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High Affinity Central Benzodiazepine Receptor Ligands: Synthesis and Structure–Activity Relationship Studies of a New Series of Pyrazolo[4,3-*c*]quinolin-3-ones

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Abstract—A large series of 2-aryl(heteroaryl)-2,5-dihydropyrazolo[4,3-c]quinolin-3-(3*H*)-ones, carrying appropriate substituents at the quinoline and N_2-phenyl rings, were prepared and tested as central benzodiazepine receptor ligands. Results from structure–affinity relationship studies were in full agreement with previously proposed pharmacophore models and, in addition, quantitative structure–activity analysis gave further significant insight into the main molecular determinants of high benzodiazepine receptor affinity. The intrinsic activity of some active ligands was also determined and preliminary discussed.

Introduction

Ligands that interact with the benzodiazepine receptor (BzR) and allosterically modulate the action of GABA on neuronal chloride ion flux have a *continuum* of intrinsic activity, ranging from full agonists (anxiolytic, hypnotic, and anticonvulsant agents), through antagonists (nil efficacy), to inverse agonists (proconvulsant and anxiogenic agents).[1–3] Diazepam, β-CCM, and Ro15-1788 are typical representative of an agonist, an inverse agonist and an antagonist, respectively.[4] The heterogeneity, and the functional and structural complexity of the $GABA_A$ receptor/Cl^- ionophore complexes have strongly hampered up to now a clear detection and definition of the stereoelectronic requirements necessary for eliciting a specific intrinsic activity. A total of at least 14 subunits (α1 to α6, β1 to β3, γ1 to γ3, δ and ε)[5,6] of the $GABA_A$ receptor have been identified by molecular cloning, and these subunits are taught to assemble into a pentameric structure to form a Cl^- channel. Most functional subtypes of $GABA_A$ receptors contain α, β, and γ subunits, with different subtypes showing high sensitivity to different benzodiazepine receptor ligands.[7–9] Unfortunately, the key structural and dynamic properties of the BzR cannot be directly measured, or modeled, because of the limited current data and methodologies and therefore, several indirect studies have been carried out to detect the common structural features of diverse classes of BzR ligands most likely responsible for high affinity and possibly for specific intrinsic activity and definite pharmacological profile.[10–17]

Despite the recent significant advances in the structural and functional studies of BzR,[6] and in the molecular pharmacology and physiopathology of anxiety disorders,[3] much remains to be achieved for the design and development of truly innovative drugs, lacking the numerous side effects of benzodiazepines.[18]

From a medicinal chemistry view point, the recent years have seen the proposal and refinement of several pharmacophore models,[10–17] which may constitute an useful guide for the design of new potent ligands. However,

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they have not achieved yet the ability to correctly predict the intrinsic activity.

An interesting contribution in this field came also from some recent 2-D and 3-D QSAR studies[19,20] of a new class of BzR ligands, namely the 2-aryl-2,5-dihydropyridazino[4,3-*b*]indol-3-(3*H*)-ones (PIs), which are structurally related to the well known 2-aryl-2,5-dihydropyrazolo[4,3-*c*]quinoline-3-(3*H*)ones (PQs).

Since the discovery of PQs as high affinity BzR ligands[21] interest in their peculiar pharmacological activity has continued to grow. The lack of some unwanted side effects found in classical benzodiazepines as well as the net shift of intrinsic activity caused by small structural changes, render the PQs a fascinating and intriguing class of BzR ligands. It is very surprising therefore that only few papers dealing with SAR studies of PQs had appeared in the literature.[22–26]

Inasmuch as PQ ligands show higher affinity than the corresponding PI analogs, and moreover small structural modifications lead to a complete span of the intrinsic activity (from agonists to inverse agonists), we decided to carry out an extensive structure–activity relationships study by designing and testing a large series of new PQ analogs. Thus, the molecular skeleton of PQs was properly substituted aiming at the identification of the primary ligand–receptor interactions all around the quinoline and the N_2-phenyl rings responsible for recognition and different activation of BzR. To reach this important objective, a high number of PQ analogs (listed in Table 1) have been synthesized and their BzR affinity measured by radioligand binding assay. In particular, several mono and polysubstituted derivatives on the quinoline ring and monosubstituted *ortho, meta*, and *para* congeners at the N_2-phenyl ring were prepared. The aryl substituents at position 2 were replaced also by aza-heterocycles. The overall structural modifications were such that the substituent physicochemical space resulted more completely explored for *meta* and *para* congeners and for the positions 6 and 8 at the quinoline moiety.

The synthesis and BzR affinity of some PQs listed in Table 1 have been already reported by some of us.[27,28] Unfortunately, due to an undetected and not systematic error in the experimental procedure of the binding assay, those data were wrong and therefore they have been correctly redetermined for the present study.

With few exceptions, the biological data listed in Table 1 come from the present work and constitute a very homogeneous and well suited data set for SAR and QSAR studies. Literature data used in the actual study were normalized with respect to internal standards (as shown).

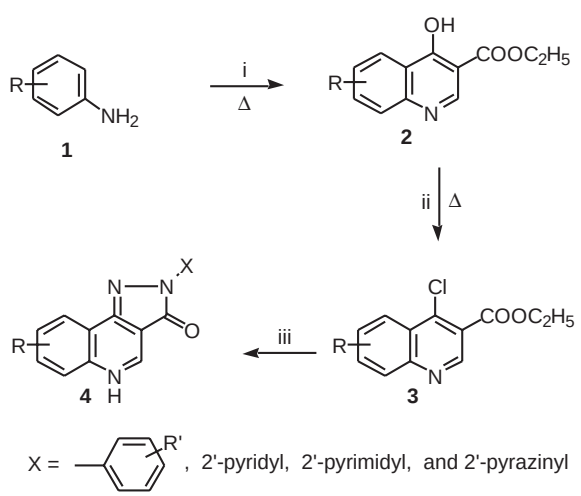
Chemistry

The pyrazolo[4,3-*c*]quinolin-3-ones (**4**) were prepared through the synthetic pathway shown in Scheme 1, according to reported literature procedures;[21,29] thus, properly substituted anilines (**1**) were reacted with diethyl(ethoxymethylene) malonate (EMME) in refluxing Dowtherm A to give ethyl 4-hydroxyquinoline-3-carboxylates (**2**) via thermal ring closure. Treatment of (**2**) with phosphorus oxychloride led to the corresponding chloroderivatives (**3**), which were cyclized with the appropriate hydrazines (hydrazine hydrate 85%, R′-substituted phenylhydrazines, and α-*N*-heterocyclichydrazines to afford PQs (**4**).

All the synthesized PQs were isolated in satisfactory yields (40–60%) and their chemical structures were confirmed by means of IR and ^{1}H NMR data.

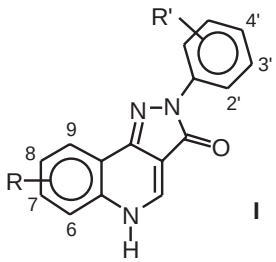
For all compounds (**4**), a strong band between 1620 and 1680 cm^{-1} and a weak band at 3400 to 3100 cm^{-1}, due to the stretching vibration of the carbonyl and the NH groups, respectively, were observed in the IR spectra. In the ^{1}H NMR spectra the characteristic signals for compounds (**4**), in good accordance with the literature data,[21] were a singlet between 8.31 and 9.52 δ, attributable to H-4 proton, and a broad singlet, exchangeable with D_2O, between 12.3 and 13.2 δ due to the N_5-H proton.

In Table 3, the IR and ^{1}H NMR spectral data of some representative PQ **4** have been reported.



Scheme 1. (i) Diethyl(ethoxymethylene)malonate, Dowtherm A; (ii) $POCl_3$; (iii) hydrazine, R′-phenylhydrazine (ethanol reflux); α-(*N*)-heterocyclichydrazines (rt).

