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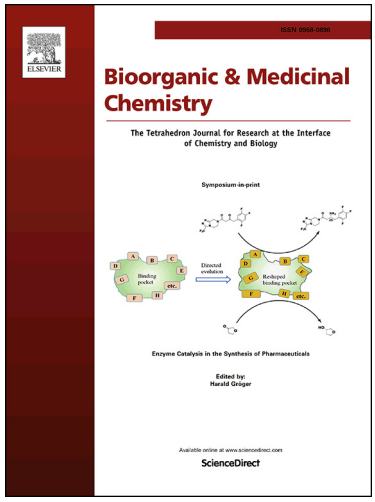

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Synthesis and pharmacological evaluation of pyrazolo[4,3-c]quinolinones as high affinity $GABA_A$-R ligands and potential anxiolytics

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Dedicated to our beloved Prof. Gloria Inés Yranzo on the tenth anniversary of her physical departure.

Abstract: The synthesis, *in vitro* ligand binding study and *in vivo* Elevated Plus Maze test (EPM) of a series of pyrazolo[4,3-*c*]quinolin-3-ones (PQs) are reported. Multistep synthesis of PQs started from anilines and diethyl 2-(ethoxymethylene)malonate to give the quinolin-4-one

nucleus, via the Gould-Jacobs reaction. These quinolinones were transformed to 4-chloroquinolines, which react with aryl-hydrazines affording the final compounds. PQs exhibited different potency in displacing specific [H^3]Flunitrazepam binding from the benzodiazepine binding site at the γ-aminobutiric acid receptor ($GABA_A$-R) depending on the substitution of the pyrazoloquinolone nucleus. PCA helped determine how different substituents contributed to the differential behavior of the PQs studied. Compounds with high affinity for the $GABA_A$-R were tested regarding their anxiolytic properties in Wistar adult male rats using the Elevated Plus Maze (EPM). Thus, PQs with a *p*-methoxy phenyl group at N-1 (**7b-*ii*** and **7c-*ii***) displayed a remarkable anxiolytic activity at low doses (0.5-1.0 mg/kg). Meanwhile, PQs featuring an unsubstituted phenyl (**7b-*i***) or *p*-fluoro phenyl group (**7b-*iii***) at the N-1 showed anxiogenic effects in the EPM test.

Keywords: pyrazoloquinolinones; elevated plus maze; $GABA_A$ receptor; benzodiazepine binding

1. Introduction

Among the pyrazole derivatives, the condensed compounds having the pyrazoloquinolin-3-one (PQ) core have extensively been studied for their biological activities e.g. acetylcholinesterasa inhibitors, anti-inflammatory agents, antimalarial and antiviral activities, between others [1-3].

In particular, PQs have been known for their high affinity for the benzodiazepine binding site (BZD_{BS}) at the ionotropic γ-aminobutiric acid (GABA) receptor ($GABA_A$-R) [4-6]. After the performance of PQs was described, there has been a growing interest in their peculiar pharmacological activity. These target compounds constitute a biologically interesting class of

ligands, lacking some unwanted side effects (e.g. sedative effects) found in classical benzodiazepines [7], as well as the net shift of intrinsic activity from agonist, through antagonist, to inverse agonist, caused by small structural changes render the PQs a fascinating and intriguing class of $GABA_A$-R ligands [5,8-9]. $GABA_A$-R belongs to the "Cys-loop ligand-gated ion channels" family, specifically a chloride channel gated by the γ-aminobutiric acid, the most widely distributed inhibitory neurotransmitter in the central nervous system (CNS) of vertebrates [10,11]. Thus, $GABA_A$-R is the target of many pharmacologically relevant drugs and possess several allosteric binding sites which modulate in diverse ways the effect of its endogenous agonist GABA [12]. These allosteric modulators recognize own specific binding sites in the receptor, different from the specific GABA site [13]. Thus, the chloride flux is increased by several types of depresant drugs, fundamentally benzodiazepines (BZDs), barbiturates, steroids and anaesthetics, whereas it is reduced by convulsant agents like bicuculline and picrotoxinin [14,15].

The most important drugs in clinical use for $GABA_A$-R are the BZDs, which mainly have anxiolytic, anticonvulsivant, muscle relaxant and sedative-hypnotic-effects [11,15]. For this reason, the BZDs and other chemical compounds capable of bindings with high affinity to $GABA_A$-R are considered therapeutic agents of the upmost importance to treat specific diseases in the CNS [16]. Since the discovery of chlordiazepoxide and diazepam [17] the 1,4-benzodiazepines, have been fruitful source of research however, anxioselective compounds, which do not possess the undesirable side effects of BZDs are needed [18,19].

In this regard, 2-aryl-pyrazolo[4,3-*c*]quinolin-3-ones represent promising synthetic targets [3] that have held the attention of researchers in the fields of medicine, pharmacology and chemistry, for several years. Cook and co-workers, have developed a comprehensive pharmacophore model for BZD_{BS} at $GABA_A$-R based on structure-activity relationship studies of 136 different ligands from 10 structurally different classes of compounds [20]. The proposed agonist/antagonist pharmacophore model, based on 2-aryl-pyrazolo[4,3-*c*]quinolin-3-ones,

involves the interaction sites between the receptor and ligand necessary for activity, [21,22] whereas the structure–activity relationship (SAR and QSAR) findings indicate the importance of substitutions on the pyrazolo [4,3-*c*]quinolin-3-one skeleton which determine ligand affinities for BZD_{BS} at $GABA_A$-R [9, 21,23].

Regarding the tests of anxiety, elevated plus maze test (EPM) is one of the most widely used animal models for screening anxiolytic and anxiogenic agents. It is an exploratory based approach–avoidance paradigm measuring non-conditioned responses. This model was validated by Pellow et al based on the natural aversion of rodents for open spaces and uses an elevated plus-maze with two open and two closed arms [24,25]. Two indices of anxiety are obtained: the number of entries into open arms expressed as a percentage of the total number of arm entries, and the amount of time spent on the open arms expressed as a percentage of the total time on both open and closed arms. The test is rapid and was found to be sensitive to the effects of both anxiolytic and anxiogenic agents, anxiolytic agents increasing and anxiogenic agents decreasing the two measures. Its simplicity and relatively low cost made it essential in the preclinical phase of industrial research for central nervous system drugs.

