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2-Aryl-2,5-Dihydropyridazino[4,3-*b*]Indol-3(3*H*)-ones: Novel Rigid Planar Benzodiazepine Receptor Ligands

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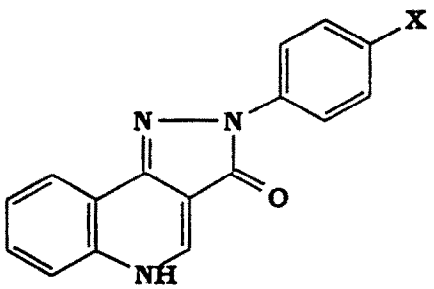
Abstract—A series of 2-aryl-2,5-dihydropyridazino[4,3-*b*]indol-3(3*H*)-ones **5** were prepared and evaluated for their ability to inhibit radioligand binding to BZR, and to prevent sound and pentylenetetrazole (PTZ) induced seizures in mice. The biological and pharmacological results are discussed in the light of some recently proposed pharmacophore models and compared through molecular orbital and molecular modeling studies to those obtained from the close pyrazoloquinoline analogs **6**.

Introduction

Benzodiazepine receptor (BZR) ligands modulate the action of GABA at $GABA_A$–receptor/Cl^- ionophore supramolecular complex,[1,2] eliciting a wide variety of pharmacological effects.[3] Full agonists ("GABA-positive" ligands) have sedative/hypnotic, muscle relaxant, anticonvulsant and anxiolitic activities, while inverse agonists ("GABA-negative" ligands) display (pro)convulsant and anxiogenic activities; antagonists, on the other hand, do not exhibit, *per se*, any relevant biological effects but could be used to selectively prevent or reverse pharmacological effects mediated by benzodiazepines.[3]

During the past several years many efforts have been focused on discovering the essential common structural features of the diverse classes of BZR ligands, responsible for binding to and different modulations of BZR.[4–15] Unfortunately, the numerous pharmacophore models proposed so far, coming from different initial assumptions (for instance same/diverse binding site(s) for the three major pharmacological classes of BZR ligands) and methodological approaches, are neither convergent nor conclusive. Therefore, additional experimental and theoretical investigations still seem necessary to gain more insight on the molecular determinants responsible for high *in vitro* activity and for more specific and selective pharmacological profiles. Within this frame and moving towards those goals, we have just begun a systematic structural modification of high affinity receptor ligands, choosing pyrazoloquinolines **6**[16] as reference structures (Figure 1). The interest in pyrazoloquinolines **6** resides not only in their outstanding BZR affinity, which remains almost the highest known so far, but especially in their peculiar, dramatically different activities caused by a simple, single change of the *para* substituent on the 2-phenyl ring. In fact, while **6a** (X = H, CGS 8216) is a partial inverse agonist,[17,18] **6b** (X = OCH_3, CGS 9895) and **6c** (X = Cl, CGS 9896) have been described as partial agonists with different degrees of intrinsic activity.[19–23]