Table 1. Physicochemical and binding data of pyrazolo[4,3-*c*]quinolin-3-ones($\mathbf{4}_{1–68}$)



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Compound	R_6	R_7	R_8	R_9	R_2'	R_3'	R_4'	mp(°C)[a]	Crystallization solvent[b]	pIC_{50}[c]	Intrinsic efficacy[d]
$\mathbf{4}_1$[e]	H	H	H	H	unsubstituted at N2			> 300	A	6.30	
$\mathbf{4}_2$[f,g]	H	H	H	H	H	H	H	—		9.35	
$\mathbf{4}_3$[f,g]	H	H	H	H	H	H	Cl	—		9.00	
$\mathbf{4}_4$[f,g]	H	H	H	H	H	H	OCH_3	—		9.17	
$\mathbf{4}_5$[g,h]	H	H	H	H	H	H	H	—		6.84	
$\mathbf{4}_6$[g,h]	H	H	H	H	H	H	Cl	—		6.75	
$\mathbf{4}_7$	F	H	H	H	H	H	H	> 300	A[m]	8.16	
$\mathbf{4}_8$[k]	CH_3	H	H	H	H	H	H	> 300	A	7.18	
$\mathbf{4}_9$	CF_3	H	H	H	H	H	H	294–296	B	5.73	
$\mathbf{4}_{10}$[k]	OCH_3	H	H	H	H	H	H	288–290	A	5.66	
$\mathbf{4}_{11}$[l]	H	H	F	H	H	H	H	> 300	A	9.54	
$\mathbf{4}_{12}$	H	H	F	H	H	NO_2	H	> 300	D[m]	8.70	
$\mathbf{4}_{13}$	H	H	F	H	H	NH_2	H	> 300	A	9.26	
$\mathbf{4}_{14}$[l]	H	H	F	H	H	H	OCH_3	290(dec)	A	9.48	
$\mathbf{4}_{15}$	H	H	F	H	H	H	OH	> 300	A[m]	9.34	
$\mathbf{4}_{16}$[f,g]	H	H	Cl	H	H	H	H	—		9.37	
$\mathbf{4}_{17}$[f,g]	H	H	OCH_3	H	H	H	H	—		9.17	
$\mathbf{4}_{18}$	H	H	OC_2H_5	H	H	H	H	> 300	B	8.85	
$\mathbf{4}_{19}$[i]	H	H	n.C_4H_9	H	H	H	H	—		9.00	
$\mathbf{4}_{20}$	H	H	n.C_4H_9	H	H	H	COOH	> 300	E	5.93	
$\mathbf{4}_{21}$	H	H	n.C_4H_9	H	2-pyrid-2′-yl			263–267	B[m]	9.50	
$\mathbf{4}_{22}$	H	H	n.C_4H_9	H	2-pyrimid-2′-yl			> 300	A[m]	8.77	
$\mathbf{4}_{23}$	H	H	n.C_4H^9	H	2-pyrazin-2′-yl			270(dec)	B[m]	9.22	
$\mathbf{4}_{24}$[i]	H	H	cC_6H_{11}	H	H	H	H	—		8.35	
$\mathbf{4}_{25}$	H	H	cC_6H_{11}	H	H	H	COOH	> 300	A[m]	5.55	
$\mathbf{4}_{26}$	H	H	cC_6H_{11}	H	2-pyrid-2′-yl			> 300	A	8.60	**AG**
$\mathbf{4}_{27}$	H	H	cC_6H_{11}	H	2-pyrimid-2′-yl			> 300	Cm	8.36	
$\mathbf{4}_{28}$	H	H	cC_6H_{11}	H	2-pyrazin-2′-yl			> 300	B	8.16	
$\mathbf{4}_{29}$[g]	H	H	OBn	H	H	H	H	—		7.75	
$\mathbf{4}_{30}$	H	H	OCF_3	H	unsubstituted at N2			> 300	B[m]	6.94	
$\mathbf{4}_{31}$	H	H	OCF_3	H	H	H	H	> 300	A	9.15	**AG**
$\mathbf{4}_{32}$	H	H	OCF_3	H	F	H	H	268–271	F	9.40	
$\mathbf{4}_{33}$	H	H	OCF_3	H	Cl	H	H	291–294	B[m]	8.60	
$\mathbf{4}_{34}$	H	H	OCF_3	H	CH_3	H	H	290–292	B	8.47	
$\mathbf{4}_{35}$	H	H	OCF_3	H	H	Br	H	> 300	A	7.46	
$\mathbf{4}_{36}$	H	H	OCF_3	H	H	CH_3	H	295(dec)	B	8.20	
$\mathbf{4}_{37}$	H	H	OCF_3	H	H	Cl	H	> 300	A	7.62	
$\mathbf{4}_{38}$	H	H	OCF_3	H	H	F	H	> 300	A	9.40	
$\mathbf{4}_{39}$	H	H	OCF_3	H	H	NO_2	H	> 300	D	7.20	
$\mathbf{4}_{40}$	H	H	OCF_3	H	H	NH_2	H	> 300	B	9.62	
$\mathbf{4}_{41}$	H	H	OCF_3	H	H	H	Br	> 300	B	7.82	
$\mathbf{4}_{42}$	H	H	OCF_3	H	H	H	CH_3	> 300	A	8.79	
$\mathbf{4}_{43}$	H	H	OCF_3	H	H	H	Cl	> 300	A	7.90	**AG**
$\mathbf{4}_{44}$	H	H	OCF_3	H	H	H	F	> 300	A	9.00	

Table 1—*contd*

Compound	R_6	R_7	R_8	R_9	R_2'	R_3'	R_4'	mp(°C)[a]	Crystallization solvent[b]	pIC_{50}[c]	Intrinsic efficacy[d]
$\mathbf{4}_{45}$	H	H	OCF_3	H	H	H	NO_2	> 300	C	7.40	
$\mathbf{4}_{46}$	H	H	OCF_3	H	H	H	NH_2	> 300	B[m]	9.10	
$\mathbf{4}_{47}$	H	H	OCF_3	H	H	H	OCH_3	> 300	B[m]	9.22	**AG**
$\mathbf{4}_{48}$	H	H	OCF_3	H	H	H	OH	284–287	A[m]	9.63	
$\mathbf{4}_{49}$[g]	H	H	H	OH	H	H	H	—		9.62	
$\mathbf{4}_{50}$[g]	H	H	H	OCH_3	H	H	H	—		8.84	
$\mathbf{4}_{51}$[j]	F	H	F	H	H	H	H	—		7.87	
$\mathbf{4}_{52}$[j]	F	H	F	H	H	F	H	—		8.02	
$\mathbf{4}_{53}$[j]	F	H	F	H	H	H	Br	—		6.79	
$\mathbf{4}_{54}$[j]	F	H	F	H	H	H	OCH_3	—		8.12	
$\mathbf{4}_{55}$	F	H	F	H	2-pyrid-2′-yl			> 300	A	7.82	**IA**
$\mathbf{4}_{56}$	F	H	F	H	2-pyrimid-2′-yl			> 300	A[m]	6.47	
$\mathbf{4}_{57}$	F	H	F	H	2-pyrazin-2′-yl			> 300	A[m]	6.94	**IA**
$\mathbf{4}_{58}$[j]	H	Cl	H	Cl	H	H	H	—		8.43	
$\mathbf{4}_{59}$	F	F	F	H	H	H	H	> 300	A	7.70	
$\mathbf{4}_{60}$	F	F	F	H	H	H	CH_3	> 300	A[m]	7.15	
$\mathbf{4}_{61}$	F	F	F	H	H	H	Cl	> 300	A	7.13	
$\mathbf{4}_{62}$	F	F	F	H	H	H	F	> 300	A	7.68	
$\mathbf{4}_{63}$	F	F	F	H	H	H	OCH_3	> 300	A	8.14	
$\mathbf{4}_{64}$	H	OCH_3	OCH_3	OCH_3	H	H	H	298–301	A	8.90	
$\mathbf{4}_{65}$	H	OCH_3	OCH_3	OCH_3	H	H	COOH	> 300	D[m]	5.52	
$\mathbf{4}_{66}$	H	OCH_3	OCH_3	OCH_3	2-pyrid-2′-yl			> 300	A	8.50	
$\mathbf{4}_{67}$	H	OCH_3	OCH_3	OCH_3	2-pyrimid-2′-yl			297(dec)	A[m]	7.24	
$\mathbf{4}_{68}$	H	OCH_3	OCH_3	OCH_3	2-pyrazin-2′-yl			267(dec)	A[m]	8.94	**IA**

