Tricyclic Heteroaromatic Systems. 1,2,4-Triazolo[4,3-*a*]quinoxalines and 1,2,4-Triazino[4,3-*a*]quinoxalines: Synthesis and Central Benzodiazepine Receptor Activity

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***Key Words**: 1,2,4-Triazolo[4,3-a]quinoxalines; 1,2,4-triazino[4,3-a]quinoxalines, synthesis; benzodiazepine receptor activity*

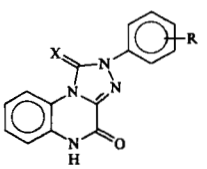
Summary

Some 1,2,4-triazolo[4,3-*a*]quinoxalines **1–10**, and 1,2,4-triazino[4,3-*a*]quinoxalines **11–12** were prepared and biologically evaluated for their binding at the benzodiazepine receptor (BZR) in rat cortical membranes. The BZR affinity of **1–10** demonstrates that the presence of a proton acceptor at position-1 is important for the potency of a BZR ligand. On the other hand, the BZR inactivity of the 1,2,5-trione derivatives **11–12** shows that the right collocation of the essential L_2 lipophilic substituent is of paramount importance for receptor-ligand interaction.

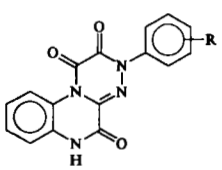
Introduction

Benzodiazepines (BDZs) are the most widely prescribed class of psychoactive drugs in current therapeutic use, despite the important side effects that they produce such as sedation, myorelaxation, ataxia, amnesia, ethanol and barbiturate potentiation and tolerance. Searching for safer BDZ-receptor (BZR) ligands, some research in our laboratory has been directed toward the synthesis, BZR binding activity, and structure-activity relationship (SAR) studies of some 6,6,5 tricyclic heteroaromatic systems, leading to the formulation of a schematic representation of some optional (a_1 and d) and essential (L_1, L_2, and a_2) pharmacophoric descriptors for receptor recognition of our 6,6,5-tricyclic BZR ligands [1–6].

With the aim of evaluating the importance of the presence of an exocyclic proton acceptor at position-1 of our tricyclic derivatives, in this paper we report on the synthesis, BZR binding activity, and SAR of some 1,2,4-triazolo[4,3-*a*]quinoxaline-1,4-diones **1–9**, 1,2,4-triazolo[4,3-*a*]quinoxalin-4-one **10**, and 1,2,4-triazino[4,3-*a*]quinoxaline-1,2,5-triones **11–12** (see Chart 1).



