL of the same Tris buffer, and the retained radioactivity was determined in pico vials in 4 mL of Ready Protein Beckman liquid scintillation cocktail. The imidazobenzoxazole derivative **3d** was solubilized in 50% ethanol/Tris-HCl buffer and added to the assay mixture. Blank sample was carried out in the same conditions to determine the effect of ethanol on the total binding. Non-specific binding was defined as binding of [*N*-methyl-^{3}H]-Ro 5-4864 in the presence of 10 µM of diazepam. Specific binding was obtained by subtracting non-specific binding from the total binding and was approximately 90% of the total binding. Six to eight concentrations of the drugs in triplicate were used to determine IC_{50} values with an iterative curve-fitting program.

Ligand binding to mouse cortical membranes
Male CD-1 mice (Charles River, Como, Italy) with body weights 25–30 g were kept under a 12 h light/dark cycle at a temperature of 23 ± 2 °C and 65% humidity. Upon arrival at the animal facilities there was a minimum of 7 days of acclimatization during which the animals had free access to food and

water. The animals were sacrificed by cervical dislocation in the middle of the light phase. The brains were rapidly removed, the cerebral cortex was dissected out and was used for the measurement of [^{35}S]TBPS binding.

[^{35}S]TBPS binding

Cerebral cortices were homogenized with a polytron PT 10 (setting 5, for 20 s) in 50 volumes of ice-cold 50 mM Tris-citrate buffer (pH 7.4 at 25 °C) containing 100 mM NaCl. The homogenate was centrifuged at 20 000 x *g* for 20 min and reconstituted in 50 volumes of 50 mM Tris-citrate buffer without NaCl. [^{35}S]TBPS binding was determined in a final volume of 500 µL, consisting of 200 µL tissue homogenate (200–300 µg of protein), 50 µL 2 nM [^{35}S]TBPS, 50 µL 0.2 M NaCl, 50 µL drugs (dissolved in dimethylsulfoxide and serial diluitions made up in buffer) or solvent (total and non-specific samples). The incubation (25 °C) were started by the addition of tissue homogenate and were terminated 90 min later by rapid filtration through glass-fiber filter strips (Whatman GF/B) with a filtration manifold (Model M-24, Brandel). The filters were rinsed with two 4 mL portions of ice-cold 50 mM Tris-citrate buffer. Filter-bound radioactivity was quantitated by liquid scintillation spectrometry. Non-specific binding was defined as binding in the presence of 100 mM picrotoxin, and represented about 10% of total binding.

Protein concentration was assayed by the method of Lowry [10] with bovine serum as standard. Biochemical data were analyzed using Student's *t* test and IC_{50} values were determined from displacement curves with the Ligand program [11].

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