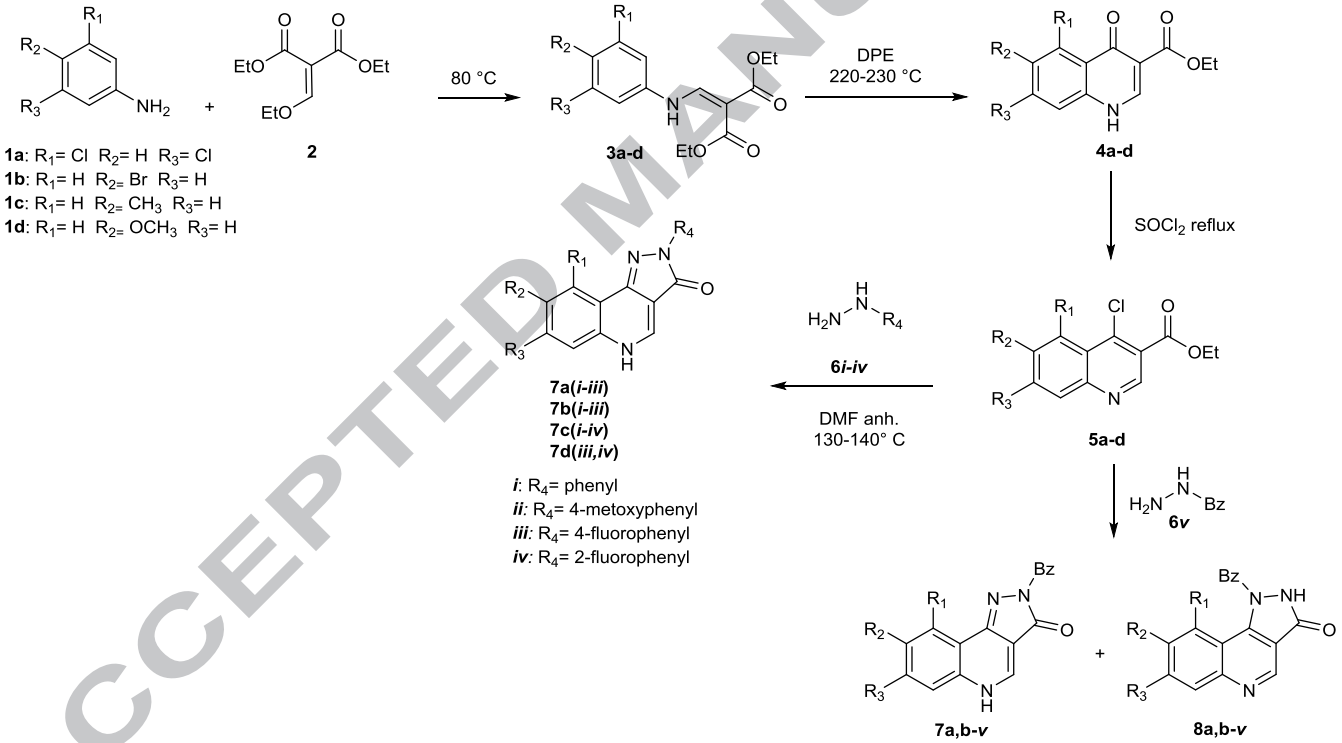
The aim of the present study is to report the synthesis of a series of 2-substituted-pyrazolo[4,3-*c*]quinolin-3-ones (PQs), the *in vitro* binding affinities of these compounds to the BZD_{BS} at $GABA_A$-R through displacement [^{3}H]-Flunitrazepam ([^{3}H]-FNZ) experiments and the *in vivo* anxiolytic properties of selected PQs in Wistar adult male rats by Elevated Plus Maze (EPM).

2. Results and discussion

2.1. Synthesis of pyrazolo[4,3-c]quinolin-3-ones (PQs)

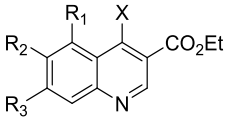
The synthesis of PQs (**7-9**) were performed through the route outlines in Scheme 1, according to procedures previously described [26-28] consisting in a Gould-Jacobs modified

protocol [5,9,29]. The first step involved the addition of the anilines **1a-d** to diethyl 2-(ethoxymethylene) malonate **2**, followed by ethanol elimination, to provide the corresponding anilinomethylenemalonic esters **3a-d** in quantitative yields (> 95%) and in conditions to be used in the next step without purification (see Scheme 1) [26]. The second step consisted on the thermal cyclization of **3** in diphenylether (DPE) to give ethyl-4-oxo-1,4-dihydroquinoline-3-carboxylates **4a-d** [5,29,30]. In the next step the chlorination of quinolones **4a-d** was carried out using $SOCl_2$ to furnish the chloro-derivatives **5a-d** in a straightforward way. Quinolone compounds **4a-d** and **5a-d** were obtained in very good to excellent yields (see Table 1).



Scheme 1. Multistep synthesis of pyrazolo[4,3-*c*]quinolin-3-ones

Table 1. Yields of quinolines **4** and **5**

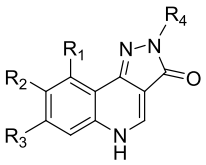
General structure	Compound	X	R_1	R_2	R_3	Yield (%)[a]
	**4a**	OH	Cl	H	Cl	78
	4b	OH	H	Br	H	74
	4c	OH	H	CH_3	H	77
	4d	OH	H	OCH_3	H	58
	5a	Cl	Cl	H	Cl	98
	5b	Cl	H	Br	H	99
	5c	Cl	H	CH_3	H	98
	5d	Cl	H	OCH_3	H	98

[a]Yields of isolated product

In the last step, the reaction of chloroquinolines **5a-d** with aryl-hydrazines (**6*i-iv***) and benzylhydrazine (**6*v***) gave 2-aryl-pyrazoloquinolinones and 2-benzyl-pyrazoloquinolinones, respectively (see Table 2). In all reactions, anhydrous DMF was used as solvent and cyclization were completed over 1-4,5 h at 130-140 °C. In the case of the cyclizations with hydrazine monohydrochloride (**6 *ii-iv***), and dihydrochloride (**6*v***) a previous neutralization with triethylamine (TEA) was required. The formation of aryl-PQs was good in the most of cases (41-85%, see Table 2) however, for the synthesis of benzyl-PQs yields were modest taking place the formation of two isomers (**7a,b-*v*** and **8a,b-*v*** in Scheme 1). These findings could be attributed to the comparable nucleophilicity of both nitrogens in the benzylhydrazine substrate, which allows the displacement of chlorine by both hydrazinic nitrogens followed by cyclization to give the

pyrazolone ring. The ratio of isomers **7a,b-*v*** and **8a,b-*v*** obtained in the reaction mixture depends on the amount and type of base used in the neutralization step [26].