1-9: X = O
10: X = H_2

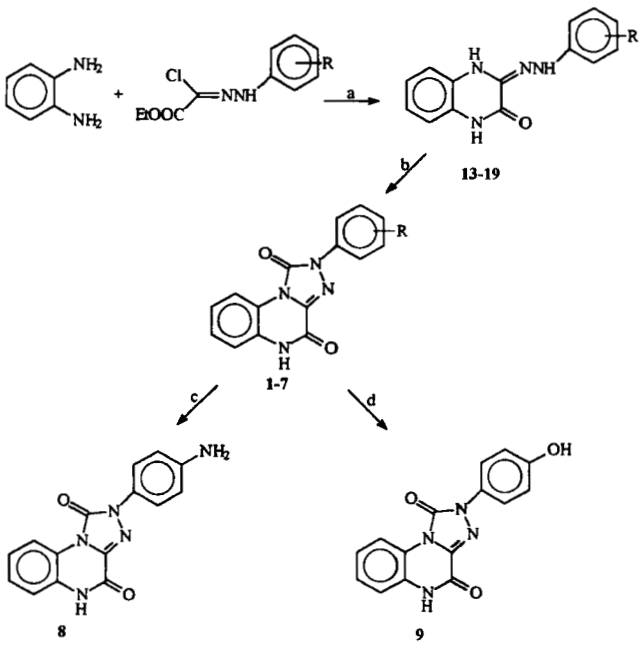


11-12

Chart 1

Chemistry

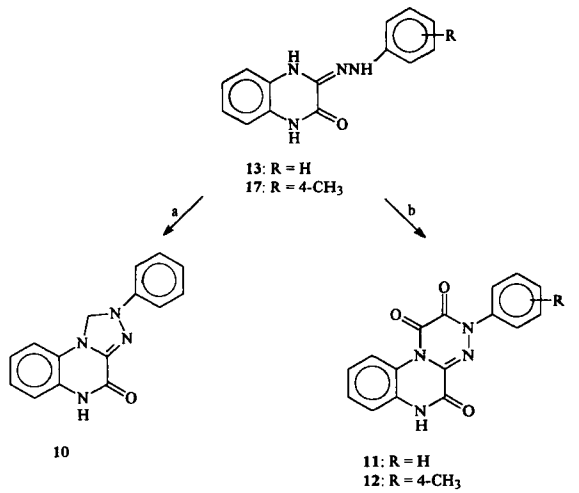
The synthesis of compounds **1–12** is illustrated in Schemes 1–2.



	R
13, 1	H
14, 2	2-F
15, 3	3-F
16, 4	3-CH_3
17, 5	4-CH_3
18, 6	4-OCH_3
19, 7	4-NO_2

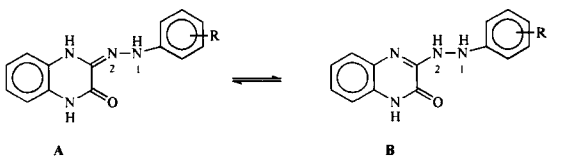
Scheme 1. a: Et_3N, EtOH. b: $(Cl_3CO)_2CO$, THF. c: 10% Pd/C, H_2, DMF. d: AcOH, 48% HBr.

  0365-6233/97/1212/0387 $17.50 +.50/0



Scheme 2. a: 40% HCHO, ethylene glycol. b: oxalyl chloride, THF.

By reacting *ortho*-phenylendiamine with ethyl N^1-arylhydrazono-N^2-chloroacetates [7–9] the corresponding 3-arylhydrazonoquinoxalin-2-ones **13**[10]**–19** were isolated (Scheme 1). These intermediates may exist in either one of the two tautomeric forms **A** and **B**:



The ^{1}H NMR spectra of **13–19** reveals the existence of both tautomers since there are six protons that exchange with deuterium oxide. Moreover, in compounds **16** (R = 3-CH_3), **17** (R = 4-CH_3), and **18** (R = 4-OCH_3) the existence of both tautomers is stressed by the presence of two singlets for each methyl group. In compounds **13–18** tautomer **A** is easily distinguished from tautomer **B** since in the former each exchangeable proton is present as singlet, while in the latter the two hydrazine protons appear as doublets. On the contrary, in the ^{1}H NMR spectrum of the 2-(4-nitrophenyl) derivative **19,** the two tautomers **A** and **B** cannot be differentiated since no doublets, due to the two hydrazine protons, can be detected (see Experimental Section).

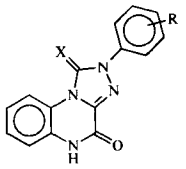
Cyclization with triphosgene of compounds **13–19** yielded the final tricyclic derivatives **1–7**. Catalytic reduction of 2-(4-nitrophenyl)- **7** and dealkylation of 2-(4-methoxyphenyl)- **6** afforded compounds **8** and **9**, respectively.

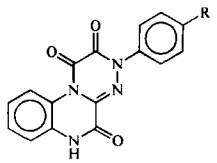
Finally, hydrazono-quinoxaline **13** was cyclized with formaldehyde to yield the fused triazoline **10**, while cyclization of **13** and **17** with oxalyl chloride gave the 3-aryl-1,2,4-triazino[4,3-*a*]quinoxaline-1,2,5-triones **11–12** (Scheme 2).

Results and Discussion

The binding activities of the reported compounds at the BZR in rat cortical membranes was determined by displacement experiments with radiolabeled [^{3}H]flunitrazepam and are listed in Table 1.

Table 1. Binding activity of 1,2,4-triazolo[4,3-*a*]quinoxalines **1–10** and 1,2,4-triazino[4,3-*a*]quinoxalines **11–12**.