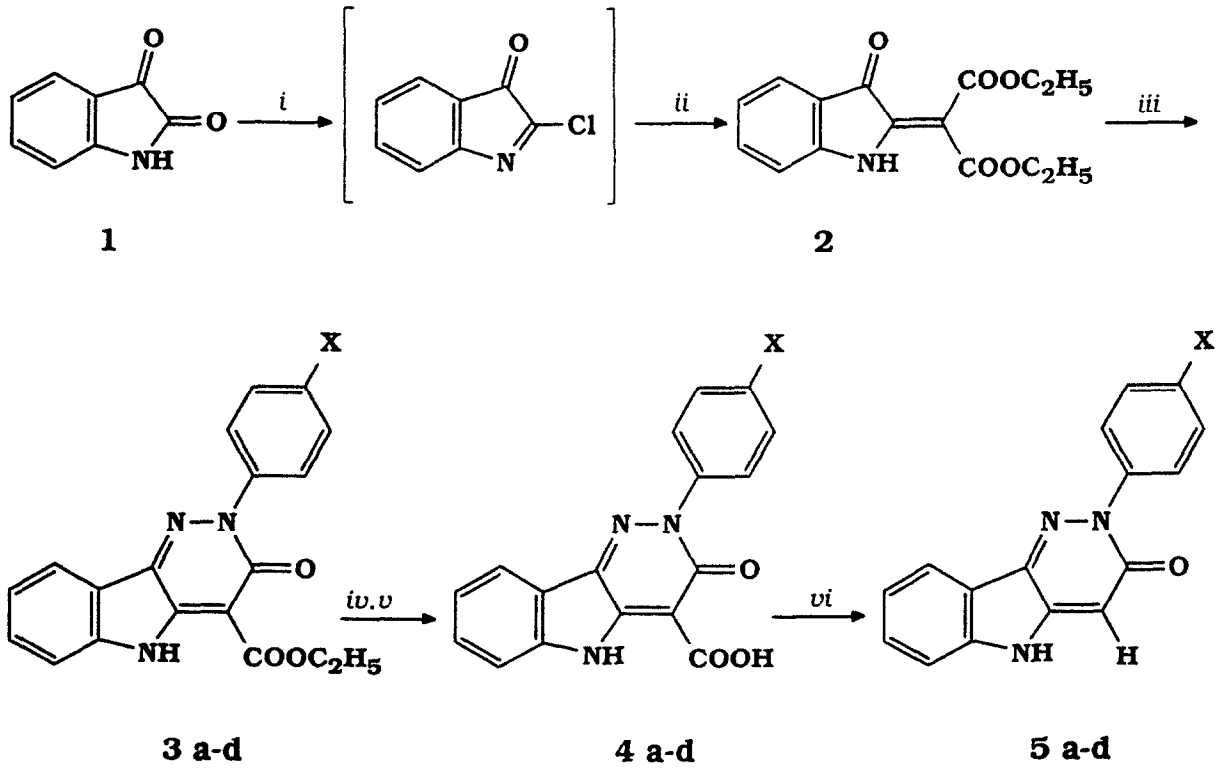
6 a-c

Figure 1. X: **a** = H: **b** = OCH_3: **c** = Cl

In the present paper we report on the synthesis, BZR binding and preliminary pharmacological characterization of pyridazinoindolones **5a–d**.

Results and Discussion

Scheme I outlines the method used to prepare 2-X-phenyl-2,5-dihydropyridazino[4,3-*b*]indol-3(3*H*)-ones **5a–d**. The key intermediate 2-biscarbethoxymethylene-indolin-3-one **2** was obtained in 25% overall yield according to the synthetic pathway and reaction conditions reported in Scheme I.[24] The condensation of **2** with X-phenylhydrazine hydrochlorides furnished the pyridazinoindolone esters **3a–d**,[25] which were then hydrolyzed to the corresponding carboxylic acids **4a–d** in 85–90% yield. Title compounds **5a–d** were obtained by thermal decarboxylation of **4a–d** in a sealed glass tube in 70–85% yield. Physical and spectroscopic properties of compounds **2–5** are listed in Table 1.



Scheme I. Reagents: (i) Triphosgene NEt_3, anhydrous dioxane, reflux; (ii) $(COOC_2H_5)_2CH^-Na^+$, r.t.; (iii) X–PhNHNH$_2$·HCl, EtOH/H_2O, reflux; (iv) NaOH, EtOH, reflux; (v) H^+, r.t.; (vi) Δ, 350 °C.

The binding affinities of intermediate esters **3a–d** were measured up to a final 20 μM concentration and no significant radioligand displacement was detected; the corresponding acids **4a–d** were not tested because of their poor solubility in the assay medium.

In Table 2 the binding affinities and the GABA ratios of compounds **5** are reported together with similar data determined on the reference pyrazoloquinoline congeners **6**. The latter were measured aiming at a more straightforward and reliable comparison with our newly synthesized compounds, since the "classical" experimental conditions used for the displacement analysis of BZR radioligand, had to be considerably changed. As can be inferred from the analysis of data in Table 2, our method led to IC_{50} values, 4–10 times higher than those reported in the literature[7,26] and, in the case of the agonists, also led to lower GABA ratios. The analysis and comparison of experimentally homogeneous affinity data in Table 2, point out several interesting and somewhat unexpected differences between similarly substituted congeners of the two series and, even more unexpectedly, within the pyridazinoindolone congener **5** series. The binding affinities of compounds **5b,c** are about one order of magnitude lower than those observed for the corresponding pyrazoloquinolines **6b,c** and an even more striking difference of about two orders of magnitude resulted for the unsubstituted analogues **5a** and **6a**. Considering the structural analogy of compounds **5** and **6**, the above results are quite surprising and called for further structural investigations aimed at the identification of the molecular features responsible for the different affinities observed. Molecular orbital (AM_1) and modeling studies were therefore carried out on the parent compounds **5a** and **6a**; some selected MO theoretical descriptors and geometrical characteristics derived from the two optimized molecules are listed in Tables 3 and 4, respectively. Despite their apparent structural similarity, the data collected reveal some significant differences, both at molecular and atomic levels. Data in Table 3 indicate that the dipole moments differ in intensity and direction, and in particular the ability of N_1 to make a hydrogen bond, as indicated by its charge, electrophilic superdelocalizability and heat of protonation,[27] is much greater for **6a** than **5a**; these features could play a prominent role in the correct orientation of the ligand in the binding step. Also data from Table 4 show different distances and relative positions of the supposed anchoring points to the BZR. According to the most accepted hypothesis on the BZR pharmacophore model(s),[11,13] at least three attachment sites of the ligand seem necessary to elicit BZR affinity: two HB accepting sites, usually two nitrogen atoms or a nitrogen and a carbonyl oxygen, and a more or less planar lipophilic moiety. The additional presence of one NH group in the benzofused moiety is not a stringent requisite for BZR