Table 2. Yields of 2-aryl/benzyl-pyrazolo[4,3-*c*]quinolin-3-ones **7**.

General structure	Compound	R_1	R_2	R_3	R_4	Yield (%)[a]
	**7a-*i***	Cl	H	Cl	Ph	71
	7a-*ii*	Cl	H	Cl	*p*-$OMeC_6H_4$	41
	7a-*iii*	Cl	H	Cl	*p*-FC_6H_4	41
	7a-*v*	Cl	H	Cl	Bz	15
	7b-*i*	H	Br	H	Ph	61
	7b-*ii*	H	Br	H	*p*-$OMeC_6H_4$	55
	7b-*iii*	H	Br	H	*p*-FC_6H_4	72
	7b-*v*	H	Br	H	Bz	10
	7c-*i*	H	CH_3	H	Ph	65
	7c-*ii*	H	CH_3	H	*p*-$OMeC_6H_4$	80
	7c-*iii*	H	CH_3	H	*p*-FC_6H_4	60
	7c-*iv*	H	CH_3	H	*o*-FC_6H_4	60
	7d-*iii*	H	OCH_3	H	*p*-FC_6H_4	85
	7d-*iv*	H	OCH_3	H	*o*-FC_6H_4	73

[a]Yields of isolated products. BZ refers to benzyl group.

2.2. Specific interaction of PQs with $GABA_A$-R

2.2.1. Determination of [^{3}H]FNZ kinetic binding parameters B_{max} and K_d

The kinetics of the [^{3}H]FNZ specific binding to the $GABA_A$-R contained in purified synaptosomal membranes (SMs) followed a hyperbolic behavior (Fig.1A) characteristic of a

single type of binding sites. These values came from the difference calculated between data obtained in saturation experiments in the presence (nonspecific binding) and absence (total binding) of a non-labeled displacement agent (Diazepam, DZ) (Fig.1A, inset). The linearity of the former and its quantitative relation with the latter (less than 25%) reflected the good quality of the experimental system. The fitness of Eq. 1 (Section 3) to the experimental data in the specific binding curve, let calculating the kinetic parameters for the [^{3}H]FNZ / GABA-R specific binding, K_d = 2.3 ± 0.2nM and B_{max} = 1702 ± 32 fmol/mg protein (Figure 1A). These values agree with previous reports [31,32].

2.2.2. Radioligand competition experiment

The potencial GABAergic activity of PQs was tested by investigating their ability to displace [^{3}H]FNZ from the BDZ_{BS} at the $GABA_A$-R through a radioligand competition assay. A typical experiment performed with compound **7b-*ii*** is depicted in Fig.1B showing a plot of percent of [^{3}H]FNZ specifically bound in the presence of PQ with respect to the control without PQ (%B) vs. log PQ concentration (see Supporting Information for a full set of displacement curves for the other PQs). Fitting Eq. 2 to each displacement plot let determine the relative binding affinities of non-labelled ligands (IC_{50}) (Eq. 2, section 3). Then, from the IC_{50} values for PQs, the corresponding K_i could be calculated according to Eq. 2 [33]. The resulting IC_{50} and K_i values (Table 4) showed that, with the exception of dichloro derivatives **7a** and the benzyl derivative **7b-*v***, PQ compounds were able to displace the [^{3}H]FNZ bound to SM at different extents.

We found a clear distinction between displacing and not displacing compounds as shown in the displacement curves depicted in the Supporting Information. Additionally, among the displacing compounds there was a progression in their potency. The $LogIC_{50}$ parameter values were analysed using one-way ANOVA followed by post hoc analysis Tukey to enable specific group comparisons ($P < 0.05$ was significant). The ANOVA showed a significant difference between IC50 values between PQ **7b-*ii*** and **7d-*iii***, **7d-*iv***, **7b-*iii*** and **7c-*iii***; **7c-*iv*** compared with

7d-*iii*, **7d-*iv*** and **7b-*iii*** as well as **7c-*ii*** compared with **7d-*iii*** (see Supporting information for further details).

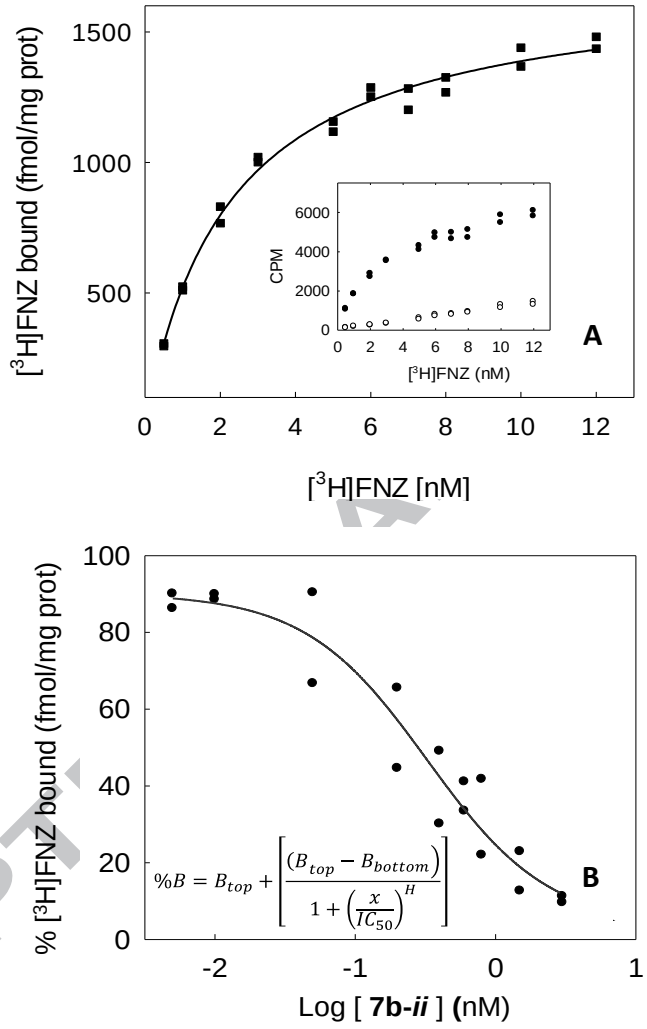
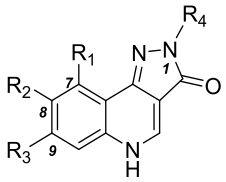


Figure 1. Binding of [^{3}H]FNZ at $GABA_A$-R in synaptosomal membranes. **A**: Typical saturation curve. The inset shows the TB (●) and the NB (○). The $GABA_A$-R source was bovine brain cortex SM. [^{3}H]FNZ and protein concentration were 2 nM and 0.25 mg protein/mL, respectively; **B**: representative [^{3}H]FNZ displacement experiment as a function of **7b-*ii*** concentration. Data were fitted to Eq. 2 (inset).