Cpd	R	X	K_i ± SEM (nM)[a]
1	H	O	159 ± 17
2	2-F	O	16.3 ± 0.8
3	3-F	O	1536 ± 221
4	3-CH_3	O	3129 ± 387
5	4-CH_3	O	6293 ± 500
6	4- OCH_3	O	545 ± 49
7	4-NO_2	O	>10000
8	4-NH_2	O	>10000
9	4-OH	O	203 ±18
10	H	H_2	2168 ± 770
11	H		>10000
12	4-CH_3		>10000

[a] *Ki* values are means ± SEM of 3–5 separate determinations.

The binding activities of **1–10** are on the whole higher than those of some previously reported pyrazolo[3,4-*c*]quinolin-4-ones [6] which are devoid of the optional proton acceptor at position-1, with the exception of the 2-(4-nitrophenyl)- **7** and 2-(4-aminophenyl)-derivative **8** which are completely inactive. This supports our hypothesis of the beneficial effect of the presence of the exocyclic oxygen atom at position-1 as optional proton acceptor able to reinforce receptor-ligand interaction. This suggestion is confirmed by the decreased BZR affinity of the reduced derivative **10**. However, the beneficial effect of the exocyclic proton acceptor at position-1 is not so good as an endocyclic one, as evidenced by the higher BZR affinity of some previously reported 1,2,4-triazolo[1,5-*a*]quinoxalines [1, 3] and 1,2,4-triazolo[1,5-*c*]quinazolines [11] .In the triazolo-quinoxalines **1–10** the appended 2-phenyl moiety does not support any substitution, with the exception of an *ortho*-fluorine atom. Indeed, the 2-(2-fluorophenyl)-1,2,4-triazolo[4,3-*a*]quinoxalin-1,4-dione **2** is the most active among the reported compounds (K_i = 16.3 nM). Comparison of BZR affinity of **2** with that of the corresponding triazolo[1,5-*c*]quinazolin-5-one (IC_{50} = 0.4 nM)[11] evidences the beneficial effect of an endocyclic proton acceptor at position-1 with respect to an exocyclic one.

The beneficial effect of the presence of an *ortho*-fluoro substituent on the 2-phenyl moiety is in agreement with previous data [3–4, 11] .

It is difficult to rationalise the effect of the other substituents on the appended 2-phenyl. In fact, the 4-hydroxyphenyl- **9** has the same order of BZR affinity as the unsubstituted **1**, while the 4-aminophenyl- **8** is completely inactive, although the amino and hydroxy groups have similar hydrophobicity and electronic properties. The BZR inactivity of compound **8** cannot be attributed to the different bulk of the 4-amino group, with respect to the 4-hydroxy one, since the 4-methoxyphenyl- **6** is 10-fold more active than the 4-methylphenyl- **5**. It has to be noted that the presence on the 2-phenyl substituent of two groups with opposite electronic effects, such as 4-nitro (compound **7**) and 4-amino (compound **8**), produce the same drammatic effect on the binding activity.

Finally, the synthesis of the 6,6,6-tricyclic triazino-quinoxalines **11–12** was performed to evaluate whether a reinforced hydrogen bond in the a_1 region could enhance the BZR affinity. Compounds **11–12** have in the a_1 region two carbonyl oxygens which, due to the lone pair orientation, could engage in a strong hydrogen bond with the optional proton donor of the receptor site. However, the enlargement of the five-membered ring to a six-membered one shifts the aryl substituent into a position which can no longer be accommodated in the L_2 lipophilic receptor area (see Figure 1). The lack of the occupation of the L_2 essential lipophilic area justifies the inactivity of compounds **11–12**.

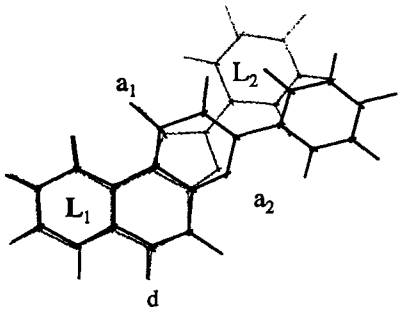


Figure 1. Superposition of 2-phenyl-1,2,4-triazolo[1,5-*a*]quinoxaline-1,4-dione **1** (light line) and 3-phenyl-1,2,4-triazino[4,3-*a*]quinazoline-1,2,5-trione **11** (dark line).

