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1-Substituted pyrazolo[1,5-*c*]quinazolines as novel Gly/NMDA receptor antagonists: Synthesis, biological evaluation, and molecular modeling study

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Abstract—A new set of 5,6-dihydro-pyrazolo[1,5-*c*]quinazoline-2-carboxylates (**2**–**18**), bearing different substituents (COOEt, Cl, Br, CH_3, and COOH) at position-1, were synthesized in order to investigate the influence of various groups at this specific position on Gly/NMDA receptor affinity and/or selectivity. All the herein reported compounds were evaluated for their binding at the Gly/NMDA, AMPA, and KA receptors. Some selected compounds were also tested for their functional antagonistic activity at both the AMPA and NMDA receptor-ion channels.
The results obtained in this study have highlighted that a C-1 lipophilic substituent on the pyrazolo[1,5-*c*]quinazoline-2-carboxylate core shifts selectivity toward the Gly/NMDA receptor, while a C-1 anionic carboxylate residue is able to increase affinity toward this receptor subtype. In particular, the 2-carboxylic acids **15** and **16**, bearing a chlorine atom at position-1, are not only potent (K_i = 0.18 and 0.16 μM, respectively), but also highly Gly/NMDA versus AMPA selective (selectivity ratio > 500). Furthermore, the 1,2-dicarboxylic acids **13** and **14** are endowed with the highest Gly/NMDA receptor binding activity (K_i = 0.09 and 0.059 μM, respectively), among the pyrazoloquinazoline series of derivatives.
A molecular modeling study has been carried out to better understand receptor affinity and selectivity of these new pyrazoloquinazoline derivatives.

1. Introduction

Glutamate, the most important excitatory neurotransmitter in mammalian brain, acts through the activation of either ionotropic (iGluRs) (NMDA, AMPA, and Kainate subtypes) or metabotropic (mGluRs) receptors.[1,2]

It is now widely accepted that over-activation of iGluRs, especially of the NMDA receptor, is one of the major causes of Ca^{2+} overload in cells, potentially leading to cell damage and death. This excitotoxic mechanism is thought to be involved in several neurodegenerative disorders, such as Parkinson's, Huntington's, and Alzheimer's diseases, as well as in brain ischemia and epilepsy. Therefore, a pharmacological intervention with iGluRs antagonists has been suggested to have a potential therapeutic benefit.[1,3,4] In particular, the non-competitive NMDA antagonists acting at the glutamate coagonist glycine binding site on the NMDA receptor complex (Gly/NMDA)[5] have been recognized as potential therapeutic agents for the above mentioned disorders.[6–10] In fact, selective Gly/NMDA receptor antagonists are endowed with a better side-effect profile than competitive and other non-competitive NMDA receptor antagonists.[1,10] Furthermore, it is worth mentioning that increasing evidence supports the hypothesis that the NMDA receptor plays a pivotal role in the

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development and maintenance of pain hypersensitivity. Following such a hypothesis, Gly/NMDA receptor antagonists should also be effective for the treatment of chronic pain.[6]

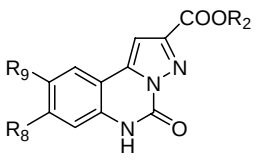
In recent years, some research in our laboratory has been directed toward the synthesis of tricyclic heteroaromatic systems as Gly/NMDA and/or AMPA receptor antagonists.[11–18] Structure–activity relationship (SAR) studies on some of these tricyclic derivatives have pointed out that introduction of suitable substituents on precise positions of the benzofused moiety shifts selectivity toward AMPA or Gly/NMDA receptors.[13,14,16–18]

As a part of this research project, we reported the synthesis and binding activity at Gly/NMDA, AMPA, and kainate (KA) receptors of some 5,6-dihydro-pyrazolo[1,5-*c*]quinazoline-2-carboxylates (**1** PQZ) (Chart 1) bearing various substituents on the benzofused moiety.[17]

The binding results showed that some of these compounds are potent and/or selective AMPA or Gly/NMDA receptor antagonists, depending on the benzofused substitutions. In particular, in accordance with our previously reported data,[13,14,16] the presence of both a chlorine atom at position-8 and a N^3-nitrogen-containing heterocycle at position-9 is required for potent and highly selective AMPA receptor antagonists. Moreover, some Gly/NMDA receptor selectivity is obtained when the benzofused moiety of the PQZ derivatives is unsubstituted or when it bears chlorine atom(s).

Continuing with the study on PQZ derivatives, in the present paper we report the synthesis of a new set of 1-substituted pyrazolo[1,5-*c*]quinazoline-2-carboxylates **2**–**18** (Chart 2), in order to investigate the influence of different groups (COOEt, Cl, Br, CH_3, and COOH) at position-1 of the PQZ tricyclic system on Gly/NMDA receptor affinity and/or selectivity.

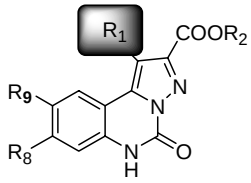
Since our goal was to obtain potent and/or selective Gly/NMDA receptor antagonists, the herein reported pyrazoloquinazoline-2-carboxylates are either unsubstituted on the benzofused moiety or bear chlorine atom(s). Moreover, a molecular modeling study has been carried out to better understand receptor affinity and selectivity of these new pyrazoloquinazoline derivatives.



1 (PQZ)

R_2 = Et, Me, H
R_8 = H, Cl
R_9 = H, Cl, 

Chart 1. Previously reported pyrazolo[1,5-*c*]quinazoline-2-carboxylates.



2-18

R_1 = COOEt, Cl, Br, CH_3, COOH
R_2 = Et, H
R_8, R_9 = H, Cl

Chart 2. C-1 substituted pyrazolo[1,5-*c*]quinazoline-2-carboxylates.

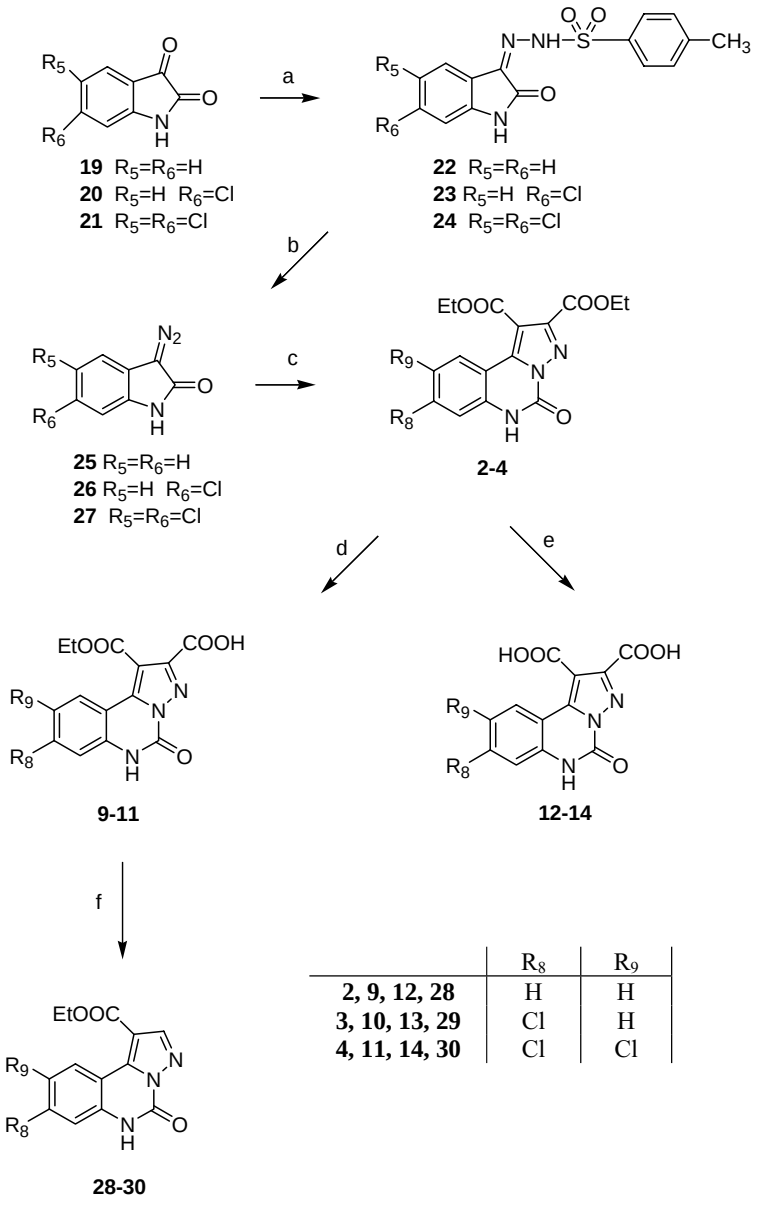
2. Chemistry

Compounds **2**–**18** were synthesized as depicted in Schemes 1–4. The diethyl 1,2-dicarboxylate derivatives **2**–**4** were obtained starting from the commercially available isatine **19** and its 5-chloro- and 5,6-dichloro-analogues **20**–**21**,[19,20] respectively, which when treated with an equimolar amount of *p*-toluenesulfonohydrazide, yielded the corresponding sulfonylhydrazones **22**–**24**[21,22] (Scheme 1). The latter, by hydrolysis in alkaline medium, led to the 3-diazo-1,3-dihydro-indol-2-one derivatives **25**–**27**,[21,22] which yielded the tricyclic diethyl pyrazolo[1,5-*c*]quinazoline-1,2-dicarboxylates **2**–**4** after reaction with an excess of diethyl acetylenedicarboxylate.