Considering the substituents on the quinoline ring, it was observed that compounds having two chlorine atoms at positions 7 and 9 (R_1 = Cl and R_3 = Cl respectively) did not

displace the radioactive ligand from the BZD_{BS}, independently of the *N*-aryl substitution on the pyrazolone ring. This could be related with a steric and/or electrostatic disturbance area at the receptor regions described previously as LDi and H2 according to the pharmacophoric model of Cook and co-workers [20]. Probably the presence of the chlorine atom in position 9 (R_1 = Cl) interferes in the formation of hydrogen bond between the N-1 of the PQ and the H2 receptor site. In addition, the presence of the two acceptors groups diminish the hydrophobic π-π interaction by decreasing the electron density on the phenyl ring of the quinolone nucleus, which would reduce the interaction with the receptor site LDi. In this regard, Savini et al. [6] have tested several pyrazoloquinolinone compounds as central benzodiazepine receptor ligands and their results of structure-affinity relationship studies would indicate that the substitution with chlorine at positions 7 and 9 results unfavourable with respect to the hydrogen substituent.

Table 4: Binding affinities of PQs.[a]

PQs					Log IC_{50} (nM)	IC_{50} (nM)	K_i (nM)
	R_1	R_2	R_3	R_4			
7a-*i*	Cl	H	Cl	Ph	-	-	-
7a-*ii*	Cl	H	Cl	*p*-$OMeC_6H_4$	-	-	-
7a-*iii*	Cl	H	Cl	*p*-FC_6H_4	-	-	-
7a-*v*	Cl	H	Cl	BZ	-	-	-
7b-*i*	H	Br	H	Ph	-0.084 ± 0.133	0.823	0.437
7b-*ii*	H	Br	H	*p*-$OMeC_6H_4$	-0.487 ± 0.137	0.326	0.173
7b-*iii*	H	Br	H	*p*-FC_6H_4	0.167 ± 0.065	1.468	0.780
7b-*v*	H	Br	H	BZ	-	-	-
7c-*i*	H	CH_3	H	Ph	-0.230 ± 0.060	0.589	0.313
7c-*ii*	H	CH_3	H	*p*-$OMeC_6H_4$	-0.266 ± 0.040	0.542	0.288
7c-*iii*	H	CH_3	H	*p*-FC_6H_4	0.062 ± 0.095	1.152	0.612
7c-*iv*	H	CH_3	H	*o*-FC_6H_4	-0.433 ± 0.047	0.370	0.196
7d-*iii*	H	OCH_3	H	*p*-FC_6H_4	0.266 ± 0.057	1.845	0.980
7d-*iv*	H	OCH_3	H	*o*-FC_6H_4	0.176 ± 0.111	1.500	0.797

[a] The $GABA_A$-R source was bovine synaptosomal membranes, [^{3}H]FNZ and protein concentration used were 2 nM and 0.25 mg/mL, respectively. The IC_{50} values were determined from the % [^{3}H]FNZ bound *vs.* log PQ concentration (nM) plot, as the PQ concentration that

displace the 50% of the [^{3}H]-FNZ bound. K_i was calculated by means of the Cheng and Prusoff equation (Eq. 2, Ref. 33). BZ refers to benzyl group.

The remaining compounds have displaced [^{3}H]FNZ from the BZD_{BS} at the $GABA_A$-R at different extents according to the substituents at positions 8 and/or the nature of the aryl substituent at N1, being **7b-*ii*** and **7c-*iv*** two of the most active ligands (Figure 2).

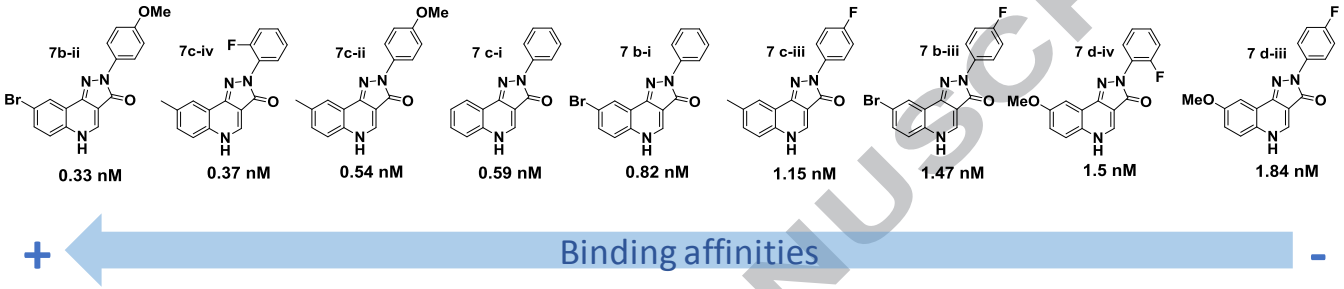


Figure 2. Values of IC_{50} listed in descending order of ligand–receptor binding affinity for PQs **7**

The compounds bearing a methyl group at position 8 (R_2) have showed very good affinity, although the compound which has the greatest affinity has a bromine atom in this position (**7b-*ii***). In this regard, the presence of a methoxy group in C-8 (R_2) notably reduces the affinity of the PQ for this receptor. These results agree with the QSAR relations previously proposed [6], where the authors have postulated the equation 4, considering the Van der Waals volume (VW) as an important parameter involved in the biological activity.

$$\mathbf{pIC_{50} = -0{,}32\ (\pm 0{,}10\ vW) + 9{,}63\ (\pm 0{,}28)} \qquad \text{Eq. 4}$$

$n=9$, $r^2=0{,}883$, $Q^2=0{,}745$, $s=0{,}208$, $F= 52{,}77$

The negative sign of the VW coefficient means that there is a negative steric contribution for the VW greater substituents. VW values for methyl, bromine and methoxy substituents have been calculated as 1.01, 1.32 and 1.49 respectively. Considering these values, the methoxy group promotes the greatest steric effect leading to the increase of the negative character of the equation term and decreasing the biological activity, as it was observed in the evaluation of PQs **7d**.