In conclusion, the synthesis and the binding data of the 1,2,4-triazolo-quinoxalines **1–10,** and that of their enlarged derivatives 1,2,4-triazino-quinoxaline-1,2,5-triones **11–12** shed some more light on the structural requirements of tricyclic heteroaromatic system BZR ligands. They, in fact, confirm our hypothesis that the presence of an exocyclic optional a_1 proton acceptor enhances the ligand potency, even though to a lesser extent than an endocyclic one, and that the right collocation of the essential L_2 appended aromatic substituent is of paramount importance for receptor-ligand recognition.

Acknowledgement

Valuable assistance in animal handing was provided by MG Giovannini.

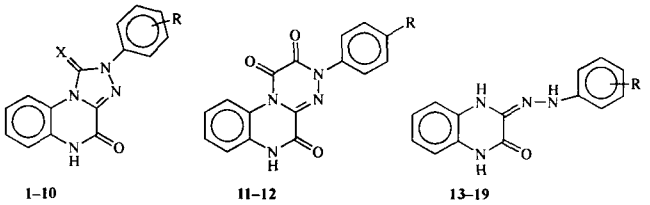
Experimental Section

Chemistry

Silica gel plates (Merck F_{254}) 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 analyser for C, H, N, and the results were within ± 0.4% of the theoretical values. The IR spectra were recorded with a Perkin-Elmer 1420 spectrometer in Nujol mulls and are expressed in cm^{-1}. The ^1H NMR spectra were obtained with a Varian Gemini 200 instrument at 200 MHz. The chemical shifts are reported in δ (ppm) and are relative to the central peak of the solvent. The following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad, and ar = aromatic protons. The physical data of the newly synthesised compounds are shown in Table 2.

The molecular geometry of compounds **1** and **11** shown in Figure 1 were minimised by the MOPAC 6.0 semiempirical MO package [12] using AM1 parametrization running on a IBM RISC 6000 3CT workstation.

Table 2. Physical data of triazolo-quinoxalines **1–10**, triazino-quinoxalines **11–12**, and of intermediates **13–19**.



1–10 **11–12** **13–19**

Cpd	R	X	mp, °C	solv[a]	% yield
1	H	O	>300	A	94
2	2-F	O	>300	B	18
3	3-F	O	>300	C	73
4	3-CH_3	O	>300	C	31
5	4-CH_3	O	>300	D	72
6	4-OCH_3	O	>300	B	94
7	4-NO_2	O	>300	B	92
8	4-NH_2	O	>300	D	62
9	4-OH	O	>300	B	92
10	H	H_2	271–273 dec	E	80
11	H		>300	D	22
12	4-CH_3		297–299 dec	F	50
13[b]	H		257–258 dec	G	72
14	2-F		238–240 dec	H	60
15	3-F		256–257 dec	C	69
16	3-CH_3		221–223 dec	I	73
17	4-CH_3		258-259 dec	J	78
18	4-OCH3		231–233 dec	I	67
19	4-NO_2		278–280 dec	C	80

[a]Recrystallization solvents: A = dioxane. B = ethanol/dimethylformamide. C = glacial acetic acid. D = dimethylformamide. E = ethylene glycol. F = glacial acetic acid/water. G = nitromethane. H = methanol. I = acetonitrile. J = glacial acetic acid/ethanol. [b]Ref.[10]: mp 260 °C (dimethylformamide).

General procedure for the synthesis of 1,2,3,4-tetrahydro-3-arylhydrazono-quinoxalin-2-ones **13**[10]**–19**

A mixture of the suitable ethyl N^1-arylhydrazono-N^2-chloroacetate [7–9] (2 mmol), *ortho*-phenylenediamine (2 mmol) and triethyl amine (2.4 mmol) in ethanol (30 mL) was refluxed for 3 h. Upon cooling the mixture yielded a solid which was collected, washed with water (10–15 mL) and ethanol (5–6 mL), and recrystallized. Compounds **13–19** displayed the following spectral data:

13: ^{1}H NMR (DMSO-d$_6$): 6.66–7.30 (m, ar), 7.82 (d, N_1-H of tautomer **B**, J = 1.2 Hz), 8.76 (s, NH of tautomer **A**), 9.38 (d, N_2-H of tautomer **B**, J = 1.2 Hz), 9.58 (s, NH of tautomer **A**), 11.09 (s, lactam NH of tautomer **A**), 12.28 (s, lactam NH of tautomer **B**).