Table 1. Physical and spectroscopic data of compounds **2–5**

Compound	Mp, °C (Crystallization solvent)	IR ν_{max}, cm^{-1}	^{1}H-NMR,[a] δ[ppm, J(Hz)]
2	117-118[b] (cyclohexane)	3380, 1740, 1720,1685, 1610	1.22(t,3H,CH_3,J=7.1), 1.24(t,3H,CH_3, J=7.1), 4.23(q,4H,2CH_2, J= 7.1), 6.90-7.05(m,1H, Arom.), 7.20-7.30(m,1H,Arom.),7.50-7.65(m,2H,Arom.), 10.46(s,1H,NH).
3 a	217-219 (ligroin)	3395, 1680, 1670, 1655, 1615	1.44(t,3H,CH_3, J=7.2), 4.46(q,2H,CH_2, J=7.2), 7.25-7.65(m,8H, Arom.), 7.95-8.0(m,1H, Arom.), 9.84(s,1H,NH)
3 b	220-222 (ligroin)	3370, 1680, 1670, 1655, 1615	1.43(t,3H,C-CH_3,J=7.1), 3.86(s,3H,O-CH_3), 4.46(q,2H,CH_2, J= 7.1), 6.95-7.05(m,2H, Arom.), 7.25-7.30(m,2H,Arom.), 7.44-7.54(m, 3H,Arom.), 7.95-8.05(m,1H,Arom.), 9.86(s, 1H,NH).
3 c	257-258 (ligroin)	3390, 1680, 1670, 1655, 1615	1.44(t,3H,CH_3, J=7.1), 4.47(q,2H,CH_2, J=7.1), 7.20-7.60(m,7H,Arom.) 7.95-8.0(m,1H, Arom.), 9.85(s,1H,NH).
3 d	264-265 (ligroin)	3380, 1680 1670, 1655, 1615	1.45(t,3H, CH_3,J=7.1), 4.47(q,2H,CH_2,J=7.1), 7.20-7.35(m,2H,Arom.), 7.45-7.70(m,5H, Arom.), 7.95-8.05(m,1H,Arom.), 9.86(s,1H, NH).
4 a	325-327dec. (AcOH)	3320, 1720, 1645, 1620	7.30-7.40(m,1H,Arom.), 7.55-7.75(m,7H, Arom.), 8.07(d,1H,Arom., J=7.5), 12.0-12.50(br,1H,NH), 14.0-14.50(br,1H,COOH).
4 b	326-328dec. (AcOH)	3300, 1730 1650, 1620	3.77(s,3H,CH_3), 7.05-7.15(m,2H,Arom.), 7.25-7.70(m,5H,Arom.), 8.02(d,1H,Arom., J=7.5), 12.13(s,1H,NH), 14.50-14.70(br,1H,COOH).
4 c	334-335dec. (AcOH)	3320, 1720, 1650,1620	7.25-7.35(m,1H,Arom.), 7.55-7.75(m,6H, Arom.) 8.00(d,1H,Arom., J=7.6), 11.90-12.50(br,1H,NH), 13.80-14.30(br,1H,COOH).
4 d	341-342dec. (AcOH)	3315, 1710, 1650, 1615	6.95-7.15(m,1H,Arom.), 7.30-7.60(m,6H, Arom.), 7.75(d,1H,Arom.,J=7.7), 11.90-12.10 (br,1H,NH), 13.70-14.30(br,1H,COOH).
5 a	348-350dec. (dioxane)	3180, 1650, 1610, 1550	6.49(s,1H,CH-C=O), 7.10-7.65(m,8H,Arom.), 7.85-7.95(m,1H,Arom.), 11.22(s,1H,NH)
5 b	301-302 (dioxane)	3100, 1655, 1610, 1560	3.81(s,3H,CH_3), 6.46(s,1H,CH-C=O), 6.95-7.55 (m,7H,Arom.), 7.85-7.95(m,1H,Arom.), 11.19 (s,1H,NH).
5 c	345-347dec. (dioxane)	3165, 1650, 1615, 1555	6.49(s,1H,CH-C=O), 7.10-7.70(m,7H,Arom.), 7.85-7.95(m,1H,Arom.), 11.24(s,1H,NH).
5 d	338-340dec. (dioxane)	3190, 1675, 1610, 1550	6.55(s,1H,CH-C=O), 7.20-7.80(m,7H,Arom.), 7.85-8.0(m,1H,Arom.), 11.31(s,1H,NH).

[a]The ^{1}H-NMR spectra were recorded in $CDCl_3$ (**3**) and in DMSO-d_6 (**2, 4, 5**); abbreviations used: *s*, singlet; d, doublet; t, triplet; q, quartet; m, multiplet(*s*); br, broad signal.

[b]Reported melting points: 111–112 °C from EtOH[24a] and 121.5–122.5 °C from benzene–ligroin.[24b]

Table 2. Inhibition of [^{3}H]flunitrazepam binding and GABA ratio

Compound	X	IC_{50}, nM (±SD)	GABA/ratio[a]
5 a	H	238(±18)	0.94
5 b	OCH_3	23.9(±1)	1.02
5 c	Cl	31.1(±1)	1.12
5 d	Br	39.4(±2)	1.13
6 a	H	1.85(±0.4)	n.d (0.94)
6 b	OCH_3	2.31(±0.7)	n.d. (0.86)
6 c	Cl	2.07(±0.5)	1.06(1.32)
DIAZEPAM		14.2(±0.6)	1.27 (1.82)

[a]Literature GABA ratios were reported in parentheses.[7] Note that the different experimental conditions used by Villar,[7] led always, as stated by the same author, to higher GABA ratio values for agonists; n.d., not determined.