The examination of the nature of substituents on the phenyl ring attached to N-1 (R_4), indicates that the methoxy group is present in the most active compounds (**7b-*ii*** and **7c-*ii***). The activity decreases for the 2-phenyl unsubstituted compounds (**7b-*i*** and **7c-*i***) and the behaviour of fluorine derivatives would depend on the position of fluorine in the phenyl ring. Thus, when the fluoro substituent is located in the *para* position of the aryl ring the compounds showed low activities (**7b-*iii***, **7c-*iii*** and **7d-*iii***) while the *ortho*-fluoro phenyl derivatives displayed a dissimilar behaviour depending on the substitution at C-8. Thus, the 8-methyl-PQ **7c-*iv*** was one of the most active compounds while the 8-methoxy-PQ **7d-*iv*** showed a reduced activity. In this regard, Catarzi et al. [34] have proposed that the presence of a halogen atom at C-8 in combination with a methoxy group at the *para*-position on phenyl ring produces a steric effect that improves the interaction of these compounds with the BZD_{BS} at the $GABA_A$-R. Moreover, Francis et al. [35] have found that the replacement of the H atom at the phenyl ring attached at N-1 by a methoxy group (*ortho* and *para* position), a hydroxy group (*para* position) or a fluoro atom increase the compounds affinity. The positive effect of the methoxy group on the *para* position of the phenyl ring was also demonstrated in the study of the lead compounds of series CGS [36]. More specifically, these investigations have indicated that the presence of a fluorine atom in the ortho position of phenyl substituent improve the affinity of the PQs for the BZD_{BS} due to the minor size, good electronegativity and the possibility of this halogen to be an acceptor of H-bond with the H2 region of the receptor [9,34,35]. This behaviour could explain the high affinity observed for compound **7c-*iv***.

It is noticeable that the derivatives having a benzyl substituent at N-1 (**7a-*v*** and **7b-*v***) did not show interaction with the BZD_{BS}, probably due to a volume related steric hindrance of this group limiting the access to the BZD_{BS} cavity in the R-$GABA_A$.

2.3. Elevated Plus Maze experiments

The anxiety-like behaviour of PQs was evaluated in the EPM experiment, which is a widely used assay for rodents and it has been validated to assess the anti-anxiety effects of pharmacological agents [24,37]. Rodents are placed in the centre of the four arms of the EPM, and their behaviour was recorded for 5 min. Anxiogenic drugs reducing time spent on the open arms and anxiolytic drugs increasing the time spent on the open arms of the EPM.

The brominated PQs **7b-*i*-*iii*** were tested regarding their anxiolytic properties in Wistar adult male rats using the EPM experiments. We decided to study these compounds in order to correlate the results with the structural changes in the PQ nucleus, focussing on the substitution of phenyl ring attached to N1 (R_4). In the Figures 3a-c, the percentage of time in open arms *versus* the oral doses provided is shown.

For compound **7b-*i***, rats treated with doses of 0.25 and 0.5 mg/kg exhibited an increase in anxiety compared with the rats treated with other doses and vehicle treated control animals (Figure 3a, ANOVA $F(4,28)=3.0521$ $p=0.0331$). These results would indicate an anxiogenic effect of this PQ at both doses and this behavior could be related to an inverse agonism.

In the experiments performed with **7b-*iii***, the administration of this compound also shortened the time spent in the open-sided arms of the plus-maze (Figure 3b). The effect gradually increased with the increment on doses with the peak of the effect observed at 3 mg/kg (ANOVA $F(4,28)=4.6784$ $p=0.0062$). It is worthy to note that a great reduction of entries of animals to the closed arms was evidenced, which could reflect a reduced capacity of locomotion

(ANOVA F(4,24)=4.7678 p=0.0056). In this case, it is difficult to attribute the reduced permanence in open arms to a purely anxiogenic effect because significant changes in motor activity were observed. Further studies are needed to determine the exact mechanism for this effect which might be due to a sedative or toxic action of this compound.

The administration of **7b-*ii*** resulted in a significant increase in the time spent in the open-sided arms, with the peak anxiolytic-like effect observed at 0.50 mg/kg (Figure 3c ANOVA F(4,26)=7.5553 p=0.0004). In this case, the motor activity of animals was not suppressed at any evaluated doses (ANOVA F(4,25)=1.2986 p=02975).

Considering that the methoxy derivative **7b-*ii*** presented a remarkable anxiolytic effect, the EPM experiments with the PQ **7c-*ii*** (Figure 3d) were carried out to compare the effect of substituent in C-8 keeping the *p*-methoxy group in the *N*-phenyl ring. In this case, **7c-*ii*** caused an increment in the time spent in the open-sided arms (50%) at 1mg/kg compared with a 20% response rate in vehicle treated control animals (ANOVA F(4,24)= 5.0932 p=0.0040) without any significant effect on the locomotor activity (ANOVA F(4,24)=1,1750 p=0.3467). Although the administration of **7c-ii** was effective in producing an anxiolytic-like effect, the doses were two times the required for the same effect with the compound **7b-*ii***. Preliminary, these PQs could be an agonist in the BZD_{BS} at $GABA_A$-R.