14: ^{1}H NMR (DMSO-d$_6$): 6.71–7.51 (m, ar), 7.72 (d, N_1-H of tautomer **B**, J = 1.4 Hz), 8.57 (s, NH of tautomer **A**), 9.46 (d, N_2-H of tautomer **B**, J = 1.4 Hz), 10.08 (s, NH of tautomer **A**), 11.20 (s, lactam NH of tautomer **A**), 12.32 (s, lactam NH of tautomer **B**).

15: ^{1}H NMR (DMSO-d$_6$): 6.46–6.64 (m, H ar), 6.88–7.31 (m, H, ar), 8.14 (d, N_1-H of tautomer **B**, J = 1.4 Hz), 9.04 (s, NH of tautomer **A**), 9.45 (d, N_2-H of tautomer **B**, J = 1.4 Hz), 9.68 (s, NH of tautomer **A**), 11.17 (s, lactam NH of tautomer **A**), 12.29 (s, lactam NH of tautomer **B**).

16: ^{1}H NMR (DMSO-d$_6$): 2.21 (s, CH_3 of tautomer **B**), 2.28 (s, CH_3 of tautomer **A**), 6.54–6.70 (m, H, ar), 6.80–7.31 (m, H, ar), 7.74 (d, N_1-H of tautomer **B**, J = 1.6 Hz), 8.72 (s, NH of tautomer **A**), 9.35 (d, N_2-H of tautomer **B**, J = 1.6 Hz), 9.56 (s, NH of tautomer **A**), 11.11 (s, lactam NH of tautomer **A**), 12.28 (s, lactam NH of tautomer **B**).

17: ^{1}H NMR (DMSO-d$_6$): 2.18 (s, CH_3 of tautomer **B**), 2.23 (s, CH_3 of tautomer **A**), 6.69–7.29 (m, ar), 7.65 (d, N_1-H of tautomer **B**, J = 2.7 Hz), 8.66 (s, NH of tautomer **A**), 9.35 (d, N_2-H of tautomer **B**, J = 2.7 Hz), 9.54 (s, NH of tautomer **A**), 11.06 (s, lactam NH of tautomer **A**), 12.27 (s, lactam NH of tautomer **B**).

18: ^{1}H NMR (DMSO-d$_6$): 3.65 (s, OCH_3 of tautomer **B**), 3.70 (s, OCH_3 of tautomer **A**), 6.77–7.30 (m, ar), 7.48 (d, N_1-H of tautomer **B**, J = 3.0 Hz), 8.50 (s, NH of tautomer **A**), 9.37 (d, N_2-H of tautomer **B**, J = 3.0 Hz), 9.48 (s, NH of tautomer **A**), 11.05 (s, lactam NH of tautomer **A**), 12.26 (s, lactam NH of tautomer **B**).

19: ^{1}H NMR (DMSO-d$_6$): 6.83–7.27 (m, H, ar), 8.05–8.07 (m, H, ar), 9.30 (br s, NH), 9.80 (br s, 2NH), 11.40 (br s, NH), 12.0 (br s, lactam NH), 12.4 (br s, lactam NH).

General procedure for the synthesis of 2-aryl-1,2,4,5-tetrahydro-1,2,4-triazolo[4,3-a]quinoxaline-1,4-diones **1–7**

Method A. A mixture of **13–14, 16–18** (2 mmol) and triphosgene (2 mmol) in anhydrous tetrahydrofuran (20 mL) was stirred at room temperature for 3 h. Dilution with water (50 mL) yielded crude **1–2, 4–6**, which were collected and recrystallized. Compounds **1–2, 4–6** displayed the following spectral data:

1: ^{1}H NMR (DMSO-d$_6$): 7.28–7.41 (m, 4H, ar), 7.53–7.61 (m, 2H, ar), 8.02 (d, 2H, ar, J = 7.4 Hz), 8.62 (d, 1H, ar, J = 7.9 Hz), 11.97 (s, 1H, NH).– IR: 1690, 1720, 3050, 3140.