[a]Reported only for the newly synthesized compounds and for compounds already described but lacking those data.
[b]Crystallization solvent(s): A, EtOH; B, EtOH + H_2O; C, DMF; D, DMF + H_2O; E, MeOH; F, AcOEt.
[c]Binding data from the displacement of [^{3}H]-flunitrazepam.
[d]Intrinsic efficacy from [^{35}S]TBPS binding assay (see text and Figure 2). AG, agonist; IA, inverse agonist.
[e]Ref 30.
[f]Ref 25; pIC_{50} has been redeterminated for molecules $\mathbf{4}_2$ and $\mathbf{4}_3$. Compounds $\mathbf{4}_2$, $\mathbf{4}_3$, and $\mathbf{4}_4$ are better known as CGS-8216, CGS-9896, and CGS-9895, respectively.
[g]Ref 24.
[h]N_5-Methyl derivatives.
[i]Ref 27.
[j]Ref 28.
[k]Ref 31.
[l]Ref 32.
[m]Crystallized with *n*-water molecules; *n* was determined by Karl Fischer method.

The known compounds CGS-8216 ($\mathbf{4}_2$) and CGS-9896 ($\mathbf{4}_3$) were also resynthesized as biological standards and their physical and spectral properties are consistent with literature values.[21]

The hydroxyderivatives ($\mathbf{4}_{15}$), and ($\mathbf{4}_{48}$) were conveniently obtained from the corresponding methoxyderivatives ($\mathbf{4}_{14}$), and ($\mathbf{4}_{47}$) respectively, by hydrolysis with 48% HBr in glacial acetic acid.

Catalytic reduction of compounds ($\mathbf{4}_{12}$), ($\mathbf{4}_{39}$) and ($\mathbf{4}_{45}$) afforded the target aminoderivatives ($\mathbf{4}_{13}$), ($\mathbf{4}_{40}$) and ($\mathbf{4}_{46}$), respectively.

Biochemistry

The compounds listed in Table 1 were tested for their ability to displace [^{3}H]flunitrazepam binding from rat brain membranes. The pIC_{50} values reported in Table 1 have been normalized using CGS-8216 ($\mathbf{4}_2$) and CGS-9896 ($\mathbf{4}_3$) as reference compounds. Also literature data (Table 1) were normalized by the same method. The intrinsic activity of some selected compounds in enhancing $GABA_A$ receptor function was evaluated by measuring their efficacy in reducing ^{35}S-*t*-butylbicyclophosphorotionate (^{35}S-TBPS) binding in unwashed membranes (where GABA is present) from rat cerebral

cortex. In fact, by measuring ^{35}S-TBPS binding in the above membrane preparation it is possible to characterize the pharmacological profile (agonist, inverse agonist, antagonist) as well as the efficacy of different benzodiazepine receptor ligands at the $GABA_A$ receptor level.[33–35]

Results and discussion

In agreement with and as integration of previous findings (see Figure 1 for the sake of clarity) the following general remarks on SAR can be made:

- The presence of an aryl or heteroaryl substituent at N_2 (occupation of the L_1–L_2 regions) was crucial for a high affinity (see the low activity of compds $\mathbf{4}_1$ and $\mathbf{4}_{30}$).
- The substitution at N_5 with a methyl group strongly diminished the affinity (compare $\mathbf{4}_2$ and $\mathbf{4}_3$ versus $\mathbf{4}_5$ and $\mathbf{4}_6$: elimination of an HB with A_2)
- *Ortho*-substitution at the 2-phenyl ring reduced the activity (partial occupation of the 'sterically unaccessible' S_1 region) with the notable exception of the fluoro derivative $\mathbf{4}_{32}$, which, in contrast, presented an activity slightly higher than the unsubstituted congener $\mathbf{4}_{31}$. This result may be explained considering both the small dimensions of the fluorine atom and its possible involvement in a three-centered hydrogen bonding with the N_1 atom and a hydrogen bond donor in the receptor, as recently hypothesized in other regions for similar BzR ligands.[37]
- *Para*-substitution at the 2-phenyl ring afforded congeners more active than the corresponding *meta* analogs with two clear exceptions, the amino (compare $\mathbf{4}_{46}$ versus $\mathbf{4}_{40}$) and the fluoro (compare $\mathbf{4}_{44}$ versus $\mathbf{4}_{38}$) derivatives. The *para*-carboxy substituent always caused a drastic decrease of activity (see $\mathbf{4}_{20}$, $\mathbf{4}_{25}$, and $\mathbf{4}_{65}$).
- The replacement of the 2-phenyl ring with azaheterocycles gave interesting results; higher affinity arise for the 8-cC_6H_{11} and 8-nC_4H_9 substituted congeners (compare $\mathbf{4}_{26}$ versus $\mathbf{4}_{24}$ and $\mathbf{4}_{19}$ versus $\mathbf{4}_{23}$ and $\mathbf{4}_{21}$) whereas a lower affinity was detected for the 7,8,9-trimethoxy substituted congeners (compare $\mathbf{4}_{64}$ versus $\mathbf{4}_{66}$ and $\mathbf{4}_{67}$). The 2-pyridyl congeners were generally more active than 2-pyrimidyl and 2-pyrazinyl ones.
- Substitution at position 6 was less tolerated than in positions 7, 8 and 9. Most likely, the formation of an efficient hydrogen bond of the N_5-H group with an HB acceptor group in the receptor (A_2) might be prevented or limited by the presence of bulky groups at position 6. A similar effect was very recently reported at the same topological position for imidazo[1,5]quinoxalin-4-ones.[38]
- Mono substitution at the quinoline ring afforded ligands more active than those deriving from multiple substitutions. The substitution at position 9, in particular, gave the most satisfactory results.

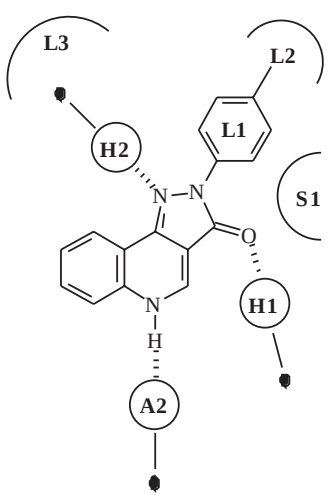

Figure 1. Proposed pharmacophore model[17] for BzR. The main binding sites for pyrazoloquinoline CGS-8216 ($\mathbf{4}_2$) are indicated as H_1 and H_2 (HB donor sites), A_2 (HB acceptor site), L_1 and L_2 (hydrophobic sites). L_3 is another lipophilic region reached by the 5-phenyl ring of classical benzodiazepines. S_1 represents a sterically unaccessible region. Binding to H_2 and A_2 is not necessary for inverse agonist activity.[36]