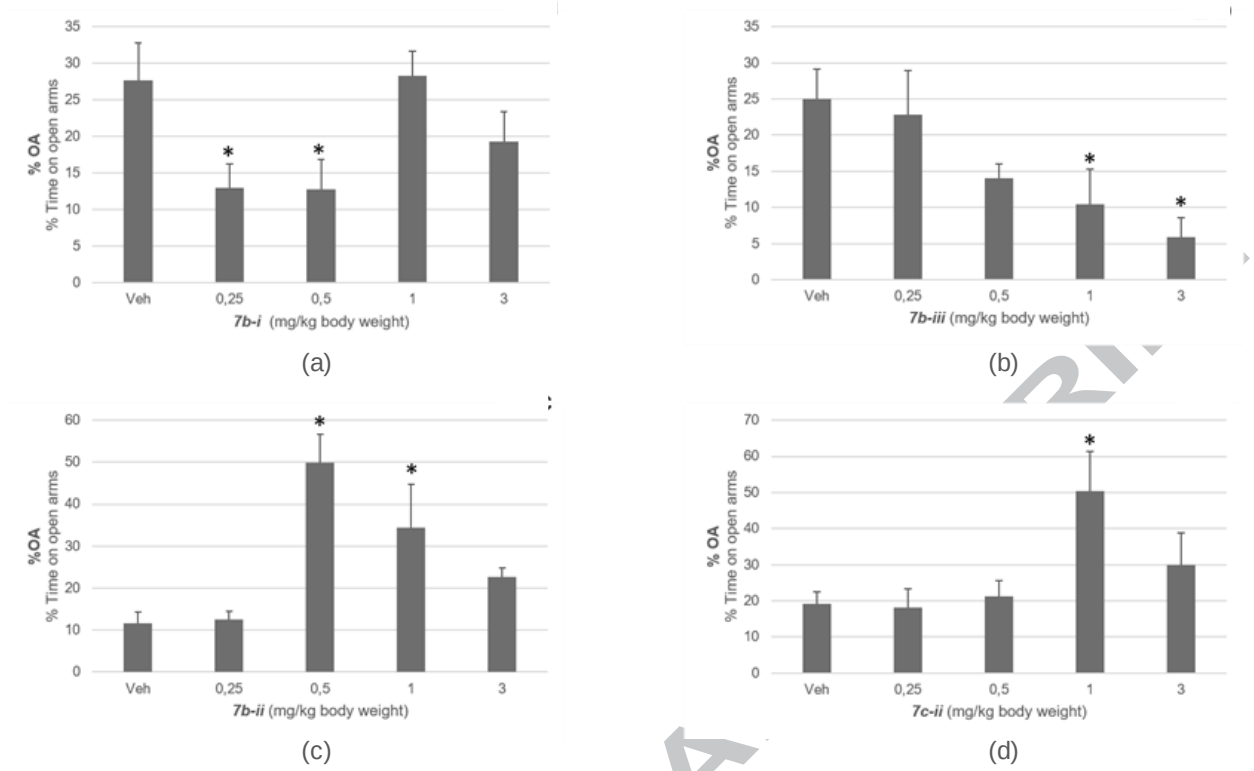


Figure 3. Elevated plus maze tests for PQs **7b-*i*** (a), **7b-*ii*** (b), **7b-*iii*** (c) and **7c-*ii*** (d). Data represent the mean ± SEM of the percentage of time spent on open arms relative to total time spent on all four arms. □p≤ 0.05 vs. other groups (n=6-8).

2.4. Principal component Analysis.

Principal component analysis (PCA) is a multivariate type analysis here applied to ascertain which molecular property or properties combination of PQs were associated with the differential behavior of these compounds on displacement and EPM experiments (see Table S33, in Supporting Information). The contribution of a molecular parameter or effect to each Principal Component (PC) is reflected by the loading value (eigenvalue) derived from PCA analysis (see Table S34, in Supporting Information).

The PCA graphical representation is shown in Figure 4 (data taken from Table S35, Supporting Information). The first two PCs explained 56% of total variance. The first principal component (PC1) explained 42% of total variance and was mainly influenced (loading values

~|0.39|) by the variables 1 (R_1-Cl), 3 (R_2-H), 7 (R_3-Cl), 11 (R_4-BZ), 14 (IC_{50}). PC1 separated the PQs unable to displace FNZ from $GABA_A$-R_{BS} (bearing a chloride atom at positions 7 (R_1) and 9 (R_3) from the rest of PQs studied. The second principal component (PC2, which explained 14% of total variance), was mainly influenced by the variables 5 (R_2-CH_3), 10 (R_4-*p*-$OMeC_6H_4$), 15 (EPM-tOA) and separated the PQs that induced an increase or decrease in the time spent on the open arms.

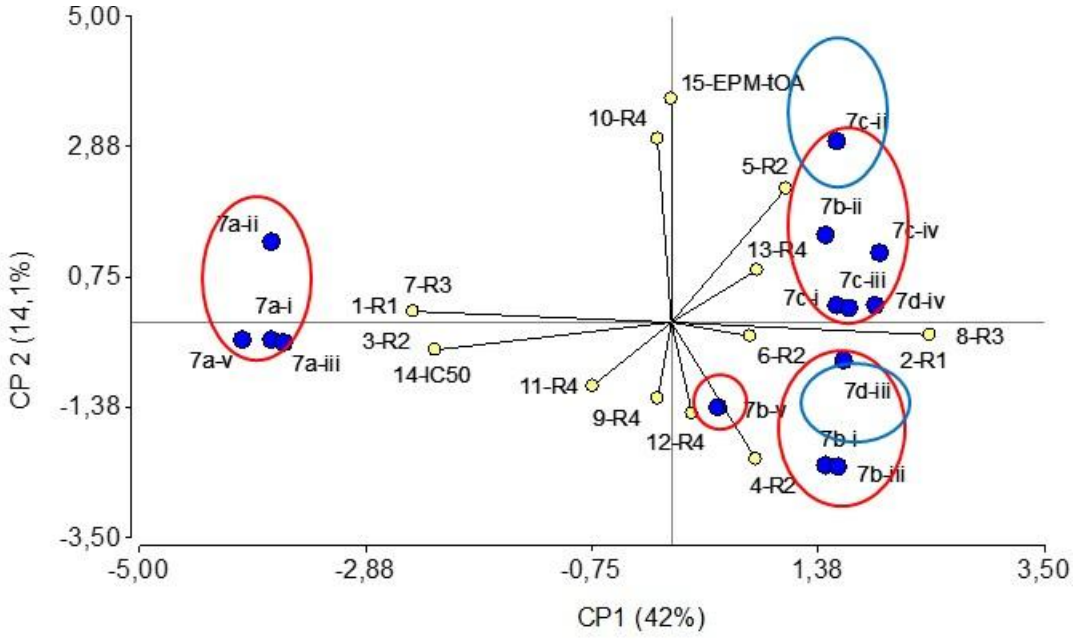


Figure 4. Graphical representation of the Principal Component Analysis. The first two principal components were plotted in a phase space where the grouping of related variables become clear. Data points are represented by the same identification numbers given to variables in Table S1 (Supporting Information). Red circles identify PQs with variables exhibiting negative and positive loadings for PC1 and associated with high and low IC_{50} values, respectively. Blue circles identify PQs with variables exhibiting positive and negative loadings for PC2 and associated with high and low EPM-tOA values, respectively.

