Structure-Activity Relationship Studies of Novel Pyrazolo[1,5-*c*][1,3]benzoxazines: Synthesis and Benzodiazepine Receptor Affinity

Flavia Varano[a], Daniela Catarzi[a], Vittoria Colotta[a], Lucia Cecchi[a]*, Guido Filacchioni[a], Alessandro Galli[b], and Chiara Costagli[b]

[a] Dipartimento di Scienze Farmaceutiche, Università di Firenze, Via Gino Capponi 9, 50121 Firenze, Italy

[b] Dipartimento di Farmacologia Preclinica e Clinica, Università di Firenze, Viale G.B. Morgagni 65, 50134 Firenze, Italy

***Key Words**: pyrazolo[1,5-c][1,3]benzoxazines; benzodiazepine receptor ligands; pharmacophoric descriptors*

Summary

Some 2-arylpyrazolo[1,5-*c*][1,3]benzoxazin-5-ones **1** and 5-oxopyrazolo[1,5-*c*][1,3]benzoxazin-2-carboxylates **2** were prepared and biologically evaluated for their binding at benzodiazepine receptor (BZR) in rat cortical membranes. Structure-activity relationship studies suggest that, although proton donor d and proton acceptor a_1 are both optional pharmacophoric descriptors, at least one of them must be present for good BZR affinity. When the proton donor d is not present, the heteroatom acceptor a_1 is necessary either in the tricyclic core or in the appended substituent at the C-2 to obtain sub-micromolar BZR affinity.

Introduction

The tranquillizing and anticonvulsant activity of benzodiazepines (BDZ) in clinical use is due to the allosteric modulation of the major inhibitory neurotransmitter, the γ-aminobutyric acid (GABA) at its $GABA_A$ receptor. BDZ and non-BDZ agonists positively modulate the action of GABA response by binding at the so-called benzodiazepine receptor (BZR). However, the modulation of the GABA response may also be negative, and thus anxiogenic and convulsive, via the binding of an inverse agonist to BZR. The interaction of both agonists and inverse agonists with the BZR is inhibited by the interaction of an antagonist with this receptor. It follows that all three classes of BZR ligands bind to the same location of the receptor. Although the binding sites corresponding to each of these functional states of the receptor may overlap only partially, agonists, antagonists and inverse agonists must share common pharmacophoric descriptors consistent with a single binding domain[1–2].

An enormous amount of structure-activity relationship (SAR) data available for a large number of diverse structural classes of ligands has resulted in the formulation of several models for the pharmacophore for BZR binding[3]. Recently we proposed a two-dimensional schematic representation for the binding to the BZR of some 6,6,5-tricyclic heteroaromatic compounds in which some essential and optional pharmacophoric descriptors (see Figure 1) were identified[4–5].

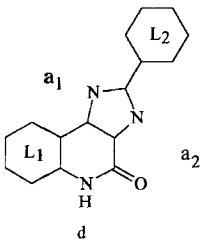


Figure 1. Schematic two-dimensional representation of the essential and optional pharmacophoric descriptors of a BZR ligand. Essential pharmacophoric descriptors are: L_1 and L_2 lipophilic areas, and a_2 proton acceptor site. Optional binding sites, only affecting the potency of a ligand, are: d proton donor and a_1 proton acceptor sites.

The essential pharmacophoric descriptors were thought to be two lipophilic substituents called L_1 and L_2 and a proton acceptor atom designated a_2, while the optional descriptors, which were not necessary for receptor-ligand interaction but only affected the potency of a ligand, were a proton acceptor site called a_1 and a proton donor site called d. Our hypothesis on the optionality of the donor site d agrees with other models[1–2] and is also supported by the synthesis and binding activity of compounds devoid of the pharmacophoric descriptor d[6–11]. The optionality of the acceptor a_1 has been demonstrated by the synthesis and BZR affinity of some pyrazolo-quinazolines[5].

However, only two compounds not bearing both d and a_1 optional sites are known to bind with micromolar affinity to BZR, namely 2-phenylpyrazolo[1,5-*c*][1,3]benzoxazin-5-one **1a**[4] and ethyl 5-oxopyrazolo[1,5-*c*][1,3]benzoxazin-2-carboxylate **2a**[4]. Thus, in order to ascertain whether compounds devoid of both d and a_1 pharmacophoric descriptors could bind to the BZR, a hypothesis which cannot be based on the binding activity of just two compounds, the synthesis and BZR binding activity of further pyrazolobenzoxazines **1b–f** and **2b–e**, analogs of **1a** and **2a**, respectively, are reported.