Quantitative structure–activity relationship (QSAR) studies

In order to gain useful indications at a quantitative level on the topography of the BzR all around the molecular skeleton of PQ, a QSAR study by the classical Hansch approach[39] was carried out. Recently, an extensive review on the quantitative structure–activity relationships of several classes of BzR ligands, not inclusive of PQ compounds, was carried out by Hansch himself.[40] In our case, the lack of parameters for some substituent in the data set and the presence of vicinal polisubstituted congeners, quite difficult to correctly parametrize, prevented a safe application of the classical QSAR approach to the overall data set. The Hansch approach, on the other hand, is well suited for congeneric series and for this reason our QSAR analysis was limited to the 8 and 6 substituted 2-aryl congeners and to the *meta* and *para* substituted congeners of the 8-OCF_3 derivative $\mathbf{4}_{31}$. A multiple regression analysis with cross-validation was performed on the binding data of Table 1 using the chemical descriptors listed in Table 2. The electronic and hydrophobic effects of substitutents were assessed by the Hammett (σ) and Hansch (π) substituent constants,

Table 2. Substituent parameters used for the derivation of regression eqs (1)–(3)[a]

Substituent	σ[b]	π	MR[c]	vW	L	B_1	B_5
H	0.00	0.00	0.10	0.08	2.06	1.00	1.00
OCF_3	0.35	1.04	0.79	1.60	4.57	1.35	3.61
$n.C_4H_9$	−0.16	2.13	1.96	3.12	6.17	1.52	4.54
cC_6H_{11}	−0.22	2.51	2.67	4.26	6.17	1.91	3.49
Cl	0.23 (0.37)	0.71	0.60	1.07	3.52	1.80	1.80
OCH_3	−0.27	−0.02	0.79	1.49	3.98	1.35	3.07
OBn	−0.23	1.66	3.17	4.95	8.20[d]	1.61[d]	4.44[d]
OH	−0.37	−0.67	0.28	0.49	2.74	1.35	1.93
F	0.06 (0.34)	0.14	0.09	0.36	2.65	1.35	1.35
OC_2H_5	−0.24	0.38	1.25	2.42	4.80	1.35	3.36
CH_3	−0.17 (−0.07)	0.56	0.57	1.01	2.87	1.52	2.04
CF_3	0.54	0.88	0.50	1.11	3.30	1.99	2.61
Br	0.23 (0.39)	0.86	0.89	1.32	3.82	1.95	1.95
NO_2	0.78 (0.71)	−0.28	0.74	1.20	3.44	1.70	2.44
NH_2	−0.66 (−0.16)	−1.23	0.54	0.67	2.78	1.35	1.97

[a]Data taken from ref 41. See text for the significance of parameter symbols.
[b]Values for *meta* substituents are reported in parentheses.
[c]Scaled by 0.1.
[d]Referring to the benzyloxy group in a planar conformation.

whereas the molar refractivity (MR), the van der Waals volume (vW) and the STERIMOL Verloop parameters L, B_1, and B_5 were employed to model bulkiness and polarizability effects.[41]

The following equations were derived:

$$\begin{gathered}8\ \text{substituted},\ 2-\text{phenyl congeners}\\ \mathrm{pIC_{50}} = -0.32(\pm0.10)vW + 9.63(\pm0.28)\\ n = 9,\ r^2 = 0.883,\ Q^2 = 0.745,\ s = 0.208, F = 52.77\end{gathered} \quad (1)$$

$$\begin{gathered}6\ \text{substituted},\ 2-\text{phenyl congeners}\\ \mathrm{pIC_{50}} = -2.63(\pm0.47)vW + 9.35(\pm0.45)\\ n = 5,\ r^2 = 0.912,\ Q^2 = 0.801,\ s = 0.543,\ F = 31.23\end{gathered} \quad (2)$$

$$\begin{gathered}8-\mathrm{OCF_3},\ 2-\text{phenyl substituted congeners}\\ \mathrm{pIC_{50}} = -1.40(\pm0.29)\sigma - 1.55(\pm0.42)MR + 9.46(\pm0.24)\\ n = 15,\ r^2 = 0.803,\ Q^2 = 0.723,\ s = 0.418,\ F = 24.51\end{gathered} \quad (3)$$

In eqs (1)–(3), n is the number of compounds, r and Q are the correlation coefficient and the cross-validated correlation coefficient, respectively; s is the standard deviation; F is the F statistic. Eqs (1)–(3) present good statistics both in terms of fitting and predictive power. Eqs (1) and (2) point out a significant steric hindrance on both 8 and 6 positions. The negative coefficient with vW is much higher in eq (2) than in eq (1) and this suggests a stronger steric effect in position 6, which may limit the formation of an HB of the N_5-H group. The negative sign with σ and MR in eq (3) indicates that the interaction of the 2-phenylsubstituted ring at the L_1 (L_2) receptor regions is favoured by small electron donor substituents at both *meta* and *para* positions. An equation of comparable statistical value can be obtained by substituting MR with vW.

The lipophilic character of the substituents on the phenyl ring seems to play no or marginal role in the receptor-ligand interaction and this casts some doubt about the lipophilic nature assigned in several pharmacophore models to the L_2 receptor regions.

In addition eq (3) evidences a limited steric accessibility of the L_2 region, as noticed also in our previous study.[20] Finally, eq (3) suggests that a π–π stacking interaction,[42] in which the substituted phenyl ring acts as π electron donor, might take place at the L_1 receptor region.

Structure–efficacy relationships: preliminary results

The most used in vitro methods to predict the intrinsic activity of a BzR ligand are the GABA ratio (IC_{50} without GABA/IC_{50} with GABA) and the binding assay with [^{35}S]TBPS.

To estimate the antagonist or inverse agonist activity of BzR ligands **4**, we decided to use the binding assay with [^{35}]TBPS which is considered one of the best biochemical tool, sometimes superior to GABA shift,[43,44] to

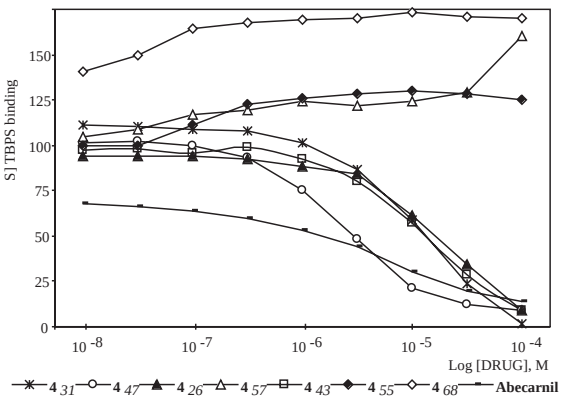

Figure 2. Effects of modulation of the indicated PQ **4** and Abecarnil on [^{35}S]TBPS binding in rat cerebral cortex.

evaluate the intrinsic activity of both positive and negative modulator of $GABA_A$ receptor complex.[45] Accordingly, GABA agonists decrease the total number of [^{35}S] TBPS binding sites in a dose-dependent manner whereas an opposite effect can be seen for GABA inverse agonists.[33–35,41] No significant changes have been observed for antagonists.

In this preliminary phase of our study on structure–efficacy relationships we limited our analysis to seven compounds, which were chosen to test mainly the effect of the *para*-substitution at the 2-phenyl ring (as originally made with CGS congeners)[21] and of the replacement of the phenyl ring with nitrogen heterocycles. The results obtained are listed on the last column of Table 1 and represented graphically in Figure 2.

Unlike what has been found in the original PQ series (compds $\mathbf{4}_2$, $\mathbf{4}_3$ and $\mathbf{4}_4$)[21], no change in the functional effects on BzR was observed for the corresponding 8-OCF_3 congeners $\mathbf{4}_{31}$, $\mathbf{4}_{43}$, and $\mathbf{4}_{47}$, all eliciting a clear agonist activity.

That was unexpected since also in our recent investigation on the BzR activity of pyridazino-indolones, a net influence of the nature of *para*-substituents on the intrinsic activity has been detected.[19,20]

With the only exception of compound $\mathbf{4}_{26}$, the presence of an heterocyclic substituent at position 2 (compds $\mathbf{4}_{55}$, $\mathbf{4}_{57}$, and $\mathbf{4}_{68}$) elicited an inverse agonist activity. These findings are quite interesting and in good agreement with those obtained for 2-thienyl substituted PQs.[22,23] Further investigations to evaluate whether the presence of a nitrogen heterocycles at position 2 may induce an inverse agonist activity and how such an activity may be modulated or reversed by the nature (and position?) of other substituents in the quinoline ring are warranted.