Partial hydrolysis of esters **2**–**4** at room temperature in the presence of a hydro-methanolic solution of KOH gave the corresponding 1-ethoxycarbonyl-pyrazoloquinazoline-2-carboxylic acid derivatives **9**–**11**. On the contrary, by refluxing esters **2**–**4** in the presence of a hydro-ethanolic solution of KOH, the corresponding pyrazoloquinazoline-1,2-dicarboxylic acid derivatives **12**–**14**[23] were obtained. The 1-ethoxycarbonyl-2 carboxylic acid structure of compounds **9**–**11** were assigned by transforming them into their corresponding decarboxylate analogues. Thus, upon heating of **9**–**11** at a temperature just over their melting points, the corresponding ethyl 5,6-dihydro-5-oxo-pyrazolo[1,5-*c*]quinazoline-1-carboxylates **28**–**30** were isolated. The 1-ethyl carboxylate structure of **28**–**29** was unambiguously assigned, since the corresponding ethyl pyrazolo[1,5-*c*]quinazoline-2-carboxylate regio-isomers had already been obtained by an univocal synthesis.[17] The 1-ethyl carboxylate structure of **30** was attributed on the basis of the ^{1}H NMR signal of the pyrazole proton: in fact, the chemical shift value of this proton (8.45 ppm) is very close to that of the H-2 proton of **28** and **29** (8.43, 8.44 ppm, respectively).

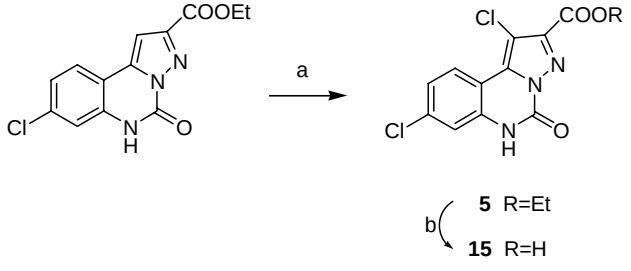
Reaction of ethyl 8-chloro-4,5-dihydro-pyrazolo[1,5-*c*]quinazoline-2-carboxylates[17] with sulfuryl chloride in glacial acetic acid gave the 1-chloro derivative **5**, which was easily transformed into its corresponding acid **15** (Scheme 2).

The ethyl 8,9-dichloro-5,6-dihydro-pyrazolo[1,5-*c*]quinazoline-2-carboxylates **6** and **7**, 1-chloro and 1-bromo substituted, respectively (Scheme 3) were prepared



	R_8	R_9
2, 9, 12, 28	H	H
3, 10, 13, 29	Cl	H
4, 11, 14, 30	Cl	Cl

Scheme 1. Reagents and conditions: (a) *p*-toluenesulfonohydrazide, MeOH; (b) 0.35 M NaOH; (c) diethyl acetylenedicarboxylate, anhydrous toluene; (d) 1.5 M KOH, MeOH, room temperature; (e) 1.5 M KOH, EtOH, reflux; (f) heating over the melting points.

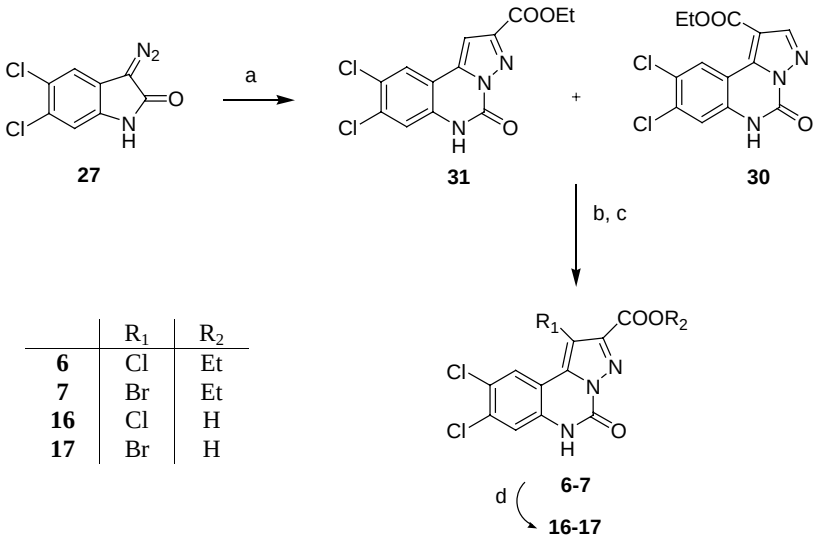


Scheme 2. Reagents and conditions: (a) SO_2Cl_2, glacial acetic acid; (b) 1.5 M KOH, MeOH, reflux.

starting from the 5,6-dichloro-3-diazoindole derivative **27** obtained as above and depicted in Scheme 1.

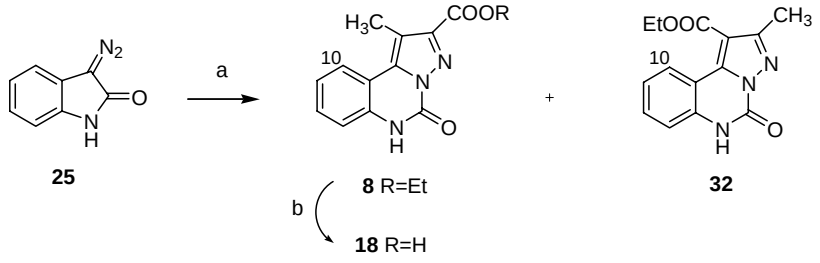
By reacting **27** with an excess of ethyl propiolate, a mixture (1:1, ^{1}H NMR analysis) of the two regio-isomers **31** and **30** was obtained. We were unable to separate **31** from **30** with satisfactory yield; thus their combined mixture was used for the next step. Reaction of this mixture with either sulfuryl chloride or bromine in glacial acetic acid gave a solid in which only the 1-chloro- or 1-bromo derivatives **6** and **7** were present, respectively; in both, cases the less reactive ethyl pyrazolo[1,5-*c*]quinazoline-1-carboxylate **30** was recovered unmodified in the mother liquor. The esters **6** and **7** were then hydrolyzed in alkaline medium to their corresponding acids **16** and **17**.

The tricyclic ethyl 1-methyl-pyrazoloquinazoline-2-carboxylate derivative **8**[24,25] (Scheme 4) was obtained in mixture (1:1, ^{1}H NMR analysis) with its regio-isomer ethyl 2-methyl-pyrazoloquinazoline-1-carboxylate **32**, by reacting the 3-diazoindol-2-one intermediate **25**[21] with an excess of ethyl 2-butinoate. Pure **8** and **32** were then obtained by column chromatography.



	R_1	R_2
6	Cl	Et
7	Br	Et
16	Cl	H
17	Br	H

Scheme 3. Reagents and conditions: (a) ethyl propiolate, anhydrous toluene; (b) SO_2Cl_2, glacial acetic acid; (c) Br_2, glacial acetic acid; (d) 1.5 M KOH, MeOH, reflux.



Scheme 4. Reagents and conditions: (a) ethyl 2-butinoate, toluene; (b) 1.5 M KOH, MeOH, reflux.

The structures of the two isomers **8** and **32** were assigned on the basis of their ^{1}H NMR spectra, using as key tool the signal of the proton at position-10, which is easily identified in this class of tricyclic derivatives. In fact, the H-10 is, in general, the most deshielded aromatic proton. In both ^{1}H NMR spectra of isomers **8** and **32**, the H-10 signal appears as a doublet, but with a different chemical shift: while the H-10 signal of **8** appears at 8.08 ppm, that of **32** is at 9.01 ppm, due to the paramagnetic effect of the carbonyl function of the ester group at position-1.

Finally, by treatment of **8** in alkaline medium, the corresponding acid **18**[25] was obtained.

3. Results and discussion

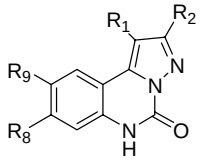
The pyrazoloquinazolines **2**–**18** were tested for their ability to displace tritiated glycine, AMPA, and KA from their specific binding sites in rat cortical membranes. The binding data are shown in Table 1, together with those of the previously reported C-1 unsubstituted derivatives **1a**–**e**,[17] included as reference compounds. Moreover, the binding data of the well-known Gly/NMDA antagonist 5,7-dichlorokynurenic acid (**DCKA**) are also reported.

In general, the binding results suggest that the nature of the substituent at position-1 of the pyrazoloquinazoline tricyclic system is important for shifting selectivity toward the Gly/NMDA receptor. In fact, derivatives **15** and **16**, bearing a chlorine atom at position-1, are the most Gly/NMDA versus AMPA selective compounds among the PQZ series, showing a selectivity ratio comparable to that of **DCKA** (selectivity ratio > 500). Moreover, **15** and **16** also show good Gly/NMDA receptor affinity (K_i = 0.18 and 0.16 μM, respectively). In contrast, derivatives **13** and **14**, bearing a carboxylic acid group at position-1, are scarcely Gly/NMDA selective (selectivity ratio ≅ 12), but they are the most potent Gly/NMDA receptor antagonists among the PQZ series (K_i = 0.09 and 0.059 μM, respectively).

This study confirms that the presence of an anionic carboxylate residue at position-2 of the pyrazoloquinazoline tricyclic system, being able to bind with a proton donor site of the receptors, is an important feature for anchoring at both AMPA and Gly/NMDA receptors.[13,14,16–18] In fact, the 2-carboxylic acids **9**–**11**, **15**–**18** are more active than their corresponding 2-carboxylate esters **2**–**8** at both receptors, with the exception of the 1-chloro-2-carboxylic acid derivatives **15** and **16**, which like their corresponding esters **5** and **6**, are inactive at the AMPA receptor, and the 1-bromo-2-carboxylic acid **17**, which is less AMPA active than its ethyl ester **7**.