2: ^{1}H NMR (DMSO-d$_6$): 7.24–7.69 (m, 7H, ar), 8.56 (d, 1H, ar, J = 8.3 Hz), 11.99 (s, 1H, NH).– IR: 1695, 1740, 3050, 3140.

4: ^{1}H NMR (DMSO-d$_6$): 2.42 (s, 3H, CH_3), 7.17–7.48 (m, 5H, ar), 7.82–7.84 (m, 2H, ar), 8.62 (d, 1H, ar, J = 8.3 Hz), 11.98 (s, 1H, NH).– IR: 1695, 1728, 3050.

5: ^{1}H NMR (DMSO-d$_6$): 2.36 (s, 3H, CH_3), 7.24–7.38 (m, 5H, ar), 7.88 (d, 2H, ar, J = 8.4 Hz), 8.61 (d, 1H, ar, J = 7.6 Hz), 11.95 (s, 1H, NH).– IR: 1690, 1720, 3050, 3140.

6: ^{1}H NMR (DMSO-d$_6$): 3.82 (s, 3H, OCH_3), 7.12 (d, 2H, ar, J = 9.1 Hz), 7.27–7.36 (m, 3H, ar), 7.80 (d, 2H, ar, J = 9.1 Hz), 8.61 (d, 1H, ar, J = 8.0 Hz), 11.95 (s, 1H, NH).– IR: 1685, 1715, 3140.

Method B. A mixture of **15, 19** (2 mmol) and triphosgene (4 mmol) in anhydrous tetrahydrofuran (30 mL) was refluxed for 5 h. Dilution with water (50 mL) yielded crude **3, 7**, which were collected and recrystallized. Compounds **3** and **7** displayed the following spectral data:

3: ^{1}H NMR (DMSO-d$_6$): 7.20–7.39 (m, 4H, ar), 7.58–7.69 (m, 1H, ar), 7.83–7.91 (m, 2H, ar), 8.60 (d, 1H, ar, J = 7.6 Hz), 11.99 (s, 1H, NH).– IR: 1690, 1735, 3050, 3140.

7: ^{1}H NMR (DMSO-d$_6$): 7.29–7.40 (m, 3H, ar), 8.33 (d, 2H, ar, J = 9.4 Hz), 9.45 (d, 2H, ar, J = 9.4 Hz), 8.58 (d, 1H, ar, J = 8.1 Hz), 12.05 (s, 1H, NH).– IR: 1695, 1720, 3120.

2-(4-Aminophenyl)-1,2,4,5-tetrahydro-1,2,4-triazolo[4,3-a]quinoxaline-1,4-dione **8**

Pd/C (10%, 140 mg) was added to a hot solution of **7** (2.1 mmol) in dimethylformamide (30 mL). The mixture was hydrogenated in a Parr apparatus at 30 psi for 2 h. The mixture was then heated and the catalyst filtered off. Upon cooling the solution yielded a solid which was collected and recrystallized..– ^{1}H NMR (DMSO-d$_6$): 5.37 (s, 2H, NH_2), 6.69 (d, 2H, ar, J = 8.7 Hz), 7.26–7.35 (m, 3H, ar), 7.53 (d, 2H, ar, J = 8.7 Hz), 8.61 (d, 1H, ar, J = 8.1 Hz), 11.87 (s, 1H, NH).– IR: 1715, 3270, 3380, 3480.

1,2,4,5-Tetrahydro-2-(4-hydroxyphenyl)-1,2,4-triazolo[4,3-a]quinoxaline-1,4-dione **9**

A suspension of **6** (1 mmol) in glacial acetic acid (3 mL) and hydrobromic acid (48%, 12.4 mL) was refluxed for 20 h. Upon cooling a solid was obtained which was collected, washed with ethanol (2–3 mL) and recrystallized.– ^{1}H NMR (DMSO-d$_6$): 6.92 (d, 2H, ar, J = 8.9 Hz), 7.25–7.34 (m, 3H, ar), 7.73 (d, 2H, ar, J = 8.9 Hz), 8.59 (d, 1H, ar, J = 8.1 Hz), 9.77 (s, 1H, OH), 11.92 (s, 1H, NH).– IR: 1670, 1725, 3140, 3200, 3330.