Table 3. Selected MO theoretical descriptors of BZR ligands **5a** and **6a**

Comps.	E_{HOMO}	E_{LUMO}	μ	qN_1 ($S^{HOMO}_{N_1}$)	qN_5 ($S^{HOMO}_{N_5}$)	qO (S^{HOMO}_{O})	ΔH_{N_1}	ΔH_{N_5}	ΔH_O
5a	-185.64	-17.26	3.776	-0.0421 (8.43)	-0.2569 (8.93)	-0.3375 (7.54)	164.34	173.63	145.81
6a	-197.69	-18.60	5.946	-0.0646 (14.44)	-0.2892 (9.61)	-0.3287 (7.79)	146.71	177.73	148.21

E_{HOMO} and E_{LUMO} are the energies (Kcal/mole) of the highest occupied and the lowest unoccupied molecular orbital, respectively; μ is the molecular dipole moment (Debye); q is the net charge of the indicated atom for which, in parentheses, the electrophilic superdelocalizability (S^{HOMO}) is also reported. ΔH is the heat of protonation at the indicated nucleophilic sites.[27]

Table 4. Selected geometrical features of optimized BZR ligands **5a** and **6a**

Comps.	AN_1	AO	AD	N_1O	N_1N_5	N_5O	AN_1O	N_5N_1O
5a	3.909	6.748	7.510	3.553	3.605	4.880	129.4	85.91
6a	3.877	6.177	7.605	3.523	4.135	4.530	113.1	72.07

Distances and angles between the indicated atoms and/or centroids A and D (see Figs 2,3) are expressed in Ångstroms and degrees respectively.

recognition, even though its involvement in the formation of a strong HB with a HB accepting site on the receptor has been largely proved. The distances and relative position in a 3D space of the different pharmacophore sites might determine the rank of affinity and most likely the pharmacological profile.[7] The subtle differences between the 3D structures of **5a** and **6a** may be better perceived by a simple visual inspection of the molecular superpositions realized via the fitting of three (Figure 2) or four (Figure 3) supposed anchoring points: N_1 and N_5, O_3, and centroids A and D, referring to the benzene of the benzofused moiety and to the 2-phenyl ring respectively. In any case, however

many putative interactions conducive to the final ligand–receptor complex there are, they will comprise hydrophobic interactions and HB formation for which strict geometrical and stereochemical requirements have to be satisfied.[28] The results we have indicate that, despite a relatively high structural homology, pyrazoloquinolines **6** fulfil the receptor molecular requirements much better than pyridazinoindolones **5**. However, considering the large variety of structurally diverse molecules that BZR can efficiently accomodate, it is hard, in the case of **5a**, to interpret the potency decrease of two orders of magnitude, even taking into account the important differences in the geometrical data, which do in fact lie within the range of angles and distances characterizing high affinity BZR ligands. One tentative explanation could be found in the different position and orientation of the 2-phenyl ring in **5a**, determined by a slightly different, strong HB anchoring, resulting in a poorer hydrophobic contact for the phenyl moiety. In other words the phenyl ring in **5a** could contact a close but slightly different domain in the receptor with respect to **6a** as can be seen in the molecular overlay reported in Figure 2. A more efficient contact could be substantially recovered in the binding of congeners **5b,c,d** through a small conformational change, possibly induced by the substituent itself in the *para* position (see Figure 3), leading ultimately to a more favorable and extended hydrophobic interaction with the receptor region referred to by Cook[13] as L_2.

For a preliminary gross classification of the novel BZR ligands **5a–d** as agonists, antagonists or inverse agonists, the GABA ratio, that is the ratio between the BZR affinity measured without and with GABA, are reported in Table 2. Previous studies have shown that GABA modulates BZR ligand binding, with affinities increasing for agonists, unaffected for antagonists, and decreasing for inverse agonists.[29] More recently[7] the validity of the GABA ratio for a correct BZR ligand classification has been questioned; only for agonists in fact has a GABA ratio systematically greater than one been found. The GABA ratios collected in Table 2, were therefore analyzed bearing in mind these findings. Compounds **5c**, **5d** and **6c** could be considered agonists whereas **5a** could behave as an inverse agonist. The GABA ratio of **5b** is too close to unity to permit any safe classification. To provide a more secure basis for the biological characterization of BZR ligands **5**, a preliminary pharmacological study of their *in vivo* activity towards sound and pentylenetetrazole (PTZ) induced seizures in mice, was undertaken. The audiogenic seizure test on DBA/2 mice has been found to be very sensitive and workable in order to distinguish between compounds acting as agonists or antagonists on BZR.[30–33]