As regards the benzofused substitutions, it is important to note that the benzo-unsubstituted derivatives **2**, **8**, **9**,

Table 1. Displacement of [^{3}H]glycine, [^{3}H]AMPA, and [^{3}H]Kainate binding[a]



1a-e; 2-18

Compound	R_1	R_2	R_8	R_9	K_i (μM)[b] or I%[c]		IC_{50} (μM)[d] or I%[c]
					[^{3}H]glycine	[^{3}H]AMPA	[^{3}H]KA
1a[e]	H	COOEt	H	H	33.3 ± 7.0	42 ± 6.8	12%
1b[e]	H	COOEt	Cl	H	26.5 ± 4.4	72 ± 2.3	15%
1c[e]	H	COOH	H	H	1.41 ± 0.3	12.4 ± 2.5	26%
1d[e]	H	COOH	Cl	H	0.48 ± 0.04	2.3 ± 0.4	60 ± 3.0
1e[e]	H	COOH	Cl	Cl	0.16 ± 0.04	2.4 ± 0.8	41 ± 3.0
2	COOEt	COOEt	H	H	2.95 ± 0.25	50 ± 6.0	27%
3	COOEt	COOEt	Cl	H	3.4 ± 0.4	30%	10%
4	COOEt	COOEt	Cl	Cl	50 ± 13	25%	0%
5	Cl	COOEt	Cl	H	25%	15%	6%
6	Cl	COOEt	Cl	Cl	22%	10%	9%
7	Br	COOEt	Cl	Cl	7.1 ± 0.7	39 ± 5	8%
8	CH_3	COOEt	H	H	30%	19%	13%
9	COOEt	COOH	H	H	1.15 ± 0.2	15.2 ± 1.3	48%
10	COOEt	COOH	Cl	H	0.86 ± 0.2	1.6 ± 0.3	78 ± 7.0
11	COOEt	COOH	Cl	Cl	0.22 ± 0.04	2.5 ± 0.1	43 ± 3.0
12	COOH	COOH	H	H	0.54 ± 0.12	34.8 ± 4.0	47%
13	COOH	COOH	Cl	H	0.09 ± 0.01	1.1 ± 0.2	11.5 ± 1.4
14	COOH	COOH	Cl	Cl	0.059 ± 0.01	0.71 ± 0.08	19.5 ± 11
15	Cl	COOH	Cl	H	0.18 ± 0.04	16%	100 ± 8
16	Cl	COOH	Cl	Cl	0.16 ± 0.01	47%	91 ± 15
17	Br	COOH	Cl	Cl	4.0 ± 0.6	100 ± 15	92 ± 8.0
18	CH_3	COOH	H	H	0.78 ± 0.13	47.5 ± 4.2	33%
DCKA					0.09 ± 0.02	5%	8%

[a] The tested compounds were dissolved in 50% DMSO and then diluted with the appropriate buffer.
[b] Inhibition constant (K_i) values were means ± SEM of three or four separate determinations in triplicate.
[c] Percentage of inhibition (I%) of specific binding at 100 μM concentration.
[d] Concentrations necessary for 50% inhibition (IC_{50}). The IC_{50} values were means ± SEM of three or four separate determinations in triplicate.
[e] Ref. 17.

12, and **18** are endowed with micromolar or submicromolar Gly/NMDA receptor binding affinity, the only exception being the 1-methyl 2 ethyl ester **8**, which is inactive. Based on these data, the presence of a substituent on the benzofused moiety is not essential for the anchoring of PQZ derivatives to the Gly/NMDA binding site. However, in accordance with our previous data,[13,17] an 8-chloro-, or better still an, 8,9-dichloro substitution is profitable for Gly/NMDA receptor affinity (compare **10** and **11** with **9**, and **12** and **13** with **14**), the only exception being the ethyl 8,9-dichloro 1,2-dicarboxylate **4**, which shows a markedly reduced Gly/NMDA binding activity compared to those of its corresponding unsubstituted and 8-chloro substituted derivatives **2** and **3**, respectively.

On the contrary, the presence of a chlorine atom at position-8 is necessary for an effective AMPA receptor–ligand interaction. Indeed, the benzo-unsubstituted 2-carboxylic acids **9** and **12** are about 10-fold and 30-fold less active than their corresponding 8-chloro-substituted 2-carboxylic acids **10** and **13**, respectively. These data confirm that the 8-chloro substituent, due to its electron-withdrawing effect, could increase the NH lactam acidity, thus reinforcing the essential hydrogen-bond receptor–ligand interaction at this level.[26–28] Furthermore, the 8,9-dichloro derivatives **11** and **14**, are equi-active compared to the 8-chloro substituted **10** and **13**, respectively, at the AMPA receptor.

The presence of a C-1 ethyl carboxylate group on the PQZ core has contrasting effects. The ethyl 1,2-dicarboxylate derivatives **2** and **3**, compared to their C-1 unsubstituted parent compounds **1a** and **1b**, respectively, are 8- and 11-fold more active at the Gly/NMDA receptor and also much more Gly/NMDA versus AMPA selective (selectivity ratio = 17 and 30, respectively). In contrast, the presence of a C-1 ethyl carboxylate group in the 2-carboxylic acid derivatives **9**–**11** does not increase the affinity for either receptor subtype; in fact, the Gly/NMDA and AMPA receptor binding activities of **9**–**11** are comparable to those of their parent C-1 unsubstituted derivatives **1c**–**e**, respectively. The behavior of **9**–**11**, especially toward the Gly/NMDA receptor, was unexpected. In fact, the micromolar Gly/NMDA binding activities of the ethyl 1,2-dicarboxylates **2** and **3** had led us to hypothesize that a C-1 ethyl carboxylate group, able to act as hydrogen bond acceptor,

could have engaged a new hydrogen bond interaction with the Gly/NMDA receptor site, thus reinforcing the receptor–ligand interaction. Trying to clarify these contrasting results, we have introduced at position-1 of our pyrazoloquinazoline scaffold, a more powerful and less hindered hydrogen bond acceptor group than the ethyl carboxylate. Thus we synthesized compounds **12**–**14** bearing a carboxylic acid function at position-1. These compounds are from 3- to 5-fold more active at the Gly/NMDA receptor than the corresponding C-1 unsubstituted derivatives **1c**–**e**, respectively, and from 2- to 9-fold more active than their corresponding C-1 esters **9**–**11**. In particular, the 1,2-dicarboxylic acid derivatives **13** and **14** ($K_i = 0.09$ and $0.059\ \mu M$, respectively), are the most potent Gly/NMDA receptor antagonists among the PQZ series, showing an inhibition constant value comparable to that of **DCKA** ($K_i = 0.09\ \mu M$). These data indicate that a C-1 hydrogen bond acceptor substituent, the best being a carboxylic acid function, positively affects the anchoring of PQZ derivatives to the binding site of the Gly/NMDA receptor, probably through a new hydrogen bond interaction. However, C-1 hydrogen bond acceptor groups, like ester or carboxylic acid, are unable to distinguish the Gly/NMDA receptor from the AMPA one. In fact, with the exception of the benzo-unsubstituted **12**, which is 70-fold Gly/NMDA versus AMPA selective, compounds **9**–**11** and **13**–**14** show a selectivity toward the Gly/NMDA receptor comparable to that of their corresponding C-1 unsubstituted derivatives **1c**–**e**, respectively.

For this reason we decided to evaluate the effect on Gly/NMDA receptor selectivity of a lipophilic substituent at position-1 of the pyrazoloquinazoline tricyclic system.

Introduction of a C-1 chlorine atom positively affects the Gly/NMDA selectivity. Indeed, while the 1-chloro-2-carboxylic acids **15** and **16** are completely inactive toward the AMPA receptor, they show Gly/NMDA binding activities comparable to those of their parent compounds **1d** and **1e**. Thus, compounds **15** and **16** are endowed with the highest Gly/NMDA versus AMPA selectivity (selectivity ratio > 500) among the PQZ series. Therefore, we hypothesized that a lipophilic substituent at position-1 on the pyrazoloquinazoline tricyclic system could be important for shifting selectivity toward the Gly/NMDA receptor. This encouraging result prompted us to further investigate in this direction. Thus we synthesized the 1-bromo-2-carboxylic acid **17**, which unexpectedly, is 25-fold less active than **1e** at the Gly/NMDA receptor. Moreover, **17** is also 25-fold less active than the corresponding 1-chloro derivative **16**, probably due to the increased steric hindrance of the bromine atom with respect to the chlorine one. However, **17** is 45-fold less active at the AMPA receptor when compared to **1e**, thus confirming the negative effect of a C-1 lipophilic substituent for the anchoring of PQZ derivatives at the AMPA receptor. Indeed, the 1-bromo derivative **17** still shows a good Gly/NMDA versus AMPA selectivity (selectivity ratio = 25).

Introduction of **1c** at position-1 of the lipophilic methyl moiety, yields compound **18**, which is highly Gly/NMDA versus AMPA selective (selectivity ratio = 60). In fact, **18** shows a comparable Gly/NMDA receptor binding activity with respect to **1c** and, due to the presence of a C-1 lipophilic substituent, it is 4-fold less active at the AMPA receptor than **1c**. Unfortunately, no data about the presence of both the methyl moiety at position-1 and chlorine atom(s) at position-8 or 8,9 are available. In fact, all attempts to synthesize the 8-chloro and 8,9-dichloro analogues of **18** by reacting the corresponding 3-diazoindole **26**–**27** with ethyl 2-butinoate were unsuccessful.