3. Materials and methods

3.1. *Chemistry*

Reagents and solvents were used from commercial sources without purification. Melting points were determined by using a Electrothermal 9100 capillary melting point apparatus and are uncorrected. 1D- and 2D NMR spectra (^{1}H, ^{13}C, ROESY, HSQC, HMBC) were recorded at room temperature on a BRUKER FT-400 MHz spectrometer at 400 MHz for ^{1}H and 100 MHz for ^{13}C, using the indicated solvents. Chemical shifts are given in parts per million (ppm) relative to central peak of the solvents. Coupling constants values are given in Hertz. Mass spectra were recorded on a SHIMATZU CGMS-QP 5050A system operating at 70 e.v. High-resolution mass spectra were recorded with an Agilent LCTOF instrument.

*3.1.1. General procedure for the preparation of pyrazoloquinolinones **7a-d***

The pyrazoloquinolinone compounds were prepared following a protocol previously described [26]. Equimolar amounts (3 mmol) of aniline and diethyl(ethoxymethylene)malonate were mixed and heated at 80°C, during 1h to give malonates **3a-d**, which were cyclized in diphenyl ether at 220-230°C during 1-5 h to afford quinoline derivatives **4a-d**. These quinolones (0.8 mmol) were mixed with excess of thionyl chloride (0.5 mL) at reflux temperature for 1-3 h to give chloroquinolines **5a-d**. Pyrazoloquinolinones **7a-d** were prepared by reaction between **5a-d** (0.8 mmol) and the hydrochloride salt of hydrazines **6*i-v*** (1 mmol), previously neutralized with triethylamine, using dimethyl formamide (DMF) as solvent and temperatures between 130-140°C. Compounds **7b-*i***, **7c-*iv*** and **7d-*iii*** were recrystallized from DMF, ethanol/water and DMF/water respectively; **7b-*ii***, **7b-*iii***, **7c-*ii***, **7c-*iii*** and **7d-*iv*** were purified by chromatography column and **7c-*ii*** was obtained as pure compound. All PQ compounds were characterized by

standard spectroscopic techniques (^{1}H NMR, ^{13}C NMR, HMBC, HSQC) and high-resolution mass spectrometry, and all data agree with the proposed structures, which have been previously reported [5,6,26-29,38].

3.2. *Biochemical Experimental Section*

3.2.1. In vitro binding experiments

3.2.1.1. Materials

The BZD diazepam (DZ) was kindly supplied by Products La Roche (Córdoba, Argentina). [^{3}H]-FNZ was purchased from New England Nuclear Chemistry (E.I. DuPont de Nemours & Co. Inc., Boston, MA, USA). Other drugs and solvents were of analytical grade.

3.2.1.2. Natural membrane preparation

Synaptosomal membranes (SM) were obtained from bovine brain cortex. Meninges were eliminated, the cortex dissected, and the SM were purified essentially according to the method of Enna and Snyder, modified by Perillo and Arce [39] immediately lyophilized and stored at -20ºC. Immediately before use, membranes were resuspended in 50 mM pH 7.4 Hepes buffer containing 100 mM NaCl at a final protein concentration of 0.25 mg/mL. This SM suspension was used as membrane receptor preparation and $GABA_A$-R source in the experiments that followed.

3.2.1.3 Radioligand binding assays.

3.2.1.3.1 Saturation Curve

All the procedures were carried out at 4ºC. The membrane receptor preparation was obtained as described above. The BZDs used as labelled and non-labelled ligands were [^{3}H]FNZ and DZ, respectively. Both BZDs compete for their reversible interaction with the same binding site at $GABA_A$-R (specific interaction). On the other hand, due to their lipophilic characteristics, BZDs interact, through hydrophobic interactions, with the $GABA_A$-R containing membrane by an

irreversible partitioning process (nonspecific binding, NB). So, only the specific binding of [^{3}H]FNZ can be displaced from the receptor with high concentrations of the (DZ) and the remaining non-specifically bound [^{3}H]FNZ represents NS. TB (total binding) is measured in the presence of the same concentration of [^{3}H]FNZ but in the absence of non-labeled ligand.

The incubation system (230 mL total volume) contained: the SM suspension at a final 0.25 mg/mL total protein concentration of and 0.5–12 nM [^{3}H]FNZ (minimum specific activity 81.4 Ci/mmol). Then, 100 mM NaCl–50 mM Tris–HCl pH 7.4 buffer, containing (NB samples set) or not (TB samples set) 9.4 mM DZ. Samples were incubated at 4 °C in the dark for 1 h and then filtered through SS filters Whatman GF/B type with a Brandel automatic filtration apparatus (Brandel, Gaithersburg, MD, USA). Then, filters were rinsed, dried in the air stream and placed in vials containing 2.5 mL of scintillation liquid (25% v/v Triton X-100, 0.3% w/v diphenyloxazole in toluene). The retained radioactivity was measured with a scintillation spectrometer Rackbeta 1214 (Pharmacia-LKB, Finland) at a 60% efficiency for tritium. Each sample was run at least in duplicate determinations.

Specific binding (B) was calculated as the difference between TB and NB determined in the absence and in the presence of DZ, respectively. Eq. 1 was fitted to the saturation curves (specific binding (B) *versus* free [^{3}H]FNZ concentration), by a non-linear regression analysis performed by a computer-aided least squares method [39].

$$B = \frac{B_{max}.F}{Kd + F} \qquad \text{Eq. 1}$$

where F is the free [^{3}H]FNZ concentrations, B and B_{max} are the ligand concentration-dependent and the maximal specific binding activities, respectively, and K_d is the equilibrium dissociation constant. Protein concentration was determined by the method of Lowry [40].

3.2.1.3.2 [^{3}H]FNZ displacement curves

The experiments were carried out using a 2nM constant radioligand ([^{3}H]-FNZ) concentration corresponding to the mean dissociation binding constant (K_d) determined in a previous experiment. The SM (0.25 mg prot/mL) was incubated with the radioligand and with increasing concentrations of unlabelled PQ at 4°C, in the dark for 1 h. After incubation, samples were filtered through SS filters Whatman GF/B type with an automatic harvester apparatus. The filters rinsed and dried in the air, were placed in vials containing 2.5 ml of scintillation liquid 25%V/V Triton X-100, 0.3% P/V diphenyloxazole in toluene. The retained radioactivity was determined with a scintillation counter Rackbeta 1214 (Pharmacia-LKB, Finland) with an efficiency of 60% for Tritium. Protein concentration was determined by the method of Lowry [40]. Each sample was run at least in duplicate determinations.