Chemistry

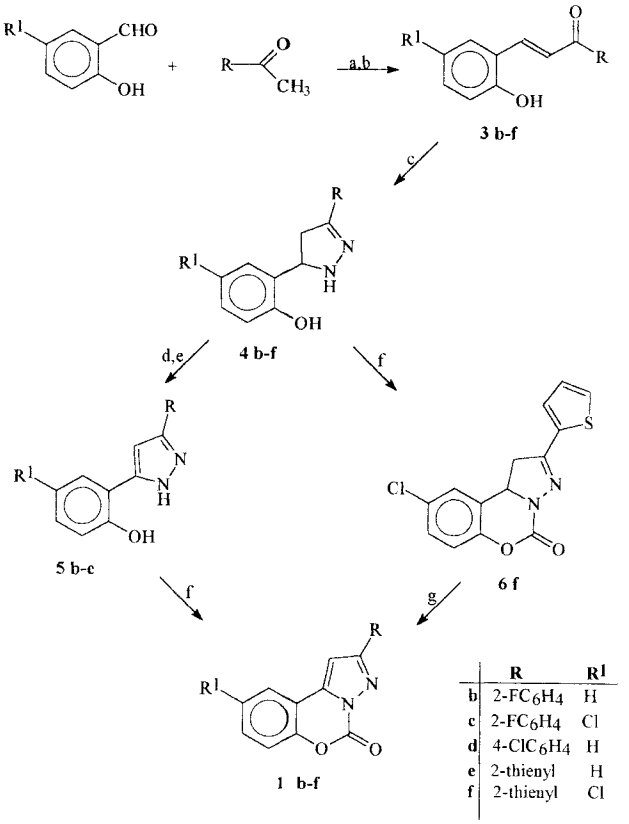
The general method for the synthesis of the pyrazolobenzoxazines **1b–f** and **2b–e** is illustrated in Schemes 1 and 2.

In Scheme 1 the synthetic pathway which led to the 2-arylpyrazolo[1,5-*c*][1,3]benzoxazin-5-ones **1b–f** is de-

  0365-6233/96/1212-0529 $5.00 + .25/0

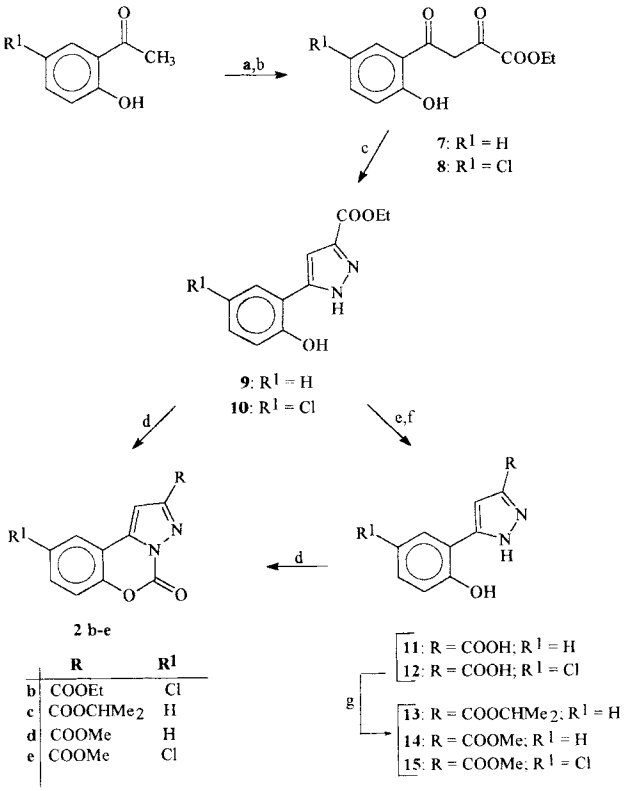
scribed. Briefly, the reaction of *ortho*-hydroxyaryl aldehydes and suitable ketones gave the 1-aryl-3-(2-hydroxyaryl)-2-propen-1-ones **3b–f** which were reacted with hydrazine to yield the 4,5-dihydro-3-aryl-5-(2-hydroxyaryl)pyrazoles **4b–f**. Dehydrogenation with lead tetraacetate of 4,5-dihydropyrazoles **4b–e** and treatment of the crude products with concentrated hydrochloric acid and ethanol afforded the key intermediate pyrazoles **5b–e**. The latter were cyclized with triphosgene to final tricyclic derivatives **1b–e**.