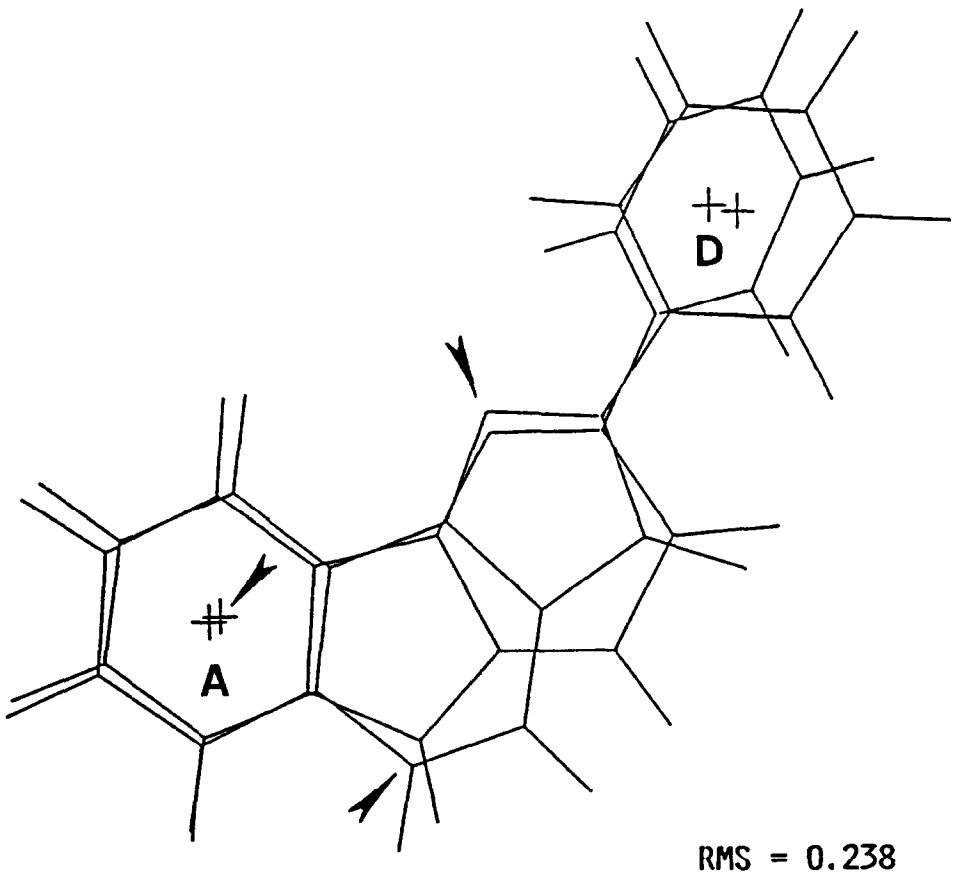


Figure 2. Superposition of **5a** and **6a** resulting from three points fitting. The reported RMS value refers to root mean squares deviation upon fitting of the points indicated by an arrow

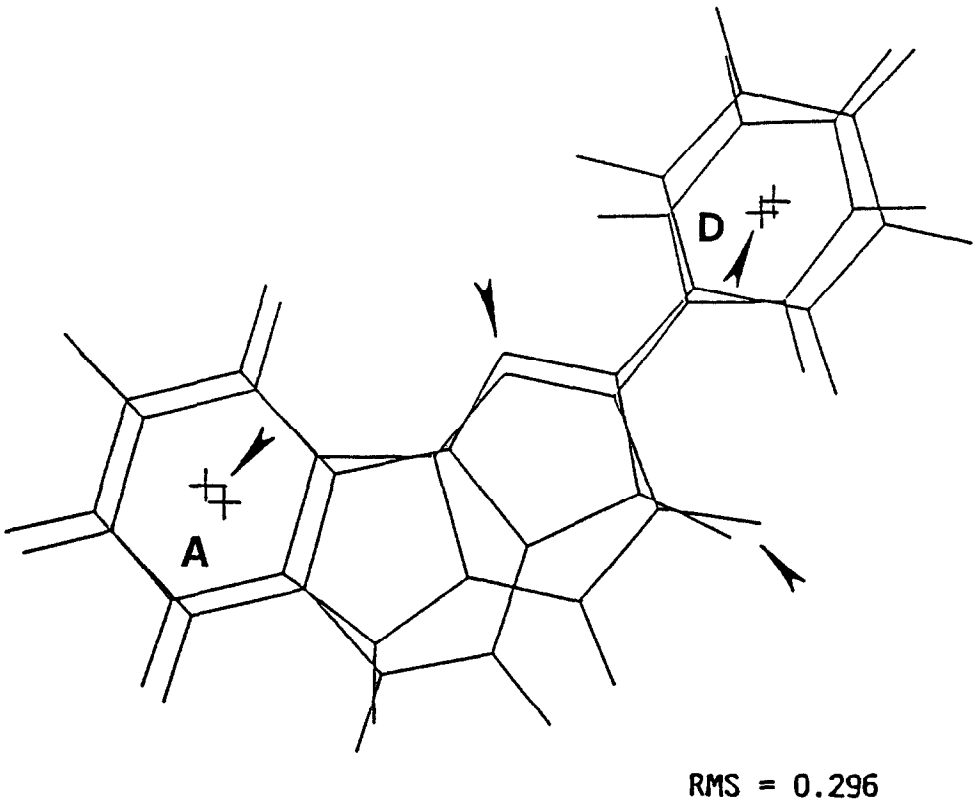


Figure 3. Superposition of **5a** and **6a** resulting from four points fitting. The reported RMS value refers to root mean squares deviation upon fitting of the points indicated by an arrow