The pyrazoloquinazoline **2**–**18** are in general, inactive or active in the high micromolar range at the KA receptor. As for the AMPA receptor–ligand interaction, in compounds **2**–**18** the contemporary presence of a chlorine atom at position-8 and a 2-carboxylic acid group on the pyrazoloquinazoline core is required for the KA receptor–ligand interaction. Furthermore, the presence of a 1-carboxylic acid group (i.e., compounds **13** and **14**) led to an increase in KA receptor binding activity with respect to their C-1 unsubstituted parent compounds **1d** and **1e**, respectively. These data confirm the high similarity of the structural requirements of the binding pockets of AMPA and KA receptors.[27,29]

Functional antagonism at the NMDA receptor-ion channel complex was evaluated by assessing the ability of some selected pyrazoloquinazolines (**2**, **3**, **8**, **10**, **12**–**16**, and **18**) to inhibit the binding of the channel-blocking agent [^{3}H]-(+)-MK801 ((+)-5-methyl-10,11-dihydro-5*H*-benzo[a,d]cyclohepten-5,10-iminemaleate) in rat cortical membranes incubated with 10 μM glutamate and 0.1 μM glycine.[10,30,31] The results, shown in Table 2, indicate that, as in the case of [^{3}H]glycine binding assays, compound **14** is the most potent tested compound with an IC_{50} value of $0.15 \pm 0.01\ \mu M$ higher than that of **DCKA** ($IC_{50} = 0.74 \pm 0.09\ \mu M$).

Furthermore, compounds **13**–**15** were also evaluated by testing their ability to inhibit depolarization induced by 5 μM AMPA or 5 μM NMDA in mouse cortical wedge

Table 2. Inhibition of stimulated [^{3}H]-(+)-MK-801 binding

Compd	IC_{50} (μM)[a] or I%[b]
2	3.2 ± 0.1
3	4.0 ± 0.4
8	43%
10	1.1 ± 0.06
12	0.85 ± 0.008
13	0.7 ± 0.08
14	0.15 ± 0.01
15	1.2 ± 0.15
16	1.0 ± 0.1
18	5.6 ± 0.2
DCKA	0.74 ± 0.09

[a] Concentration giving 50% inhibition of stimulated [^{3}H]-(+)-MK-801 binding. All assays were carried out in the presence of 10 μM glutamate and 0.1 μM glycine. IC_{50} values were means ± SEM of three or four separate determinations in triplicate.

[b] Percentage of inhibition (I%) of specific binding at 100 μM concentration.

Table 3. Functional antagonism at NMDA and AMPA sites

Compd	Mouse cortical wedge preparation: IC_{50} (μM) versus agonist-induced depolarization[a]	
	NMDA	AMPA
13	0.7 ± 0.1	7.1 ± 1.2
14	0.5 ± 0.1	6.0 ± 1.0
15	1.6 ± 0.1	38 ± 7.0
DCKA	4.7 ± 0.9	52 ± 11

[a] Concentration that inhibits 50% depolarization (IC_{50}) induced by 5 μM NMDA or AMPA. The IC_{50} values were means ± SEM of four separate determinations.

preparations (Table 3). Among the PQZ series presented in this article, compounds **13** and **14** were chosen due to their high Gly/NMDA receptor binding affinity; and compound **15** for its high selectivity.

The results obtained in these electrophysiological assays indicate that the inhibitory actions of **13**–**15** on depolarization induced by NMDA are much higher than those on AMPA-evoked response, confirming that these derivatives are more potent antagonists at the Gly/NMDA receptor. Moreover, compounds **13**–**15** are more potent Gly/NMDA receptor antagonists than the reference **DCKA**.

4. Molecular modeling studies

A molecular modeling investigation has been carried out to support the observed SAR. Molecular docking techniques have been used to explore the binding conformation of all new synthesized PQZ derivatives. The published crystallographic structures of glycine-binding domain S1S2 of NR1 subunit in complex with the antagonist **DCKA**, and of glutamate-binding domain S1S2 of GluR2 subunit in complex with the antagonist **DNQX** (6,7-dinitroquinoxalin-2,3-dione) were used to perform molecular docking studies of Gly/NMDA and AMPA receptor, respectively (see Experimental section for details).[32,33] From a structural point of view, the ligand–binding segments S1 and S2 of NR1 present in the glycine binding recombinant NR1 fusion protein display a sequence identity of 22% with the glutamate-binding domains (S1–S2) of the AMPA receptor. As in bacterial amino acid binding proteins, segments S1 and S2 form the globular lobes 1 and 2, which are connected by two hinge regions. The lobes are separated by a central cleft that provides the ligand binding pocket , and which is lined by loop regions. These loops harbor determinants of glycine site pharmacology. Computational docking of the glycine site antagonist **DCKA** and the glutamate-binding domain antagonist **DNQX** have been carried out to verify the quality of our computational docking protocol. Our docking protocol accurately reproduced the crystallographic ligand conformations solved in both receptor binding sites (data not shown).

The new series of PQZ derivatives has been analyzed using the same reference docking protocol on both receptor binding sites. Docking information has been used to explain the experimental affinity data and the selectivity of PQZ ligands between AMPA and Gly/NMDA receptors.

So far, several computational approaches have been used in order to understand the binding modes of ligands on Gly/NMDA receptor. Before the crystal structures of glycine binding core in different states were available, pharmacophore[34–36] and three-dimensional homology models[37,38] of Gly/NMDA binding site were used for explaining and rationalizing known structure–activity relationships of diverse classes of compounds. In 2003, Baskin et al. proposed CoMFA models of a set of pyrazolo[1,5-*c*]quinazolines derivatives for evaluation of binding selectivity between Gly/NMDA and AMPA receptors.[39] Our results showed to be in agreement with most of the previous models, even if the Gly/NMDA crystallographic structure availability allowed us to perform a more accurate comparison between the two different receptor binding sites.

To simplify the discussion of the computational results and the comparisons between the two receptors, we prefer to divide the receptor binding sites into *key regions*, from sub-site I to sub-site IV (Fig. 1).

As represented in Figure 1, the binding pocket of Gly/NMDA and AMPA receptors shows some conserved features important for the binding of the ligands, and some crucial structural dissimilarities, required for the selectivity of the ligands between the two receptors. Sub-site I (ss-I) seems to be the most conserved part of the receptor binding cleft and the most important in determining ligand binding affinity on both Gly/NMDA and AMPA receptors. Sub-sites II–IV seem to affect receptor selectivity the most. In fact, these regions of the receptor binding pocket interact with the substituents R_1, R_2, R_8, and R_9 of the PQZ derivatives.

Sub-site I (ss-I) contains the most important and highly conserved triad of residues crucial for binding the ligands on both receptors: an arginine (R131 in the Gly/NMDA receptor and R96 in the AMPA receptor), a threonine (T126 and T91, respectively), and a proline residue (P124 and P89, respectively). Two H-bond acceptor groups in the antagonist structure are essential for the interaction with the above mentioned arginine and threonine. Molecular docking clearly shows a good electrostatic complementarity between the structure of common pyrazolo[1,5-*c*]quinazoline moiety of PQZ derivatives and the three amino acids R131/R96, T126/T91, and P124/P89 in both the ss-I sub-sites (Fig. 2). Moreover, an aromatic residue is present in both receptors (F92 in Gly/NMDA receptor and Y61 in AMPA receptor), which can bind through π–π interaction with the aromatic scaffold of quinazoline ring.

Sub-site II (ss-II) seems to regulate the accommodation of R_8 substituent of the PQZ derivatives. In the Gly/NMDA receptor, ss-II is formed by the presence of five highly hydrophobic residues: F16, F92, F250, A226, and V227. Considering also the small size of this cavity, small and hydrophobic substituents, such as chlorine

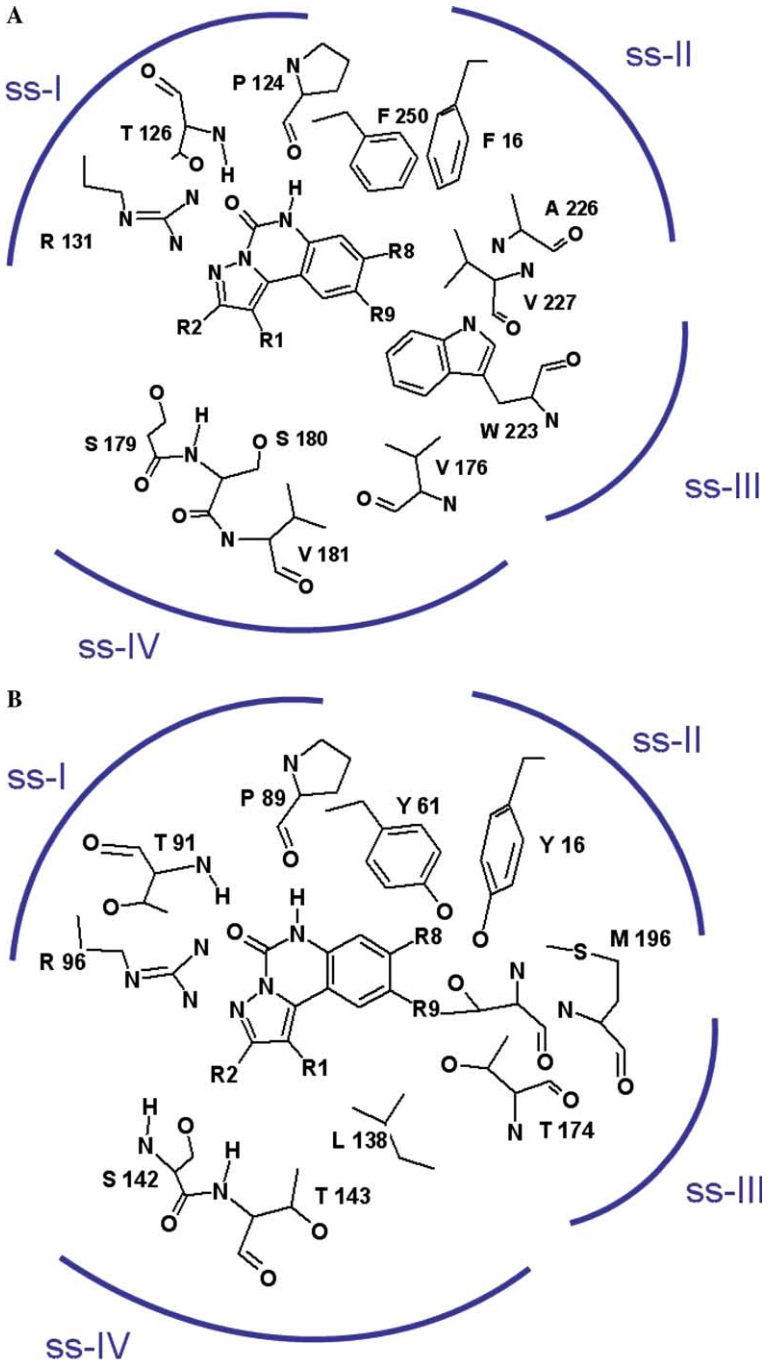


Figure 1. Schematic representation of the ligand binding pocket of the Gly/NMDA receptor (panel A) and AMPA receptor (panel B). Crucial amino acids are highlighted. Sub-sites I–IV are classified. General binding motif of PQZ derivatives is also reported.