Conclusions

Despite the relatively high number of synthesized compounds, only few of them displayed an affinity higher than the parent compound CGS-8216 ($\mathbf{4}_2$). In fact, as clearly evidenced in the SAR and QSAR analyses discussed herein, most of the substitutions, both on quinoline and 2-phenyl rings, were not tolerated primarily because of steric reasons. Evidently, the lead compound CGS-8216 ($\mathbf{4}_2$) had already an ideal chemical structure, in terms of spatial disposition of the putative pharmacophoric elements and overall physicochemical properties, to engage an efficient, strong interaction with the BzR binding sites. However, PQ derivatives bearing small substituents in definite positions, such as the fluoro derivatives $\mathbf{4}_{11}$ and $\mathbf{4}_{32}$, the hydroxy derivatives $\mathbf{4}_{15}$, $\mathbf{4}_{48}$, and $\mathbf{4}_{49}$ and the amino derivative $\mathbf{4}_{40}$, showed an outstanding affinity.

It is worth noting that the very active 8-OCF_3 congeners $\mathbf{4}_{40}$ and $\mathbf{4}_{48}$ as well as congener $\mathbf{4}_{15}$ contain highly hydrophilic substituents (OH and NH_2) and this cast some doubt on the supposedly lipophilic character of the L_2 pocket of the BzR receptors as claimed so far. Moreover, that pocket should have a limited steric accessibility as clearly pointed out by eq (3), in good agreement with a previous QSAR study on PI.[20] The strong substituent electronic effect on the BzR affinity is also indicated by eq (3): electrondonor substitutents on the 2-phenyl ring favor the ligand-receptor binding which might take place trough a π–π stacking interaction.[42]

Conversely, no electronic effect has been detected in the QSAR analysis of PI analogs and this could be due also to some different orientation of the phenyl ring in PQ and PI, as found by molecular modelling studies.[20]

A preliminary analysis of the structure–intrinsic efficacy relationships, based at the moment only on very few ligands, indicated that small structural modifications on PQ may evoke a diverse BzR activation. However, compds $\mathbf{4}_{31}$, $\mathbf{4}_{43}$, and $\mathbf{4}_{47}$ bearing H, Cl, and OCH_3 substituents at the para position of 2-phenyl ring, all elicited an agonistic activity, unlike the corresponding congeners of the PQ and PI series, for which a different intrinsic activity has been found.[19–21] These unexpected results deserve further investigation. A study on the influence of the 8-OCF_3 group on the peculiar molecular properties of compds $\mathbf{4}_{31}$, $\mathbf{4}_{43}$, and $\mathbf{4}_{47}$ compared to the corresponding 8-unsubstituted PQ and PI congeners, is underway and should help clarifying this point.

An inverse agonist profile was instead observed for compounds bearing a nitrogen heterocyclic substituent at position 2. This structural feature however seems necessary, though not sufficient, to evoke such an activity

since compd $\mathbf{4}_{26}$ which does bear a 2-heterocyclic substituent, was a BzR agonist. The testing of further, and eventually newly designed PQs, has been planned to fully evaluate the influence of the nature and position of the substituents in the quinoline ring on the intrinsic activity of congeners bearing a 2-heterocyclic substituent. Also in this case, theoretical and a molecular modelling studies based on quantomechanical and 3-D QSAR methods[47] should lead to a better interpretation of the structure–efficacy relationships data.

Chemical Experimental

Melting points were determined on a Büchi 510 capillary melting point apparatus and are uncorrected. Elemental analyses were performed on a Perkin–Elmer elemental analyzer Mod. 240 and the data for C, H, N are within ±0.40% of calculated values. Spectral analyses (IR and ^{1}H NMR) are consistent with the chemical structures indicated. IR spectra were determined in nujol mull on a Perkin–Elmer 398 or a Perkin–Elmer