Table 5. Anticonvulsant effects of BZR ligands **5a–d** and **6a–c**

Compounds	Seizure phase	ED_{50} mg/Kg Audiogenic seizures (DBA/2 mice)	ED_{50} mg/Kg Penthylentetrazole induced seizures (Swiss mice)
Diazepam	CLO	0.08(0.06-0.1)	0.33 (0.27-0.40)
	TON	0.07(0.04-0.1)	0.21 (0.17-0.26)
5a	CLO	n.a.	n.a.
	TON	n.a.	n.a.
5a + Diazepam	CLO	0.21(0.1-0.3)	n.d.
	TON	0.13(0.1-0.2)	n.d.
5b	CLO	0.91(0.5-1.5)	12.70(5.3-30.3)
	TON	0.66(0.5-0.9)	8.49(3.1-22.9)
5c	CLO	1.07(0.5-2.2)	20.31(10.3-39.9)
	TON	0.53(0.2-1.2)	15.89(6.2-40.6)
5d	CLO	0.23(0.2-0.3)	5.21(2.4-11.4)
	TON	0.09(0.1-0.2)	2.90(0.9-8.7)
Flumazenil + **5d**	CLO	0.48(0.2-0.9)	n.d
	TON	0.25(0.1-0.4)	n.d.
6a	CLO	2.71(1.2-6.2)	n.a.
	TON	1.58(0.6-3.5)	n.a.
6b	CLO	n.a.	n.a.
	TON	n.a.	n.a.
6c	CLO	1.13(0.6-2.0)	57.85(13.0-258.2)
	TON	0.83(0.6-1.1)	13.99(4.8-40.6)

CLO = Clonus; TON = Tonus; n.a., not active; n.d., not detected.
ED_{50} values with 95% confidence limits for each compound and each phase of seizure response were estimated using the Bliss method probit procedure.[44]

The anticonvulsant effects of BZR ligands **5a–d** and **6a–c** are reported in Table 5. The present results show that the new compounds **5c,d** as well as the reference compound **6c** possess good anticonvulsant properties against sound induced seizures and, in part, against PTZ induced seizures, suggesting a possible role as BZR agonists. Since the anticonvulsant effects of **5d** were reversed by flumazenil it seems likely that the actions of compounds **5** are principally mediated by BZR sites. Moreover, compound **5d** displays an anticonvulsant activity also against PTZ induced seizures in mice, strongly confirming our previous hypothesis.

No anticonvulsant activity against sound and PTZ induced seizures was instead detected for **6b**. Since this compound antagonizes the effects of diazepam it was considered a BZR antagonist. Compound **5b** seems to possess a different profile but the present study does not allow for any clear conclusion. Compound **6a** appears to be a potent antagonist with the same proconvulsant activity at high concentrations, whereas the analogue **5a** shows mild anticonvulsant activity at high doses (10 mg/kg) against PTZ induced seizures but not against sound induced seizures. In addition **5a**, at the dose of 3.3 mg/kg, was able to attenuate the anticonvulsant activity of diazepam in DBA/2 mice. These findings might suggest that **5a** is a BZR antagonist or a weak inverse agonist. However **6a** and **5a** seem to have low efficacy as inverse agonists.

The interesting anticonvulsant activity of the *para* bromo substituted compound **5d**, greater than that of the *para* chloro substituted congeners **5c** and **6c**, deserves further comment. The bromo substituent is more polarizable and lipophilic than the chloro substituent and this might determine a more favorable binding to the receptor hydrophobic region indicated as L_2 by Cook[13] and/or allow for an easier diffusion across the blood brain barrier (BBB). A more or less efficient crossing of the BBB, which may result also from a different transport mechanism, has often been invoked in the past to explain CNS *in vivo* activity that is higher than the corresponding *in vitro* data would lead us to expect.[34] This hypothesis may, at least in part, account for the higher anticonvulsant activity observed for benzodiazepines compared to other classes of BZR ligands having similar receptor affinities. The same hypothesis may hold also for the results of the present study which indicate that diazepam, our reference benzodiazepine compound, is more potent than compound **5d**, despite their comparable IC_{50} (see Tables 2 and 5). However the anticonvulsant activities of compounds **5c,d** were evident at dose levels which did not affect sedation, ataxia and in most cases body temperature (data not shown). This lower incidence of neurological side effects after administration of anticonvulsant doses of compounds **5** compared to diazepam, is indicative of a partial agonist behavior, in agreement with some previous studies.[33,35]

In conclusion the comparative analysis of the binding activities, GABA ratios and pharmacological activities of pyrazoloquinolines **6a–c** and pyridazinoindolones **5a–d** shows some distinct behavioral differences between these two classes of BZR ligands. The interesting pharmacological activity of **5a–d** calls for further studies; in particular the agonist **5d**, which possesses the best anticonvulsant activity in sound induced seizures, will be further investigated as a promising candidate for the treatment of epileptic disorders.