atom, can be efficiently allocated in it. On the other hand, ss-II of the AMPA receptor is a less hydrophobic pocket formed by Y16, Y61, Y220, T195, and M196 residues. However, the presence of a chlorine atom at the 8-position guarantees that the antagonist assumes the right conformation for an efficient interaction with the ss-I triad RTP (compare derivatives **12** and **13**).

Sub-site III (ss-III) is deputed to accommodate R_9 substituent. In the AMPA receptor, ss-III is roomier and more hydrophilic than the corresponding pocket in Gly/NMDA receptor. Residues L138, T174, L192, E193, and M196 delimit the ss-III cavity of the AMPA receptor. In comparison, Q13, W223, D224, and V227 delimit the ss-III cavity of the Gly/NMDA receptor. The presence of a chlorine atom at the 9-position of PQZ derivatives plays an important role in ligand binding by stabilizing the conformation that allows the ligand to interact with the ss-I triad RTP. Consequently, the double 8,9-dichloro substitution of PQZ derivatives is very well tolerated at both Gly/NMDA and AMPA receptors. However, considering the peculiar shape and hydrophobicity properties of the two ss-III cavities, the increase of ligand affinity versus Gly/

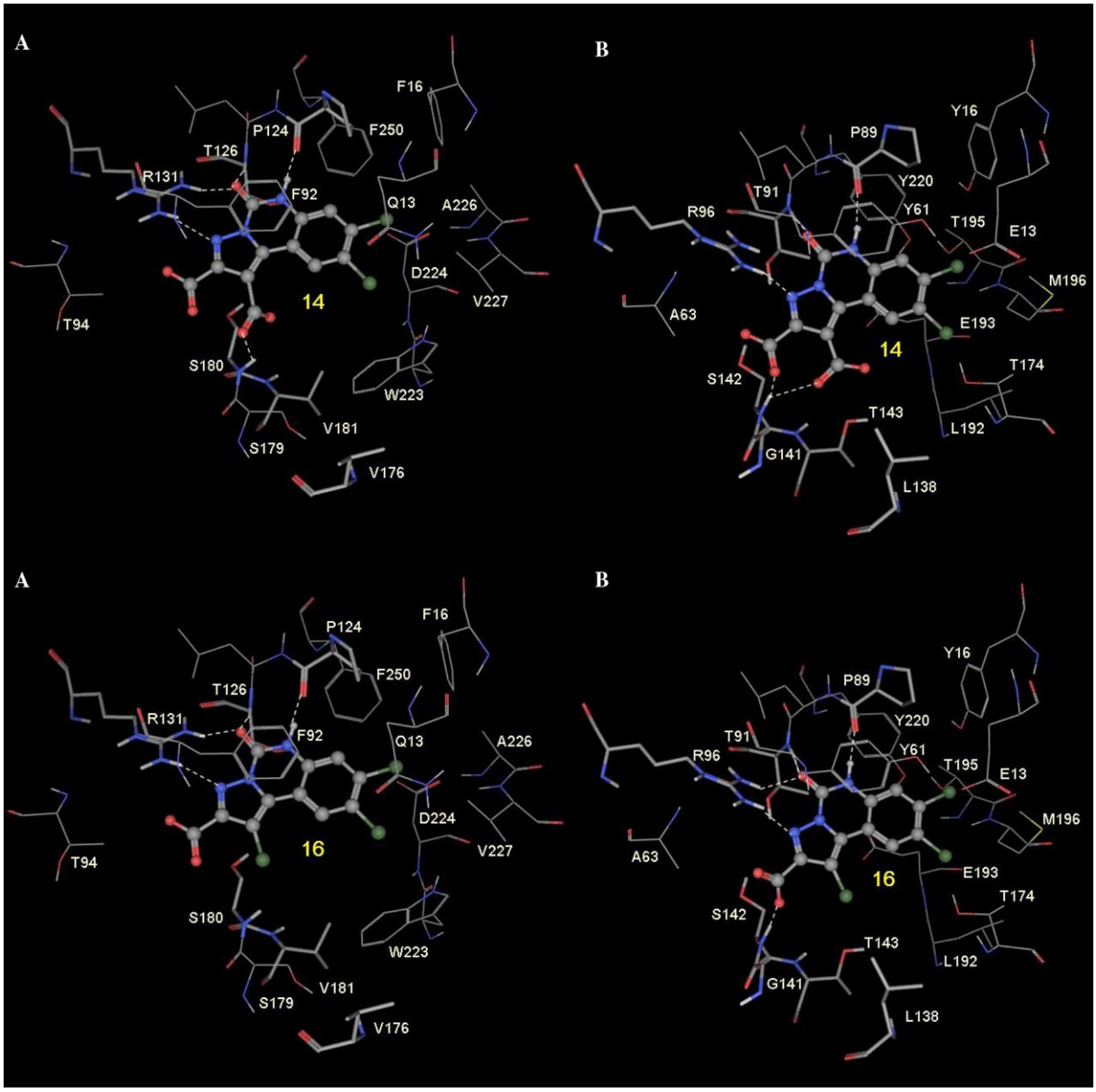


Figure 2. Best docking conformation of PQZ derivatives **14** and **16** obtained by using Gly/NMDA (on the left) and AMPA (on the right) ligand–receptor binding sites. The side chains of some very important residues in proximity ($\leqslant 5$ Å) to the docked PQZ derivatives are highlighted and labeled. Hydrogen bonding interactions are indicated (dash line).

NMDA receptor is more significant than that versus AMPA receptor (compare derivatives **13** and **14**).

Finally, sub-site IV (ss-IV) accommodates R_1 and R_2 substituents. Residues L138, S142, G141, and T143 delimit the ss-IV cavity of the AMPA receptor; V176, S 179, S180, and V181 delimit the ss-IV cavity of the Gly/NMDA receptor. As shown by docking studies, R_1 and R_2 substituents are positioned very close to a helix domain (helix F) of the S2 unit of both receptors. Moreover, R_2 substituent is also in the proximity of the crucial arginine residue of the ss-I pocket. Accordingly, the activity of PQZ derivatives increases when the carboxyl group at R_2 position is near to the crucial arginine residue of the ss-I pocket. This stabilization is due to an electrostatic interaction between the negatively charged carboxylate group and the guanidine moiety of the crucial arginine of the ss-I pocket, and the strength of this stabilization strongly depends on the final accommodation of each PQZ derivative inside the whole receptor binding cleft (from ss-I to ss-IV).

Considering R_1 substitutions and comparing Gly/NMDA and AMPA ligand binding pockets, a different steric control seems to operate. Valine 176 (V176) of Gly/NMDA receptor is substituted with the bulky leucine 138 (L138) of AMPA receptor. Analyzing our docking studies, V176 and L138 are positioned in proximity to R_1 position. This could explain the reason why bulky substituents at R_1 position of PQZ moiety are not well tolerated by AMPA receptor binding pocket (see compound **7** as an example). H-bond acceptor groups,

like ester or carboxylic acid, can interact with S180 of Gly/NMDA receptor, stabilizing the antagonist–receptor complex. Moreover, the environment created by helix F around R_1 and R_2 positions is more polar in AMPA receptor with respect to Gly/NMDA receptor. In fact, the valine residue (V181) present on Gly/NMDA receptor is replaced by a threonine (T143) on AMPA receptor. Accordingly, compounds **15** and **16**, bearing chlorine atom at R_1 position, are scarcely effective versus the more polar environment of AMPA receptor. Conversely, compounds **13** and **14**, bearing a carboxylic acid group at R_1 position, are also effective versus AMPA receptor, because they form an extra electrostatic interaction between the carboxylic moiety and the NH group of serine 142 (S142) (Fig. 2).

5. Conclusions

The synthesis of the herein reported new C-1 substituted pyrazoloquinazoline 2-carboxylates has allowed us to further investigate the SAR of tricyclic heteroaromatic systems as Gly/NMDA receptor antagonists.

The results of this study show that a C-1 lipophilic substituent on the pyrazolo[1,5-*c*]quinazoline-2-carboxylate core shifts selectivity toward the Gly/NMDA receptor. In fact, the 2-carboxylic acids **15** and **16**, bearing a chlorine atom at position-1, are the most Gly/NMDA versus AMPA selective compounds among the pyrazoloquinazoline series (selectivity ratio > 500). Furthermore, this study has highlighted that a C-1 anionic carboxylate residue leads to an increase in Gly/NMDA receptor affinity, probably through an additional hydrogen bond interaction with a proton donor site of the receptor. Indeed, the 1,2-dicarboxylic acids **13** and **14** are the most active compounds at the Gly/NMDA receptor among the pyrazoloquinazoline series.