The IC_{50} values were determined from the % [^{3}H]-FNZ bound (%B) *vs*. log PQ concentration (nM) plot. If a compound competes for a homogeneous class of binding sites, the competition curve follow a sigmoidal function with a Hill-slope (H) equals unity and the data points fit to a 4-parameter logistic equation (Eq. 2), were B_{bottom} is the %B in absence of PQ and B_{bottom} is the %B at the highest PQ concentration assayed. The IC_{50} value is obtained from the curve inflection point the and represent the PQ concentration that displace the 50% of the [^{3}H]-FNZ bound.

$$\%B = B_{top} + \left[\frac{(B_{top} - B_{bottom})}{1+\left(\frac{x}{IC_{50}}\right)^{H}}\right] \qquad \text{Eq. 2}$$

Then, if the K_d of the labeled ligand is known, K_i value can be calculated by means of the Cheng and Prusoff equation (Eq.3) [39].

$$K_i = IC_{50}/\{1+[\text{radioligand}]/K_d\} \qquad \text{Eq. 3}$$

Although IC_{50} is a very informative parameter, K_i values have the advantage over IC_{50} of being independent on the radioligand concentration used.

3.2.1.3.3 Protein quantitation

Protein concentration was quantified according to the Lowry method [40]. Briefly, the system consisted of 0.05ml of SM suspension and 0.5ml of EDTA-Cu^{2+}. The samples were incubated at room temperature by 45 min and 0.05 mL of 1:2 bidistilled water diluted Folin Ciocalteu reagent was added. The samples were incubated more 1 h at room temperature. Finally, the absorbance at 750 nm was registered using a Beckman DU 7500 spectrophotometers 0.0001 AU sensibility.

3.3. ***In vivo behavioural test***

3.3.1 Animals

Adult male Wistar rats (65–75 days), bred in our colony and weighing 280–320 g, were housed in groups of 4-5 per cage with food and water ad libitum. They were maintained in a 12 h light–dark cycle (lights on at 07:00 a.m.) at a constant room temperature of 21-22 °C. Rats were handled during the week before the experimental procedure, in order to habituate them to manipulation. This habituation consisted in the transportation of the animals to an experimental room, removing them from their cages, the handling of each animal during 1min and returning them to their home cages. This procedure was repeated during four consecutive days before the experiments. All the experiments were performed during the light cycle between 10:00 a.m. and 03:00 p.m.). An average of 7 rats was used for each dose for each compound. Procedures were conducted in accordance with the National Institutes Health Guide for the Care and Use of Laboratory Animals, as approved by the Animal Care and Use Committee of the Faculty of Chemical Sciences, National University of Córdoba, and by the National Department of Animal

Care and Health (SENASA-ARGENTINA). Efforts were made to minimize animal suffering and to reduce the number of animals used.

3.3.2 Elevated plus-maze (EPM)

The apparatus consisted of a black acrylic maze with two opposite open arms (50×10 cm) and two opposite closed arms (50×10×40 cm) disposed like a plus sign with a central square of 10 cm^2. Entire maze was elevated 50 cm above the floor. The test consisted in placing the rat in the central square of the maze facing one closed arm and allowing it to explore during 5 min. This procedure was performed in a quiet and dimly illuminated room (4 lx). The scores analysed were the time spent in open and closed arms, and the number of entries to both arms. With these data were calculated the percentage of time spent in open arms which is widely used as an anxiety index (time spent in open arms relative to the time spent in open and closed arms). The number of entries in the closed arms was also assessed, as indicative of exploratory activity [41-44].

3.3.3 Formulation and administration of compounds

Each drug was dissolved in propylenglycol by sonication and then diluted with an aqueous solution of 0.6 % v/v Tween 80. The dose tested were 0.25, 0.5, 1 y 3 mg/kg. These solutions were prepared the same day of the experiment. Each rat was injected intraperitoneally 30 min before the exposition to the behavioral test (EPM). The control rats were injected with a solution of propylenglycol/ Tween 80/ water in the same proportion that used to dissolve the drugs.

3.3.4 Statistical analysis

The data were analysed statistically using the software Statistic 6.0. The values graphed represent the means ± S.E.M (standard error of the mean). The EPM data and $LogIC_{50}$ parameter values were analysed using one-way ANOVA followed by post hoc analysis (Fischer LSD to EPM) to enable specific group comparisons or by post hoc analysis Tukey (to $LogIC_{50}$ data) [45], $P < 0.05$ was significant. Principal component analysis (PCA) was performed on the molecular properties, binding data and EPM data, previously centered and standardized since they were expressed in non-comparable units [46] using statistical InfoStat software [47].

4. Conclusions

Pyrazoloquinolinones **7a-d** and were prepared and tested as central benzodiazepine receptor ligands. The substitution on quinoline ring as well as the nature of substituent on N-2 was decisive for the affinity for $GABA_A$-R in the *in vitro* displacement experiments. The presence of chlorine in positions 7 and 9 of the quinoline moiety or the presence of a benzyl group attached at the N-2 of the pyrazolone ring led to a lack of ability of PQs to displace [^{3}H]FNZ binding from bovine brain membranes. However, PQs bearing a methyl group at C-8 of quinoline ring and *p*-methoxy phenyl group (**7c-*ii***) or *o*-fluoro phenyl group (**7c-*iv***) at N-2 showed a remarkable activity with IC_{50} (nM) values of 0.542 and 0.370 respectively. Also, the PQ having a bromine substituent at C-8 and a *p*-methoxy phenyl group at N-2 (**7b-*ii***) showed high selective affinity for the BZD binding site at $GABA_A$-R, with IC_{50} (nM) value of 0,326.

In the EPM test PQs **7b-*ii*** and **7c-*ii***, both compounds having a *p*-methoxy phenyl group at N-2 and high affinity for the $GABA_A$-R, presented a significant anxiolytic effect with the dose of 0.5 and 1.0 mg/kg. These results suggest a possible role of these compounds as agonists of the benzodiazepine binding site. Also, this behaviour would indicate that PQs were well absorbed and crossed the blood-brain barrier.

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GRAPHICAL ABSTRACT