Experimental

Chemistry

Melting points were determined by the capillary method on a Gallekamp MFB 595 010M apparatus and are uncorrected. Elemental analyses were made on a Carlo Erba 1106 analyzer; for the C, H, N the results agreed to within ± 0.40% of the theoretical values. IR spectra were recorded using potassium bromide disks on a Perkin-Elmer 283 spectrophotometer, only the most significant and diagnostic absorption bands being reported. ^{1}H NMR spectra were recorded on a Varian XL-200, chemical shifts were expressed in δ (ppm) and the coupling constants *J* in Hz. Exchange with deuterium oxide was used to identify -OH and -NH protons. Chromatographic separation was carried out on silica gel columns (230–400 mesh, Merck) by using the "flash" technique.

2-Biscarbethoxymethylene-indolin-3-one, 2. A solution of triethylamine (5.06 g, 50 mmol) in anhydrous dioxane (50 mL) was added dropwise to a stirred suspension of isatin (7.36 g, 50 mmol) and triphosgene (4.95 g, 16.67 mmol) in anhydrous dioxane (100 mL) at room temperature. The reaction mixture was refluxed for 4 h and the triethylamine hydrochloride was removed by filtration under dry nitrogen. A solution previously prepared of diethyl malonate (8.00 g, 50 mmol) and sodium (1.15 g, 50 mmol) in anhydrous dioxane (100 mL), was then added dropwise to the filtrate and the reaction mixture was stirred at room temperature for 15 h. The sodium chloride was filtered off, the dioxane solution was evaporated *in vacuo* and the residue flash chromatographed on silica gel (96:4 chloroform–ethyl acetate eluent, R_f = 0.57) to afford the title compound **2** (3.61 g, 25% yield). Mass spectrum, m/e 289.15 ($C_{15}H_{15}NO_5$ requires 289.29).

Reaction of 2 with X-phenylhydrazine hydrochlorides. A solution of X-phenylhydrazine hydrochloride (2.4 mmol) in ethanol/water 1:1 (12 mL) was added to a stirred solution of **2** (0.578 g, 2 mmol) in ethanol (40 mL), and then refluxed for 8 h. In the case of phenylhydrazine and 4-methoxyphenylhydrazine hydrochlorides, upon cooling the reaction mixture yielded a yellow precipitate which was collected and crystallized from acetic acid to give the carboxylic acids **4a** (0.244 g, 40% yield) and **4b** (0.134 g, 20% yield) respectively. The filtrate cooled in refrigerator for 48 h furnished the esters **3a** and **3b** as yellow precipitates which were collected and crystallized from ligroin (0.267 g, 40% yield, and 0.218 g, 30% yield respectively).

In the case of 4-chloro- and 4-bromophenylhydrazine hydrochlorides, upon cooling the reaction mixture gave a yellow precipitate which was collected and crystallized

from dioxane to obtain the carboxylic acids **4c** (0.102 g, 15% yield) and **4d** (0.192 g, 25% yield) respectively. The dioxane was then evaporated *in vacuo* and the residue flash chromatographed on silica gel (5:5 ethyl acetate/petroleum ether) to afford the esters **3c** (0.382 g, 52% yield, R_f = 0.35) and **3d** (0.412 g, 50% yield, R_f = 0.45) respectively.

*Hydrolysis of esters **3a–d** to carboxylic acids **4a–d**.* A solution of NaOH (0.200 g, 5 mmol) in ethanol (15 mL) was added to a stirred suspension of **3** (0.5 mmol) in ethanol (7.5 mL) and then refluxed for 1 h. The reaction mixture was cooled to room temperature and the orange precipitate was collected, washed with ethanol and dried. An aqueous (60 mL) suspension of the precipitate was acidifed with conc. HCl to pH 1 and stirred at room temperature for 2 h. The yellow solid was filtered, washed with water and dried. Crystallization from acetic acid gave pure carboxylic acid **4** in 85–90% yield.

*Thermal decarboxylation of **4a–d**.* A sealed glass tube containing the acid **4** (0.3 mmol) was heated at 350 °C for 5 min and then cooled to room temperature. The solid was crystallized from dioxane to give **5a** (0.061 g, 78% yield), **5b** (0.061 g, 70% yield), **5c** (0.076 g, 85% yield) and **5d** (0.080 g, 78% yield).