Based on our experimental and computational results, we have proposed that the interaction of the ligand with sub-site I is essential for receptor recognition. In addition, the increase of ligand affinity and selectivity necessarily involves a delicate mechanism of balancing between steric and electrostatic control, operated by the interaction of the ligand with sub-sites II–IV. Further investigations are in progress to confirm the robustness of this scenario.

6. Experimental section

6.1. Chemistry

Silica gel plates (Merck F254) and silica gel 60 (Merck; 70–230 mesh) were used for analytical and column chromatography, respectively. All melting points were determined on a Gallenkamp melting point apparatus. Microanalyses were performed with a Perkin–Elmer 260 elemental analyzer for C, H, N, and the results were within ±0.4% of the theoretical values, unless otherwise stated (Table 4). The IR spectra were recorded with a Perkin–Elmer Spectrum RX I spectrometer in Nujol mulls and are expressed in cm^{-1}. The ^1H NMR spectra were obtained with a Varian Gemini 200 instruments at 200 MHz. The chemical shifts are reported in δ (ppm), and are relative to the central peak of the solvent, which

Table 4. Analytical data of the newly synthesized compounds

Compd	Formula	C calcd–found	H calcd–found	N calcd–found
2	$C_{16}H_{15}N_3O_5$	58.35–58.54	4.60–4.72	12.76–12.80
3	$C_{16}H_{14}ClN_3O_5$	52.82–52.67	3.89–3.96	11.55–11.60
4	$C_{16}H_{13}Cl_2N_3O_5$	48.25–48.45	3.30–3.38	10.55–10.53
5	$C_{13}H_9Cl_2N_3O_3$	47.87–48.06	2.79–2.91	12.89–12.93
6	$C_{13}H_8Cl_3N_3O_3$	43.30–43.17	2.24–2.17	11.66–11.71
7	$C_{13}H_8BrCl_2N_3O_3$	38.55–38.43	1.99–2.11	10.38–10.42
8	$C_{14}H_{13}N_3O_3$	61.98–62.20	4.84–4.88	15.49–15.62
9	$C_{14}H_{11}N_3O_5$	55.81–55.93	3.69–3.76	13.95–13.79
10	$C_{14}H_{10}ClN_3O_5$	50.08–50.18	3.01–3.08	12.52–12.58
11	$C_{14}H_9Cl_2N_3O_5$	45.42–45.21	2.46–2.48	11.35–11.40
12	$C_{12}H_7N_3O_5$	52.75–52.61	2.59–2.64	15.38–15.30
13	$C_{12}H_6ClN_3O_5$	46.84–47.02	1.97–1.90	13.66–13.60
14	$C_{12}H_5Cl_2N_3O_5$	42.13–42.26	1.48–1.54	12.29–12.34
15	$C_{11}H_5Cl_2N_3O_3$	44.32–44.21	1.69–1.76	14.10–14.18
16	$C_{11}H_4Cl_3N_3O_3$	39.73–39.84	1.21–1.28	12.64–12.70
17	$C_{11}H_4BrCl_2N_3O_3$	35.04–35.12	1.07–1.10	11.15–11.23
18	$C_{12}H_9N_3O_3$	59.25–59.47	3.74–3.70	17.28–17.18
21	$C_8H_3Cl_2NO_2$	44.48–44.53	1.40–1.42	6.49–6.51
22	$C_{15}H_{13}N_3O_3S$	57.12–56.99	4.16–4.21	13.33–13.40
23	$C_{15}H_{12}ClN_3O_3S$	51.50–51.66	3.46–3.50	12.02–11.94
24	$C_{15}H_{11}Cl_2N_3O_3S$	46.88–47.02	2.89–2.93	10.94–10.90
28	$C_{13}H_{11}N_3O_3$	60.69–60.60	4.32–4.27	16.34–16.38
29	$C_{13}H_{10}ClN_3O_3$	53.52–53.46	3.46–3.51	14.41–14.36
30	$C_{13}H_9Cl_2N_3O_3$	47.87–47.93	2.79–2.83	12.89–12.94
31	$C_{13}H_9Cl_2N_3O_3$	47.87–47.98	2.79–2.84	12.89–12.94
32	$C_{14}H_{13}N_3O_3$	61.98–61.81	4.84–4.76	15.49–15.41

is always DMSO-d_6. All the exchangeable protons were confirmed by addition of D_2O.

6.2. Synthesis of 5,6-dichloro-1*H*-indole-2,3-dione (21)[20]

Sulfuryl chloride (11.6 mmol) and a crystal of I_2 as catalyst were added to a suspension of 6-chloro-1*H*-indole-2,3-dione **20**[19] (5.5 mmol) in glacial acetic acid (20 mL). The mixture was heated at 50 °C for 8 h and then cooled at room temperature. The resulting solid was collected and washed with petroleum ether. Yield 70%; mp 268–270 °C (AcOH) (lit. mp 266–272 °C). ^{1}H NMR 7.11 (s, 1H, ar), 7.78 (s, 1H, ar), 11.25 (br s, 1H, NH).

6.3. General procedure to prepare 4-methyl-*N*′-(2-oxo-1,2-dihydro-3*H*-indol-3-ylidene)benzenesulfonohydrazides 22,[21] 23,[22] and 24

To a warmed (70 °C) solution of isatin **19**–**21**[19,20] (16.2 mmol) in methanol (80 mL), an equimolar amount of *p*-toluenesulfonylhydrazide (16.2 mmol) was added. After all of the latter was dissolved, the solution was allowed to stand at room temperature for 2 h. The yellow platelets were filtered and washed with a little cold methanol.

6.3.1. 4-Methyl-*N*′-(2-oxo-1,2-dihydro-3*H*-indol-3-ylidene)-benzenesulfonohydrazide (22). Yield 88%; mp 193–200 °C dec (PhH) (lit. mp 190–200 °C from methanol). ^{1}H NMR 2.36 (s, 3H, CH_3) 6.84–6.91 (m, 1H, ar) 7.03–7.15 (m, 1H, ar) 7.35–7.51 (m, 3H, ar) 7.82–7.93 (m, 3H, ar) 10.73 (br s, 1H, NH).

6.3.2. 4-Methyl-*N*′-(6-chloro-2-oxo-1,2-dihydro-3*H*-indol-3-ylidene)benzenesulfonohydrazide (23). Yield 77%; mp 206–208 °C (AcOH). ^{1}H NMR 2.38 (s, 3H, CH_3), 6.86 (d, 1H, ar, J = 1.8 Hz), 7.13 (dd, 1H, ar, J = 8.4, 1.8 Hz), 7.43 (d, 2H, ar, J = 8.4 Hz), 7.79–7.88 (m, 3H, ar), 10.88 (br s, 1H, NH).

6.3.3. 4-Methyl-*N*′-(5,6-dichloro-2-oxo-1,2-dihydro-3*H*-indol-3-ylidene)benzenesulfonohydrazide (24). Yield 90%; mp 212–218 °C (AcOH) ^{1}H NMR 2.37 (s, 3H, CH_3) 7.09 (s, 1H, ar) 7.41 (d, 2H, ar, J = 7.7 Hz) 7.61 (s, 1H, ar) 7.85 (d, 2H, ar, J = 7.7 Hz) 11.20 (br s, 1H, NH).

6.4. General procedure to prepare 3-diazo-1,3-dihydro-indol-2-ones 25,[21] 26,[22] and 27

A mixture of **22**–**24** (4.29 mmol) and an aqueous solution of NaOH (0.35 M, 31.5 mL) was heated at 60 °C for 1 h, and then allowed to stand at room temperature for 2 h. Addition of ice (40 g) and acidification with acetic acid afforded a solid that was filtered and washed well with water. The residue was unstable upon recrystallization. Nevertheless, the crude product was pure enough and thus used without further purification.

6.4.1. 3-Diazo-1,3-dihydro-indol-2-one (25). Yield 90%; ^{1}H NMR 6.89–7.70 (m, 3H, ar) 7.41 (d, 1H, ar, J = 7.3 Hz) 10.67 (br s, 1H, NH).

6.4.2. 3-Diazo-6-chloro-1,3-dihydro-indol-2-one (26). Yield 90%; ^{1}H NMR 6.91 (s, 1H, ar) 7.03 (d, 1H, ar, J = 8.0 Hz) 7.43 (d, 1H, ar, J = 8.0 Hz) 10.81 (br s, 1H, NH).

6.4.3. 3-Diazo-5,6-dichloro-1,3-dihydro-indol-2-one (27). Yield 85%; ^{1}H NMR 7.04 (s, 1H, ar) 7.45 (s, 1H, ar) 10.90 (br s, 1H, NH).

6.5. General procedure to prepare diethyl 5,6-dihydro-5-oxo-pyrazolo[1,5-*c*]quinazoline-1,2-dicarboxylates 2–4

An excess of diethyl acetylenedicarboxylate (5.16 mmol) was added to a suspension of diazoindole **25**–**27** (2.58 mmol) in anhydrous toluene (50 mL). The resulting mixture was refluxed, under nitrogen atmosphere, until disappearance of the starting material [2–9 h, TLC monitoring, eluting system $CHCl_3$/MeOH (9:1)] and then cooled at room temperature. The resulting solid was collected by filtration and washed with diethyl ether.