Table 3. ^{1}H NMR and IR spectral data of some representative PQ **4**

Compound	^{1}H NMR(δ, ppm, J = Hz)	IR(cm^{-1})
$\mathbf{4}_1$	(DMSO-d_6) δ 7.44–7.67 (m, 3H, H-(7,8,9)), 8.08 (u dd, 1H, H-6, J_{6-7} = 7.8 Hz), 8.51 (s, 1H, H-4), 11.37 (s, 1H, NH, D_2O exchangeable), 12.43 (br s, 1H, NH, D_2O exchangeable)	1654 (CO)
$\mathbf{4}_{10}$	(DMSO-d_6): 4.00 (s, 3H, OCH_3), 7.14 (t, 1H), 7.27 (d, 1H, J_{orto} = 8.0 Hz), 7.38–7.51 (m, 3H), 7.75 (d, 1H, J_{orto} = 8.0 Hz), 8.18 (d, 2H), 8.31 (s, 1H, H-4), 12.24 (br s, 1H, NH, D_2O exchangeable).	1634 (CO)
$\mathbf{4}_{12}$	(DMSO-d_6) δ 7.60 (td, 1H), 7.71–7.85 (m, 2H), 7.95 and 8.03 (2dd, 2H), 8.67 (u dd, 1H), 8.83 (s, 1H, H-4), 9.12 (t, 1H, H-2′), 13.07 (br s, 1H, NH, D_2O exchangeable).	1654 (CO)
$\mathbf{4}_{13}$	(DMSO-d_6) δ 5.13 (br s, 2H, NH_2, D_2O exchangeable), 6.36 (u dd, 1H), 7.03 (t, 1H, H-5′), 7.33 (u dd, 1H), 7.43 (t, 1H, H-2′), 7.53 (td, 1H, H-7), 7.71–7.85 (m, 2H, H-(6,9)), 8.67 (s, 1H, H-4), 12.78 (br s, 1H, NH, D_2O exchangeable).	1654 (CO)
$\mathbf{4}_{18}$	(DMSO-d_6) δ 2.47 (t, 3H, CH_3–CH_2O), 4.16 (q, 2H, CH_3–CH_2O), 7.14 (t, 1PhH), 7.25 (dd, 1H, H-7, J_{7-6} = 9.0 Hz, J_{7-9} = 2.7 Hz), 7.42 (t, 2PhH), 7.55 (d, 1H, H-9, J_{9-7} = 2.7 Hz), 7.64 (d, 1H, H-6, J_{6-7} = 9.0 Hz), 8.21 (dd, 2PhH), 8.61 (s, 1H, H-4), 12.75 (br s, 1H, NH, D_2O exchangeable).	1625 (CO)
$\mathbf{4}_{21}$	(DMSO-d_6) δ 0.94 (t, 3H, CH_3–CH_2–CH_2–CH_2–), 1.36 (st, 2H, CH_3–CH_2–CH_2–CH_2–), 1.66 (qt, 2H, CH_3–CH_2–CH_2–CH_2–), 2.77 (t, 2H, CH_3–CH_2–CH_2–CH_2–), 7.31 (td, 1H, H-5 pyridyl), 7.56 (u dd, 1H, H-7, J_{7-6} = 8.7 Hz), 7.69 (d, 1H, H-6, J_{6-7} = 8.7 Hz), 7.95–8.05 (m, 2H, H-9 and H-4 pyridyl), 8.34 (u dd, 1H, H-3 pyridyl, J_{3-4} = 8.4 Hz), 8.56 (u dd, 1H, H-6 pyridyl), 8.75 (s, 1H, H-4), 12.70 (br s, 1H, NH, D_2O exchangeable).	1670 (CO)
$\mathbf{4}_{28}$	(DMSO-d_6) δ 1.36–1.54 and 1.73–1.88 (2m, 10H, $5CH_2$ cyclohexyl), 2.53–2.72 (m, 1H, CH cyclohexyl), 7.58–7.70 (m, 2H, H-(6,7)), 8.05 (d, 1H, H-9, J_{9-7} = 1.3 Hz), 8.48 and 8.60 (2d, 2H, H-(5,6)pyrazinyl, J_{orto} = 2.6 Hz), 8.77 (s, 1H, H-4), 9.57 (u d, 1H, H-3 pyrazinyl), 12.89 (br s, 1H, NH, D_2O exchangeable).	1665 (CO)
$\mathbf{4}_{32}$	(DMSO-d_6) δ 7.36–7.74 (m, 5H, H-7 and 4PhH), 7.88 (d, 1H, H-6, J_{6-7} = 9.2 Hz), 7.99 (u d, 1H, H-9), 8.85 (s, 1H,H-4), 12.95 (br s, 1H, NH, D_2O exchangeable).	1625 (CO)
$\mathbf{4}_{35}$	(DMSO-d_6) δ 7.37–7.49 (m, 2PhH), 7.73 (dd, 1H, H-7, J_{7-6} = 9.1 Hz, J_{7-9} = 2.3 Hz), 7.88 (d, 1H, H-6, J_{6-7} = 9.1 Hz), 8.13 (u d, 1H, H-9), 8.28 (dt, 1H, H-6′), 8.50 (s, 1PhH), 8.85 (s, 1H, H-4), 13.00 (br s, 1H, NH, D_2O exchangeable).	1633 (CO)
$\mathbf{4}_{45}$	(DMSO-d_6) δ 7.69 (dd, 1H, H-7), 7.82 (d, 1H, H-6, J_{6-7} = 9.0 Hz), 8.03 (u d, 1H, H-9), 8.30 and 8.47 (2d, 4PhH, J_{orto} = 9.5 Hz), 8.85 (s, 1H, H-4), 13.11 (br s, 1H, NH, D_2O exchangeable).	1620 (CO)
$\mathbf{4}_{46}$	(DMSO-d_6) δ 5.03 (br s, 2H, NH_2, D_2O exchangeable), 6.61 (d, 2PhH, J_{orto} = 8.8 Hz), 7.62 (dd, 1H, H-7, J_{7-6} = 9.1 Hz, J_{7-9} = 2.4 Hz), 7.73 (d, 2PhH, J_{orto} = 8.8 Hz), 7.80 (d, 1H, H-6, J_{6-7} = 9.1 Hz), 7.99 (u d, 1H, H-9), 8.69 (s, 1H, H-4), 12.85 (br s, 1H, NH, D_2O exchangeable).	1654 (CO)
$\mathbf{4}_{56}$	(DMSO-d_6) δ 7.38 (u d, 1H, H-7), 7.72 (t, 2H, H-9 and H-5 pyrimidinyl), 8.51 (s, 1H, H-4), 8.86 (u d, 2H, H-(4,6) pyrimidinyl), 12.91 (br s, 1H, NH, D_2O exchangeable).	1655 (CO)
$\mathbf{4}_{63}$	(DMSO-d_6) δ 3.77 (s, 3H, OCH_3), 7.01 (d, 2PhH, J_{orto} = 9.1 Hz), 7.97–8.04 (m, 3H, H-9 and 2PhH), 8.56 (s, 1H, H-4), 13.15 (br s, 1H, NH, D_2O exchangeable).	1635 (CO)
$\mathbf{4}_{65}$	(DMSO-d_6) δ 3.83, 3.88, and 3.97 (3s, 9H, $3OCH_3$), 7.03 (s, 1H, H-6), 7.99 (d, 2PhH, J_{orto} = 8.7 Hz), 8.37 (d, 2PhH, J_{orto} = 8.7 Hz), 8.60 (s, 1H, H-4), 12.60 (br s, 2H, NH and COOH, D_2O exchangeable).	1660 (CO)
$\mathbf{4}_{68}$	(DMSO-d_6) δ 3.82, 3.88, and 3.93 (3s, 9H, $3OCH_3$), 7.04 (s, 1H, H-6), 8.42 and 8.55 (2d, 2H, H-(5,6)pyrazinyl, J_{orto} = 2.5 Hz), 8.62 (s, 1H, H-4), 9.36 (u d, 1H, H-3 pyrazinyl), 12.61 (br s, 1H, NH, D_2O exchangeable).	1660 (CO)

FT-IR 1600 spectrophotometers. ^{1}H NMR spectra were recorded on a Varian XL-200 or AC 200 Bruker instruments. The chemical shifts (δ) are relative to Me_4Si used as internal standard. The following abbreviations were used: s = singlet, d = doublet, t = triplet, dd = double doublet, q = quartet, qt = quintet, st = sextet, m = multiplet, dt = doublet of triplets, td = triplet of doublets, u = unresolved, br = broad. The coupling constants *J* were in hertz. IR and ^{1}H NMR spectral data of some representative PQ derivatives **4** are listed in Table 3.

TLC on silica-gel plates (Merck, 60, F_{254}) was used for purity check and reaction monitoring. Column chromatography on silica-gel (Merck, 70–230 mesh) was applied, when necessary, to isolate and purify the different reaction products.

Some unknown ethyl 4-chloroquinoline-3-carboxylates (**3a**, R = 8-OCF_3; and **3b**, R = 6,7,8-F_3), and two other intermediate compounds (**A**; and **2a**, R = 8-OCF_3), are also described in this section.

Physicochemical properties of the newly synthesized PQs are summarized in Table 1.

Diethyl [(4-trifluoromethoxyanilino)methylene]malonate (A). A mixture of equimolar amounts of 4-(trifluoromethoxy)aniline and diethyl (ethoxymethylene)malonate was heated on a steam bath for 48 h. After cooling at room temperature a solid was obtained, which was purified by crystallization from EtOH–H_2O, mp 76–78° C. Anal. calcd for $C_{15}H_{16}F_3NO_5 \cdot \_H_2O$: C, 50.56; H, 4.81; N, 3.93. Found: C, 50.20; H, 4.42; N, 3.81. ^{1}H NMR ($CDCl_3$) δ 1.28–1.40 (2t, 6H, 2CH_3–CH_2O), 4.18–4.35 (2q, 4H, 2CH_3–CH_2O), 7.11–7.25 (m, 4PhH), 8.45 (d, 1H, CH = C, $J_{CH=NH}$ = 13.1); 11.03 (d, 1H, NH, $J_{NH=CH}$ = 13.1, D_2O exchangeable).

Ethyl 6-trifluoromethoxy-4-hydroxyquinoline-3-carboxylate (2a). **A** (3 g, 8.6 mmol) was refluxed in Dowtherm A (30 mL) at 260–280 °C for 40 min. After cooling to room temperature, the reaction mixture was diluted with Et_2O to give a white precipitate, which was filtered, washed with Et_2O, and purified by crystallization from DMF, mp > 300° C. Anal. calcd for $C_{13}H_{10}F_3NO_4$: C, 51.83; H, 3.35; N, 4.65. Found: C, 51.79; H, 3.24, N, 4.50. ^{1}H NMR (DMSO-d_6) δ 1.26 (t, 3H, CH_3–CH_2O), 4.20 (q, 2H, CH_3–CH_2O), 7.67–7.78 (m, 2H, H-(7,8)), 7.97 (u d, 1H, H-5), 8.59 (s, 1H, H-2), 11.78 (br s, 1H, NH/OH, D_2O exchangeable).