Radioligand binding assay

The central-type BZR affinity was determined on mitochondrial homogenate from the brains of male Sprague–Dawley rats as previously reported.[36,37] In brief: 50 µL of mitochondrial suspension (180–200 µg of protein) was incubated in quadruplicate with 0.67 nM [^{3}H]flunitrazepam (74.6 Ci/mmol, New England Nuclear, MA, U.S.A.) and ethanolic solution of inhibitor for 90 min at 4 °C in 50 mM ice-cold Tris–HCl buffer (500 µL final volume). The reaction was then terminated by the rapid addition of 5 mL of ice-cold Tris–HCl buffer (pH = 7.50), followed immediately by vacuum filtration through Wathman GF/C glass-fiber filters. The filters were washed with 5 mL of the same cold buffer and counted in pico vials in 4 mL of Ready Protein Beckman liquid scintillation cocktail. Due to the very low solubility of some tested compounds in the assay medium, a final 10% ethanol concentration had to be reached. Blank samples were analyzed to determine the effect of ethanol on the assay. Six to eight concentrations of the drugs in quadruplicate were used to generate IC_{50} values with an iterative curve fitting program (Enzfitter 1.05, Biosoft). Parallel experiments were carried out in the presence of 20 µM GABA to determine the GABA ratio which is the ratio between receptor affinity determined in the absence and presence of GABA. Non specific binding was defined as binding of [^{3}H]flunitrazepam in the presence of 10 µM diazepam. Specific binding was obtained by subtracting the non specific binding from the total binding and was approximately 90–95% of the total binding. Protein content was determined according to the method of Lowry[38] using bovine serum albumin as standard. Binding data of ligands **5,6** and diazepam are collected in Table 2.

Theoretical and molecular modeling studies

The starting geometries of the heterocyclic compounds **5a** and **6a** were taken from the fragment library of SYBYL 5.42 molecular modeling software (TRIPOS, St Louis, MO, U.S.A.), running on a Vax Station 3100 connected to a PS 390 Evans-Sutherland graphic system. A full geometry optimization, was carried out using the AM_1 Hamiltonian from the suite of programs AMPAC (QCPE 506). The geometry of compound **6a** was quite close to that resulting from its X-ray crystallographic coordinates.[39] Suitably selected molecular orbital indices and geometric parameters were derived from the optimized structures and listed in Tables 3 and 4.

Calculations of centroid of benzofused (A) and phenyl (D) aromatic rings and least square fitting were performed within SYBYL.

Pharmacology

Test of anticonvulsant activity against sound induced seizures in DBA/2 mice. DBA/2 mice (6–12 g, 22–26 days old) were purchased from Charles River (Calco, Como, Italy) and were exposed to auditory stimulation, 45 min following intraperitoneal (i.p.) administration of vehicle or drugs. For systemic injections, all compounds were given intraperitoneally (0.1 mL/10 g of body weight of the mouse) as a freshly prepared solution in 50% dimethylsulfoxide (DMSO) and 50% sterile saline (0.9% NaCl). Diazepam was administered at dose levels which affect audiogenic seizure response in DBA/2 mice.[40] Flumazenil was used at a dose level which did not affect audiogenic seizure response in DBA/2 mice.[41] Individual mice were placed under a hemispheric perspex dome (diameter 58 cm) and 60 s was allowed for habituation and assessment of locomotor activity. Auditory stimulation (12–16 kHz, 109 dB) was applied for 60 s or until tonic extension occurred. Seizure response (S.R.) as previously reported[42] was assessed on the following scale: 0 = no response, 1 = wild running, 2 = clonus, 3 = tonus, 4 = respiratory arrest. The maximum response was recorded for each animal. Rectal temperature was recorded immediately prior to auditory testing using an Elektrolaboratoriet thermometer type T.E.3. Behavior changes were observed during the period between drug administration and auditory testing.

Test of anticonvulsant activity against PTZ induced seizures in Swiss mice. Male Swiss mice (20–26 g, 42–48 days old) were purchased from Charles River (Calco, Como, Italy) and were pretreated with vehicle or drugs 30 min before the intraperitoneal administration of pentylenetetrazole. The convulsive dose 97 (CD97) of pentylenetetrazole (60 mg/kg) was injected in a volume of 0.1 mL/10 g of body weight of the mouse. The animals were then placed in isolated cages and observed for 30 min. A threshold convulsion is an episode of clonic spasms lasting for at least 5 s. Absence of this threshold convulsion over 30 min indicates that the test substance has ability to elevate pentylenetetrazole seizure threshold.[43]

Treatment with flumazenil. The anticonvulsant effects of **5d** (0.1, 0.33, 1.0, 3.3 and 10 mg/kg) was reduced by a treatment with flumazenil (2.5 mg/kg i.p.) 15 min after the administration of **5d**. In particular a significant increase in the incidence of all phases of the audiogenic seizure response was seen in the groups treated with flumazenil compared to the corresponding groups receiving **5d** alone. No behavioral changes or fall in rectal temperature were seen after flumazenil treatment in DBA/2 mice. The ED_{50} for **5d** against the audiogenic seizures increases in both phases.

Effects of 5a on the anticonvulsant activity of diazepam. The administration of **5a** (3.3 mg/kg i.p.) was able to suppress the antiseizure effects of diazepam (0.33, 0.66, 1.0, 3.3, mmol/kg).

Statistical analysis. Statistical comparisons among groups of control and drug-treated animals were made using Fisher's exact probability test (incidence of the seizure phases) or ANOVA and Dunnett's t test (rectal temperatures). The percentage incidence of each phase of the audiogenic seizure was determined for each dose of benzodiazepine administered and log dose–response curves were fitted using linear regression analysis of probit-transformed percentage response. ED_{50} values (with 95% confidence limits), for each compound and each phase of seizure response, were estimated using the method of probit analysis;[44] the relative anticonvulsant activities were determined by comparison of respective ED_{50} values.

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