6.5.1. Diethyl 5,6-dihydro-5-oxo-pyrazolo[1,5-*c*]quinazoline-1,2-dicarboxylate (2). Yield 75%; mp 192–194 °C (EtOH) ^{1}H NMR 1.28–1.37 (m, 6H, $2CH_3$) 4.33–4.44 (m, 4H, $2CH_2$) 7.34–7.42 (m, 2H, ar) 7.59–7.63 (m, 1H, ar) 8.48 (d, 1H, ar, J = 8.0 Hz) 12.36 (br s, 1H, NH); IR 3420, 1750, 1720.

6.5.2. Diethyl 8-chloro-5,6-dihydro-5-oxo-pyrazolo[1,5-*c*]quinazoline-1,2-dicarboxylate (3). Yield 70%; mp 242–245 °C (EtOH) ^{1}H NMR 1.26–1.36 (m, 6H, $2CH_3$) 4.30–4.39 (m, 4H, $2CH_2$) 7.38–7.43 (m, 2H, ar) 8.56 (d, 1H, ar, J = 8.4 Hz) 12.45 (br s, 1H, NH).

6.5.3. Diethyl 8,9-dichloro-5,6-dihydro-5-oxo-pyrazolo[1,5-*c*]quinazoline-1,2-dicarboxylate (4). Yield 75%; mp 265–268 °C (AcOH) ^{1}H NMR 1.26–1.36 (m, 6H, $2CH_3$) 4.33–4.39 (m, 4H, $2CH_2$) 7.51 (s, 1H, ar) 8.95 (s, 1H, ar) 12.58 (br s, 1H, NH); IR 3464, 1735, 1712.

6.6. General procedure to prepare 1-(ethoxycarbonyl)-5,6-dihydro-5-oxo-pyrazolo[1,5-*c*]quinazoline-2-carboxylic acids 9–11

A solution of KOH (10% w/w, 20 mL) was added to a suspension of **2**–**4** (2.75 mmol) in methanol (20 mL). The mixture was left at room temperature under stirring, until disappearance of the starting material [1–7 h, TLC monitoring, eluting system $CHCl_3$/MeOH (9:1)]. Addition of water (40 mL) and acidification with concentrated HCl afforded a suspension that was left at room temperature for 30 min. The resulting solid was collected by filtration and washed with water.

6.6.1. 1-(Ethoxycarbonyl)-5,6-dihydro-5-oxo-pyrazolo-[1,5-*c*]quinazoline-2-carboxylic acid (9). Yield 80%; mp 226–229 °C (EtOH) ^{1}H NMR 1.30 (t, 3H, CH_3, J = 7.3 Hz) 4.36 (q, 2H, CH_2, J = 7.3 Hz) 7.30–7.41 (m, 2H, ar) 7.62 (t, 1H, ar, J = 7.7 Hz) 8.38 (d, 1H, ar, J = 7.7 Hz) 12.30 (br s, 1H, NH); IR 3430, 1759, 1713.

6.6.2. 8-Chloro-1-(ethoxycarbonyl)-5,6-dihydro-5-oxo-pyrazolo[1, 5-*c*]quinazoline-2-carboxylic acid (10). Yield 90%; mp 240–243 °C (AcOH) ^{1}H NMR 1.29 (t, 3H, CH_3,

J = 7.3 Hz) 4.35 (q, 2H, CH_2, J = 7.3 Hz) 7.39–7.43 (m, 2H, ar) 8.48 (d, 1H, ar, J = 8.3 Hz) 12.39 (br s 1H, NH).

6.6.3. 8,9-Dichloro-1-(ethoxycarbonyl)-5,6-dihydro-5-oxo-pyrazolo[1,5-*c*]quinazoline-2-carboxylic acid (11). Yield 85%; mp 230–232 °C (EtOH) ^{1}H NMR 1.28 (t, 3H, CH_3, J = 7.3 Hz) 4.35 (q, 2H, CH_2, J = 7.3 Hz) 7.49 (s, 1H, ar) 8.85 (s, 1H, ar) 12.52 (br s, 1H, NH); IR 3467, 3339, 1744, 1620.

6.7. General procedure to prepare 5,6-dihydro-5-oxo-pyrazolo[1,5-*c*]quinazoline-1,2-dicarboxylic acids 12,[23] 13, and 14

A solution of KOH (10% w/w, 7 mL) was added to a suspension of **2**–**4** (0.825 mmol) in ethanol (7 mL). The mixture was heated at 100 °C for 1 h. Addition of water (15 mL) and acidification with concentrated HCl afforded an unfilterable suspension that, after heating at 100 °C for 30 min, yielded a solid, which was collected by filtration and washed well with water.

6.7.1. 5,6-Dihydro-5-oxo-pyrazolo[1,5-*c*]quinazoline-1,2-dicarboxylic acid (12). Yield 90%; mp >300 °C (EtOH)^{1}H NMR 7.30–7.41 (m, 2H, ar) 7.58–7.66 (m, 1H, ar) 8.62 (d, 1H, ar, J = 8.0 Hz) 12.68 (br s, 1H, NH); IR 3450, 3300, 1750, 1720.

6.7.2. 8-Chloro-5,6-dihydro-5-oxo-pyrazolo[1,5-*c*]quinazoline-1,2-dicarboxylic acid (13). Yield 75%; mp >300 °C (EtOH) ^{1}H NMR 7.38–7.43 (m, 2H, ar) 8.74 (d, 1H, ar, J = 8.7 Hz) 12.36 (br s, 1H, NH).

6.7.3. 8,9-Dichloro-5,6-dihydro-5-oxo-pyrazolo[1,5-*c*]quinazoline-1,2-dicarboxylic acid (14). Yield 75%; mp >300 °C (AcOH) ^{1}H NMR 7.52 (s, 1H, ar) 9.14 (s, 1H, ar) 12.45 (br s, 1H, NH); IR 3444, 3257, 1737, 1712, 1672.

6.8. General procedure to prepare ethyl 5,6-dihydro-5-oxo-pyrazolo[1,5-*c*]quinazoline-1-carboxylates 28–30

Compounds **9**–**11** (0.54 mmol) were heated in an oil bath, over their melting points for 1 h. The cooled, fused mass was worked-up with ethanol, collected, and purified.

6.8.1. Ethyl 5,6-dihydro-5-oxo-pyrazolo[1,5-*c*]quinazoline-1-carboxylate (28). Yield 45%; mp >300 °C (DMF) ^{1}H NMR 1.36 (t, 3H, CH3, J = 7.3 Hz) 4.37 (q, 2H, CH2, J = 7.3 Hz) 7.35–7.40 (m, 2H, ar) 7.63 (t, 1H, ar, j = 7.8 Hz) 8.43 (s, 1H, ar, H-2) 9.15 (d, 1H, ar, J = 7.8 Hz) 12.40 (br s, 1H, NH).

6.8.2. Ethyl 8-chloro-5,6-dihydro-5-oxo-pyrazolo[1,5-*c*]quinazoline-1-carboxylate (29). Yield 50%; mp >300 °C (DMF) ^{1}H NMR 1.35 (t, 3H, CH_3, J = 7.0 Hz) 4.33 (q, 2H, CH_2, J = 7.0 Hz) 7.38–7.43 (m, 2H, ar) 8.44 (s, 1H, ar, H-2) 9.28 (d, 1H, ar, J = 8.4 Hz) 12.38 (br s, 1H, NH).

6.8.3. Ethyl 8,9-dichloro-5,6-dihydro-5-oxo-pyrazolo[1,5-*c*]quinazoline-1-carboxylate (30). Yield 60%; mp >300 °C (AcOH) ^{1}H NMR 1.34 (t, 3H, CH_3, J = 7.3 Hz) 4.34 (q, 2H, CH_2, J = 7.3 Hz) 7.51 (s, 1H, ar) 8.45 (s, 1H, ar, H-2) 9.51 (s, 1H, ar) 12.43 (br s, 1H, NH).

6.9. Synthesis of ethyl 1,8-dichloro-5,6-dihydro-5-oxo-pyrazolo[1,5-*c*]quinazoline-2-carboxylate (5)

To a suspension of ethyl 8-chloro-5,6-dihydro-5-oxo-pyrazolo[1,5-*c*]quinazolini-2-carboxylate[17] (2.40 mmol) in glacial acetic acid (15 mL), sulfuryl chloride (5.10 mmol) was added. The reaction mixture was heated at 60 °C for 2 h, and then cooled at room temperature. The solid was collected by filtration and washed with water. Yield 90%; mp 300–302 °C (DMF/H_2O) ^{1}H NMR 1.36 (t, 3H, CH_3, J = 7.0 Hz) 4.40 (q, 2H, CH_2, J = 7.0 Hz) 7.36–7.46 (m, 2H, ar) 8.44 (d, 1H, ar, J = 8.8 Hz) 12.34 (br s, 1H, NH); IR 3220, 1770, 1700.

6.10. Synthesis of 1,8-dichloro-5,6-dihydro-5-oxo-pyrazolo[1,5-*c*]quinazoline-2-carboxylic acid (15)

To a suspension of ester **5** (0.40 mmol) in methanol (3.25 mL), a solution of KOH (10% w/w, 3.25 mL) was added, and then the mixture was refluxed for 10 min. After cooling at room temperature, addition of water (15 mL) and acidification with concentrated HCl afforded a solid that was collected by filtration and washed with water. Yield 70%; mp >300 °C (DMF/H_2O) ^{1}H NMR 7.38–7.42 (m, 2H, ar) 8.44 (d, 1H, ar, J = 8.1 Hz) 12.33 (br s, 1H, NH).