1,2,4,5-Tetrahydro-2-phenyl-1,2,4-triazolo[4,3-a]quinoxalin-4-one **10**

Formaldehyde (40%, 0.1 mL) was added to a suspension of **13** (1.2 mmol) in ethylene glycol (5 mL). The mixture was refluxed for 10 min. Upon cooling a solid was obtained which was collected, washed with water and recrystallized.– ^{1}H NMR (DMSO-d$_6$): 5.74 (s, 2H, CH_2), 6.89–7.18 (m, 7H, ar), 7.30–7.39 (m, 2H, ar), 11.50 (s, 1H, NH).– IR: 1680, 3040–3180.

General procedure for the synthesis of 3-aryl-2,3,5,6-tetrahydro-1H-1,2,4-triazino[4,3-a]quinoxaline-1,2,5-triones **11–12**

A suspension of **13, 17** (1 mmol) in anhydrous tetrahydrofuran (15 mL) at 0 °C and under nitrogen atmosphere was slowly added (1 h) to oxalyl chloride (40 mmol). The mixture was allowed to stand at room temperature for 15 min. Evaporation at reduced pressure of the solvent and of the excess of oxalyl chloride yielded a residue which was dissolved in water (10 mL). Upon alkalinization with a 2N solution of sodium carbonate a solid was obtained which was collected and recrystallized. Compounds **11–12** displayed the following spectral data:

11: ^{1}H NMR (DMSO-d$_6$): 7.20–7.69 (m, 8H, ar), 8.83 (d, 1H, ar, J = 8.4 Hz), 11.95 (s, 1H, NH).– IR: 1665, 1710, 1720, 3270.

12: ^{1}H NMR (DMSO-d$_6$): 2.40 (s, 3H, CH_3), 7.19–7.27 (m, 2H, ar), 7.35–7.47 (m, 3H, ar), 7.55 (d, 2H, ar, J = 8.1 Hz), 8.83 (d, 1H, ar, J = 8.6 Hz), 11.91 (s, 1H, NH).– IR: 1680, 1700, 1710, 3175.

Biochemistry

Crude synaptic membranes were prepared from cerebral cortices of male Sprague-Dawley rats (170–250 g). The tissue was homogenized in 15 vol of ice-cold 0.32 M sucrose, containing 20 mg/ml of phenylmethanesulfonyl fluoride, using a glass-Teflon homogenizer (clearance = 0.15–0.23 mm). The homogenate was centrifuged at 1000 × g for 10 min and the resulting supernatant further centrifuged at 20000 × g for 20 min. The final pellet was resuspended in 15 vol of ice-cold distilled water, dispersed with an Ultra-Turrax sonicator (30% of maximum speed) for 30 s, and centrifuged at 8000 × g for 20 min. The membranes were resuspended once more in distilled water, centrifuged, and frozen at –70 °C.

On the day of the experiment, appropriate amounts of membranes were thawed at room temperature, resuspended (0.5 mg of protein/mL) in 0.05 M of Tris/HCl buffer, at pH 7.4, containing 0.01% (v/v) Triton X-100, incubated at 37 °C for 60 min. The membranes were then washed with two additional resuspension and centrifugation cycles and finally resuspended in cold Tris-HCl buffer to yield 0.2–0.3 mg of protein/assay tube. [^{3}H]Flunitrazepam (83.4 Ci/mmol) binding assays were carried out in ice for 60 min at 1 nM of ligand concentration in a total of 0.5 mL vol. Bound radioactivity was separated by rapid filtration through Whatman GF/B filters using a Brandel cell harvester. Non-specific binding was determined in the presence of 10 mM of diazepam. The IC_{50} values were calculated from displacement curves based on four to six scalar concentrations of the test compound in triplicate, using the ALLFIT computer program [13], and converted to K_i values by application of the Cheng-Prusoff equation[14]. A stock of 1 mM of the test compound was prepared in ethanol. Subsequent dilutions were accomplished in buffer. Ethanol up to a final 1% concentration was seen to affect [^{3}H]flunitrazepam binding only negligibly.

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