Ethyl 6-trifluoromethoxy-4-chloroquinoline-3-carboxylate (3a). To ethyl 6-trifluoromethoxy-4-hydroxyquinoline-3-carboxylate (**2a**) (10 g, 46 mmol) was added $POCl_3$ (25 mL). The mixture was heated in a sand bath at 135° C for 45 min. After cooling, the solution was poured onto ice water and made basic with sodium carbonate. The resulting precipitate was filtered off and purified by steam distillation (mp 58–60° C). Anal. calcd for $C_{13}H_9ClF_3NO_3 \cdot \_H_2O$: C, 48.18; H, 2.95; N, 4.32. Found: C, 48.19; H, 2.76; N, 4.29. ^{1}H NMR ($CDCl_3$) δ 1.46 (t, 3H, CH_3–CH_2O), 4.51 (q, 2H, CH_3–CH_2O), 7.70 (dd, 1H, H-7, $J_{7\text{-}8}$ = 9.5, $J_{7\text{-}5}$ = 2.8), 8.20 (d, 2H, H-(5,8), $J_{8\text{-}7}$ = 9.5), 9.21 (s,1H, H-2).

Ethyl 6,7,8-trifluoro-4-chloroquinoline-3-carboxylate (3b). Compound **3b** was obtained from the 6,7,8-trifluoro-4-hydroxyquinoline-3-carboxylate and $POCl_3$ under the same conditions described above for compound **3a**. It was crystallized from ethanol (mp 106–108° C). Anal. calcd for $C_{12}H_7ClF_3NO_2$: C, 49.76; H, 2.45; N, 4.84. Found: C, 49.64; H, 2.40; N, 4.82. ^{1}H NMR ($CDCl_3$) δ 1.46 (t, 3H, CH_3–CH_2O), 4.51 (q, 2H, CH_3–CH_2O), 7.94–8.05 (m, 1H, H-5), 9.21 (s, 1H, H-2).

General procedure for the preparation of 2,5-dihydropyrazolo[4,3-*c*]quinolin-3-(3*H*)-ones (4_1, 4_{30}) and 2-aryl-2,5-dihydropyrazolo[4,3-*c*]quinolin-3-(3*H*)-ones ($4_{7–12}$, 4_{14}, 4_{18}, 4_{20}, 4_{25}, $4_{30–39}$, $4_{41–45}$, 4_{47}, $4_{59–65}$). Title compounds were prepared from ethyl 4-chloroquinoline-3-carboxylates (**3**) and hydrazine hydrate 85% or the appropriate arylhydrazines by the general method described in ref 26 (Table 1).

General procedure for the preparation of 2-heteroarylpyrazolo[4,3-*c*] quinolin-3-(3*H*)-ones ($4_{21–23}$, $4_{26–28}$, $4_{55–57}$, $4_{66–68}$). To a solution of ethyl 4-chloroquinoline-3-carboxylate (**3**) (4 mmol) in absolute ethanol, the appropriate α-(*N*)-heterocyclichydrazine (4.4 mmol) was added. The reaction mixture was stirred at room temperature for a variable time (1–12 h) until a yellow solid precipitated. It was filtered off and purified by crystallization from a suitable solvent (Table 1).

In some instances (compds $4_{21–23}$, 4_{27}, and 4_{56}), this reaction afforded the uncyclized intermediates, ethyl 4-*N,N′*-heteroarylhydrazinoquinolin-3-carboxylates, whose chemical structures were confirmed by ^{1}H NMR. They were cyclized to the corresponding pyrazoloquinolinones (**4**), without further purification, by adding an aqueous solution of 1 N sodium hydroxyde (2.2 mmol) to a suspension of compound (2 mmol) in ethanol. After 1 h stirring at room temperature, the solution was acidified with diluted acetic acid and the resulting precipitate was filtered off, washed with water and purified by crystallization (Table 1).

General procedure for the preparation of 2-(aminophenyl)pyrazolo[4,3-*c*]quinolin-3-(3*H*)-ones (4_{13}, 4_{40}, 4_{46}). To a suspension of the appropriate 2-(nitrophenyl)pyrazolo[4,3-*c*]quinolin-3-(3*H*)-one (4_{12}, 4_{39}, 4_{45}) (150 mg) in absolute ethanol (150 mL), 10% Pd/C was added. The

mixture was hydrogenated in a Parr apparatus at 40 psi at room temperature. After 2–4 h the catalyst was filtered off and the solution taken to dryness gave a solid, which was crystallized from a suitable solvent (Table 1).

General procedure for the preparation of 2-(4′-hydroxyphenyl)pyrazolo[4,3-*c*]quinolin-3-(3*H*)-ones ($\mathbf{4}_{15}$, $\mathbf{4}_{48}$). A mixture of the appropriate methoxy-pyrazoloquinolinone ($\mathbf{4}_{14}$, $\mathbf{4}_{47}$) (0.8 mmol) in glacial acetic acid (~4 mL), was stirred under nitrogen for 10 min and then heated until an homogenous solution was obtained. Aqueous hydrogen bromide (48%, 10 mL) was added and the reaction mixture was refluxed on an oil bath, and monitored to completeness by TLC (chloroform:methanol, 18:2 as eluent) for a variable time [3 h for ($\mathbf{4}_{15}$); 1 h for ($\mathbf{4}_{48}$)]. After cooling under nitrogen, the reaction mixture was diluted with water (~70 mL) to give a precipitate, which was collected, washed with water, and crystallized (Table 1).

Biochemical Experimental

Chemicals

[^{3}H]Flunitrazepam (New England Nuclear, Boston, U.S.A.) had a specific activity of 84.3 Ci/mmol and a radiochemical purity > 74.6%. [^{35}S]TBPS (New England Nuclear, Boston) had a specific activity of 72.4 Ci/mmol.

Animals

Male Sprague–Dawley rats (Charles River, Como, Italy) with body weights of 150 to 200 g were kept under a 12 h light/dark cycle at a temperature of 23 ± 2 °C and 65% humidity. Upon arrival at the animals facilities there was a minimum of seven days acclimatization, during which the animals had a free access to food and water. The animals were killed by decapitation and the brains were rapidly removed, the cerebral cortex was dissected out and was used for the measurement of [^{3}H]Flunitrazepam and [^{35}S]TBPS binding.

[^{3}H] Flunitrazepam binding

Cerebral cortices were homogenized in 50 volumes of ice-cold 50 mM Tris-HCl buffer with a polytron PT 10 (setting 5, for 20 s), centrifuged at 48,000 g for 10 min and washed one time. The pellet was resuspended in 50 volumes of 50 mM Tris-HCl buffer (pH 7.40) and aliquots of 400 µL tissue homogenate (400–500 µg of protein) were incubated in the presence of [^{3}H] Flunitrazepam at a final concentration of 0.5 nM, in a total incubation volume of 1000 µL. The compounds were dissolved in dimethylsulfoxide and serial dilutions were made up in dimethylsulfoxide and added in 5 µL aliquots. After 60 min incubation at 4° C, the assay was terminated by rapid filtration through glass-fiber filter strips (Whatman GF/B). The filters were rinsed with 2×4 mL ice-cold 50 mM Tris-HCl buffer with a cell Harvester filtration manifold (Model M-24, brandel) and transferred in plastic minivials with 3 mL scintillation fluid (AtomLight, New England Nuclear). Six to eight concentrations of the samples in triplicate were used to determine the IC_{50} values. Nonspecific binding was determined as the binding in the presence of 5 µM diazepam and was 85 to 90% of the total 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 g for 20 min and resuspended in 50 volumes of 50 mM Tris-citrate buffer without NaCl. The [^{35}S]TBPS was determined in a final volume of 1000 µL consisting of 400 µL tissue homogenate (400–500 µg protein), 100 µL 2 nM [^{35}S]TBPS, 100 µL 0.2 M NaCl, 5 µL drugs dissolved as describe above or solvent (total and nonspecific samples). The incubation (25° C) were started by the addition of tissue homogenate and terminated by rapid filtration through glass-fiber filter strips (Whatman GF/B) with a cell Harvester filtration manifold (Model M-24, brandel). The filters were rinsed with 2×4 mL ice-cold 50 mM Tris-citrate buffer. Non-specific binding was defined as binding in the presence of 100 µM picrotoxin, and represented about 10% of total binding.