6.11. Synthesis of ethyl 1,8,9-trichloro-5,6-dihydro-5-oxo-pyrazolo[1,5-*c*]quinazoline-2-carboxylate (6)

To a suspension of 3-diazoindole **27** (6.58 mmol) in anhydrous toluene (150 mL), an excess of ethyl propiolate (13.2 mmol) was added. The reaction mixture was refluxed, under nitrogen atmosphere and under stirring, for an overall time of 20 h. During this time, an equimolar amount of ethyl propiolate (13.2 mmol) was added three times at intervals of 4 h. The suspension was cooled at room temperature, and the solid, made up of a mixture of isomers **30** and **31** (overall yield 80%), was collected by filtration and washed with diethyl ether. From 100 mg of the mixture of **30** and **31**, only a few milligrams of pure ethyl 8,9-dichloro-5,6-dihydro-5-oxo-pyrazolo[1,5-*c*]quinazoline-2-carboxylate (**31**) was obtained after repeated crystallizations from acetic acid [mp >300 °C ^{1}H NMR 1.34 (t, 3H, CH_3, J = 7.0 Hz) 4.37 (q, 2H, CH_2, J = 7.0 Hz) 7.48 (s, 1H, ar) 7.81 (s, 1H, H-1) 8.55 (s, 1H, ar) 12.27 (br s, 1H, NH)]. Thus, the mixture of **30** and **31** was used without purification for the next step. Sulfuryl chloride (1.23 mmol) and a crystal of I_2 as catalyst were added to a suspension of the mixture of **30** and **31** in glacial acetic acid (15 mL). The reaction mixture was heated at 60 °C for 1 h and then, without cooling, the solid (compound **6**) was collected by filtration and washed with petroleum ether. Yield 25% (from **27**); mp >300 °C (AcOH); ^{1}H NMR 1.35 (t, 3H, CH_3, J = 7.1 Hz) 4.39 (q, 2H, CH_2, J = 7.1 Hz) 7.52 (s, 1H, ar) 8.49 (s, 1H, ar) 12.48 (br s, 1H, NH); IR 3216, 1777, 1696.

6.12. Synthesis of ethyl 1-bromo-8,9-dichloro-5,6-dihydro-5-oxo-pyrazolo[1,5-*c*]quinazoline-2-carboxylate (7)

The mixture of **30** and **31**, obtained starting from **27** (6.58 mmol) as above described for the preparation of

6, was suspended in glacial acetic acid (15 mL). The reaction mixture was heated at 70 °C, and then was added drop-wise to a solution of Br_2 (0.46 mmol) in glacial acetic acid (2 mL). Heating (70 °C) was continued for an overall time of 20 h. During this time, an equimolar amount of Br_2 (0.46 mmol) was added three times at intervals of 4 h. The resulting solid (compound **7**), without cooling, was collected by filtration and washed with petroleum ether. Yield 20% (from **27**); mp >300 °C (AcOH); ^{1}H NMR 1.35 (t, 3H, CH_3, J = 7.0 Hz) 4.39 (q, 2H, CH_2, J = 7.0 Hz) 7.54 (s, 1H, ar) 8.78 (s, 1H, ar) 12.45 (br s, 1H, NH); IR 3223, 1776, 1702.

6.13. General procedure to prepare 1,8,9-trichloro-5,6-dihydro-5-oxo-pyrazolo[1,5-*c*]quinazoline-2-carboxylic acid (16) and 1-bromo-8,9-dichloro-5,6-dihydro-5-oxo-pyrazolo[1,5-*c*]quinazoline-2-carboxylic acid (17)

The title compounds were obtained by alkaline hydrolysis of **6** and **7**, respectively, following the same experimental procedure described above to prepare **15**.

6.13.1. 1,8,9-Trichloro-5,6-dihydro-5-oxo-pyrazolo[1,5-*c*]-quinazoline-2-carboxylic acid (16). Yield 85%; mp >300 °C (EtOH); ^{1}H NMR 7.50 (s, 1H, ar) 8.47 (s, 1H, ar) 12.41 (br s, 1H, NH); IR 3650, 3451, 1747, 1684.

6.13.2. 1-Bromo-8,9-dichloro-5,6-dihydro-5-oxo-pyrazolo[1,5-*c*]quinazoline-2-carboxylic acid (17). Yield 90%; mp >300 °C (AcOH); ^{1}H NMR 7.50 (s, 1H, ar) 8.75 (s, 1H, ar) 12.41 (br s, 1H, NH); IR 3454, 3245, 1743, 1685.

6.14. Synthesis of ethyl 1-methyl-5,6-dihydro-5-oxo-pyrazolo[1,5-*c*]quinazoline-2-carboxylate (8)

To a suspension of 3-diazoindole **25** (5.99 mmol) in anhydrous toluene (40 mL), an excess of ethyl 2-butinoate (8.99 mmol) was added. The reaction mixture was refluxed, under nitrogen atmosphere and under stirring, for an overall time of 72 h. After 30 h, ethyl 2-butinoate (2.99 mmol) was added. Another addition of ethyl 2-butinoate (5.98 mmol) was made after 56 h. After the reaction was cooled at room temperature, the solid was collected by filtration. The residue, made up of a mixture of **8** and of its regio-isomer ethyl 2-methyl-5,6-dihydro-5-oxo-pyrazolo[1,5-*c*]quinazoline-1-carboxylate (**32**), was purified on silica gel column, eluting with $CHCl_3$/AcOEt/AcOH (8.5/1/0.5). Evaporation at reduced pressure of the solvent, of the first and the last elutes afforded compounds **8** and **32**, respectively.

6.14.1. Ethyl 1-methyl-5,6-dihydro-5-oxo-pyrazolo[1,5-*c*]quinazoline-2-carboxylate (8). Yield 35%; mp 293–295 °C (EtOH) ^{1}H NMR 1.35 (t, 3H, CH_3, J = 7.1 Hz) 2.68 (s, 3H, CH_3) 4.36 (q, 2H, CH_2, J = 7.1 Hz) 7.34–7.38 (m, 2H, ar) 7.50–7.53 (m, 1H, ar) 8.08 (d, 1H, ar H-10, J = 7.7 Hz) 12.04 (br s, 1H, NH); IR 3160, 1750, 1730.

6.14.2. Ethyl 2-methyl-5,6-dihydro-5-oxo-pyrazolo[1,5-*c*]quinazoline-1-carboxylate (32). Yield 20%; mp 249–251 °C (EtOH) ^{1}H NMR 1.36 (t, 3H, CH_3, J = 7.0 Hz) 2.51 (s, 3H, CH_3) 4.36 (q, 2H, CH_2, J = 7.0 Hz) 7.30–7.37 (m, 2H, ar) 7.59–7.61 (m, 1H, ar) 9.01 (d, 1H, ar H-10, J = 8.4 Hz) 12.13 (br s, 1H, NH); IR 3140, 1735, 1710.

6.15. Synthesis of 1-methyl-5,6-dihydro-5-oxo-pyrazolo[1,5-*c*]quinazoline-2-carboxylic acid (18)

The title compound was obtained by alkaline hydrolysis of **8**, following the same experimental procedure described above to prepare **15**. Yield 80%; mp 281–283 °C (EtOH) ^{1}H NMR 2.68 (s, 3H, CH_3) 7.33–7.52 (m, 3H, ar) 8.07 (d, 1H, ar H-10, J = 8.0 Hz) 11.97 (br s, 1H, NH); IR 3450, 3200, 1740.

7. Biochemistry

7.1. Binding assay

Rat cortical synaptic membrane preparation and [^{3}H]glycine, [^{3}H]AMPA, [^{3}H]-(+)-MK-801, and high-affinity [^{3}H]kainate binding experiments were performed, following the procedures described in Refs. 15, 17, 35, and 40, respectively.

7.2. Electrophysiological assay

The cortical wedge preparation described by Mannaioni et al.[41] was used, while the electrophysiolocical assays were performed following the procedures described in Ref. 17.

7.3. Sample preparation and results calculation

A stock 1 mM solution of the test compound was prepared in 50% DMSO. Subsequent dilutions were accomplished in buffer. The IC_{50} values were calculated from three or four displacement curves based on four to six scalar concentrations of the test compound using the ALLFIT computer program[42] and, in the case of tritiated glycine and AMPA binding, converted to K_i values, by application of the Cheng–Prusoff equation.[43] Under our experimental conditions, the dissociation constants (K_D) for [^{3}H]glycine (10 nM) and [^{3}H]-DL-AMPA (8 nM) were 75 ± 6 and 28 ± 3 nM, respectively.

8. Computational methodologies

All molecular modeling studies were carried out on a six-CPU (PIV 2.0–3.0 GHz) Linux cluster running under openMosix architecture.[44]

Three-dimensional structures of iGluR extracellular binding domains were obtained from the Brookhaven Protein Data Bank (PDB). For docking, the structure of glycine-binding domain S1S2 of NR1 subunit in complex with the antagonist **DCKA** (PDB code: 1PBQ)[32], and the structure of glutamate-binding domain S1S2 of GluR2 subunit in complex with the antagonist **DNQX** (PDB code: 1FTL)[33] were used. Since all X-ray crystallographic files do not contain hydrogen atoms, they need to be added to the protein by using

Molecular Operating Environment (MOE, version 2003.03) modeling suite[45], before carrying out docking studies. To minimize contacts between hydrogen atoms, the structures were subjected to Amber94 energy minimization until the rms of conjugate gradient was <0.15 kcal mol^{-1} $Å^{-1}$, keeping the heavy atoms fixed at their crystallographic positions.

PQZ derivatives were generated by using the Molecular Operating Environment suite. Atomic charges were assigned using the PEOE, implemented by MOE.[45] Each ligand was placed into the binding pockets and the semiflexible docking was achieved with the program SCHRÖDINGER/GLIDE.[46,47] The final docked complexes of ligand–receptor were selected according to the criteria of interaction energy combined with geometrical matching quality.

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