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References

1. Kerr, I. B.; Ong, *J. Med. Res. Rev.* **1992**, *12*, 593.

2. *GABA and Benzodiazepine Receptors*; Squires, R., Ed.; CRC: Boca Raton, FL, 1988; Vols. 1, 2.

3. Bernard, E. A. In *$GABA_A$ Receptor and Anxiety. From Neurobiology to Treatment*; Biggio, G.; Sanna, E.; Costa, E., Eds.; Raven: New York, 1995; pp 1–16.

4. Haefely, W.; Kyburz, E.; Gerecke, M.; Mohler, H. *Adv. Drug Res.* **1985**, *14*, 165.

5. Davies, A. P.; Hanna, C. M.; Hales, T. G.; Kirkness, F. E. *Nature* **1997**, *385*, 820.

6. Smith, G. B.; Olsen, W. R. *Trends in Pharmac. Sci.* **1995**, *16*, 162.

7. De Lorey, T. M.; Olsen, R. W. *J. Biol. Chem.* **1992**, *262*, 6747.

8. Macdonald, R. L.; Olsen, R. W. *Annu. Rev. Neurosci.* **1994**, *17*, 569.

9. McKernan, R. M.; Whiting, P. J. *TINS* **1996**, *19*, 139.

10. Borea, P. A.; Gilli, G.; Bertolasi, V.; Ferretti, V. *Mol. Pharmacol.* **1987**, *31*, 334.

11. Tebib, S.; Bourguignon, J. J.; Wermuth, C. G. *J. Comput. Aid. Mol. Des.* **1987**, *1*, 1534.

12. Allen, M. S.; Tan, Y. C.; Trudell, M. L.; Schindler, L. R.; Martin, M. J.; Schulz, C.; Hagen, T. J.; Koehler, K. F.; Codding, P. W.; Skolnick, P.; Cook, J. M. *J. Med. Chem.* **1990**, *33*, 2343.

13. Villar, H. O.; Davies, M. F.; Loew, G. H.; Maguire, P. A. *Life Sci.* **1991**, *48*, 593.

14. Diaz-Arauzo, H.; Koehler, K. F.; Hagen, T. J.; Cook, J. M. *Life Sci.* **1991**, *49*, 207.

15. Allen, M. S.; Skolnick, P.; Cook, J. M. *J. Med. Chem.* **1992**, *35*, 368.

16. Schove, L. T.; Perez, J. J.; Loew, G. H. *Bioorg. Med. Chem.* **1994**, *2*, 1029.

17. Zhang, F.; Koehler, K. F.; Zhang, P.; Cook, J. M. *Drug Des. Dis.* **1995**, *12*, 193.

18. Gardner, C. R. *Progr. Neuro-Psychopharmacol. Biol. Psychiatry* **1992**, *16*, 755.

19. Campagna, F.; Carotti, A.; Casini, G.; Palluotto, F.; Genchi, G.; De Sarro, G. B. *Bioorg. Med. Chem.* **1993**, *1*, 437.

20. Palluotto, F.; Carotti, A.; Casini, G.; Campagna, F.; Genchi, G.; Rizzo, M.; De Sarro, G. B. *Bioorg. Med. Chem.* **1996**, *4*, 2091.

21. Yokoyama, N.; Ritter, B.; Neubert, A. D. *J. Med. Chem.* **1982**, *25*, 337.

22. Takada, S.; Shindo, H.; Sasatani, T.; Chomei, N.; Matsashita, A.; Masami, E.; Kawasaki, K.; Murata, S.; Takahara, Y.; Shintaku, H. *J. Med. Chem.* **1988**, *31*, 1738.

23. Shindo, H.; Takada, S.; Murata, S.; Masami, E.; Matsushita, A. *J. Med. Chem.* **1989**, *32*, 1213.

24. Wong, G.; Zi-Qiang, G.; Fryer, R.I.; Skolnick, P. *Med. Chem. Res.* **1992**, *2*, 217.

25. Fryer, R. I.; Zhang, P.; Rios, R.; Gu, Z. Q.; Basile, A. S.; Skolnick, P. *J. Med. Chem.* **1993**, *36*, 1669.

26. Schove, L. T.; Perez, J. I.; Maguire, P. A.; Loew, G. I. *Med. Chem. Res.* **1994**, *4*, 307.

27. Savini, L.; Massarelli, P.; Pellerano, C.; Fiorini, I.; Bruni, G.; Romeo, M. R. *Il Farmaco* **1993**, *48*, 65.

28. Savini, L.; Massarelli, P.; Corti, P.; Pellerano, C.; Bruni, G.; Romeo, M. R. *Il Farmaco* **1993**, *48*, 1675.

29. Kaslow, E. E.; Clark, W. R. *J. Org. Chem.* **1953**, *18*, 55.

30. Pietrogrande, M. C.; Borea, P. A.; Biagi, G. L. *J. Chromat.* **1988**, *447*, 404.

31. Leeson, L. J. (Ciba–Geigy Corp.) U.S. Patent 4,758,427; 19 July 1988; *Chem. Abstr.* **1989**, *110*, 199215s.

32. Yokoyama, N. (Ciba–Geigy A.-G.), Eur. Pat. Appl. 22,078; 7 Jan. 1981; *Chem. Abstr.* **1981**, *95*, 7278s.

33. Squires, R. F.; Casida, J. E.; Richardson, M.; Saederup, E. *Mol. Pharmacol.* **1983**, *23*, 326.

34. Gee, K.; Lawrence, L. J.; Yamamura, H. I. *Mol. Pharmacol.* **1986**, *30*, 218.

35. Concas, A.; Serra, M.; Atsoggiu, T.; Biggio, G. *J. Neurochem.* **1988**, *51*, 1868.

36. Fryer, R. I.; Rios, R.; Zhang, P.; Gu, Z.; Wong, G.; Basile, S. A.; Skolnick, P. *Med. Chem. Res.* **1993**, *3*, 122.

37. Colotta, V.; Catarzi, D.; Varano, F.; Filacchioni, G.; Cecchi, L. *J. Med. Chem.* **1996**, *39*, 2915.

38. Mickelson, J. W.; Jacobsen, E. J.; Carter, D. B.; Im, H. K.; Im, W. B.; Schreur, P. J. K. D.; Sethy, V. H.; Tang, A. H.; McGee, J. E.; Petke, J. D. *J. Med. Chem.* **1996**, *39*, 4654.

39. Hansch, C.; Leo, A. *Exploring QSAR*; ACS: Washington, DC, 1995; Vol. 1.

40. Hadjipavlon-Litina, D.; Hansch, C. *Chem. Rev.* **1994**, *94*, 1483.

41. Hansch, C.; Leo, A.; Hoeckman, D. *Exploring QSAR*; ACS: Washington, DC, 1995; Vol. 2.

42. Jones, G. B.; Chapman, B. J. *Synthesis* **1995**, 475.

43. Trapani, G.; Franco, M.; Latrofa, A.; Carotti, A.; Genchi, G.; Serra, M.; Biggio, G.; Liso, G. Eur. *J. Med. Chem.* **1996**, *31*, 575 and references therein.

44. Ananthan, S.; Clayton, D. S.; Ealick, E. S.; Wong, G.; Evoniuk, E. G.; Skolknick, P. *J. Med. Chem.* **1993**, *36*, 479.

45. Squires, R. F.; Casida, J. E.; Richardson, M.; Saedrup, E. *Mol. Pharmacol.* **1983**, *23*, 326.

46. Biggio, G.; Concas, A.; Corda, M. G.; Giorgi, O.; Sanna, E.; Serra, M. *Pharmacol. Ther.* **1990**, *48*, 121.

47. *3D-QSAR in Drug Design*, Kubiniy, H., Ed.; Escom: Leiden, 1993.