However, dehydrogenation by the above described method of the 4,5-dihydro-3-(2-thienyl)-5-(2-hydroxy-5-chlorophenyl)pyrazole **4f** gave only traces of the corresponding pyrazole. Thus, the final tricyclic derivative **1f** was obtained following a different procedure. Compound **4f** was cyclized with triphosgene to 5,10b-dihydro-2-(2-thienyl)-9-chloro-1H-pyrazolo[1,5-*c*][1,3]benzoxazin-5-one **6f** which was dehydrogenated with tetrachloro-1,2-benzoquinone to yield the final derivative **1f**.



Scheme 1. a: KOH, EtOH/H_2O. b: 6N HCl. c: 55% N_2H_4, EtOH. d: $Pb(AcO)_4$, CH_2Cl_2. e: conc. HCl, EtOH. f: $(CCl_3O)_2CO$, Et_3N, THF. g: tetrachloro-1,2-benzoquinone, toluene.

Scheme 2 shows the general synthetic method followed to prepare the 5-oxopyrazolo[1,5-*c*][1,3]benzoxazin-2-carboxylic esters **2b–e**. Reaction of 2-hydroxyacetophenones with diethyl oxalate yielded the ethyl 4-(2-hydroxyaryl)-2,4-dioxobutanoates **7** and **8**. The latter compounds gave the pyrazoles **9** and **10** by reaction with hydrazine. Alkaline hydrolysis of **9** and **10** followed by acidification provided the carboxylic acids **11** and **12** which were transformed into esters **13–15**. Key intermediate esters **9–10, 13–15** yielded the final tricyclic derivatives **2b–e** by means of triphosgene.



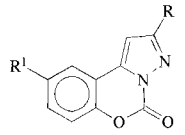
Scheme 2. a: $(COOEt)_2$, NaH. b: AcOH. c: 55% N_2H_4, EtOH. d: $(CCl_3O)_2CO$, Et_3N, THF. e: 10% NaOH, EtOH. f: 6N HCl. g: ROH, conc. H_2SO_4.

Biochemistry

Compounds **1b–f** and **2b–e** were tested for their ability to displace [^{3}H]flunitrazepam (1 nM, K_D = 2.3 nM) from its specific binding sites in rat brain cortical membranes. The BZR affinities of the tested compounds, expressed as K_i, are listed in Table 1 together with the *in vitro* efficacy trends of the most active ones (K_i < 100 nM), expressed by the GABA ratio (GR). The GR (or GABA shift) is the ratio between the receptor affinity of a ligand measured as the concentration of the displacer capable of inhibiting 50% of [^{3}H]flunitrazepam binding (IC_{50}) in the absence and in the presence of GABA. By this convenient *in vitro* method the BZR ligands can roughly be divided into agonists, inverse agonists or antagonists. In fact, the affinity of agonists is enhanced by GABA, that of inverse agonists is decreased in the presence of GABA, while that of antagonists is unaffected by the presence of GABA.

In Table 1 the K_i of 2-phenylpyrazolo[1,5-*c*][1,3]benzoxazin-5-one **1a**[4] and ethyl 5-oxopyrazolo[1,5-*c*][1,3]benzoxazin-2-carboxylate **2a**[4] are also reported.

Table 1. Binding constants at BZR[a].

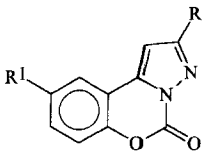


Compd	R	R^1	K_i (nM)[b]	GR[c]
1a[d]	C_6H_5	H	6573±909	
1b	2-FC_6H_4	H	6294±630	
1c	2-FC_6H_4	Cl	2517±700	
1d	4-ClC_6H_4	H	>10000	
1e	2-thienyl	H	560±42	
1f	2-thienyl	Cl	241±31	
2a[d]	COOEt	H	196±14	
2b	COOEt	Cl	92±5	0.84
2c	$COOCHMe_2$	H	861±68	
2d	COOMe	H	126±14	
2e	COOMe	Cl	58±10	0.66

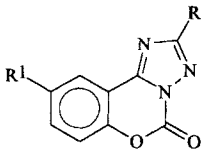
[a] The assays were carried out using a 1 mM solution of the test compound in 50% ethanol. Subsequent dilutions were accomplished in buffer. [b] K_i values are means ± SEM of 3–5 separate determinations. [c] GR (GABA ratio): IC_{50}(compound)/IC_{50}(compound + 0.1 mM GABA). [d] Ref. [4].

Results and Conclusions

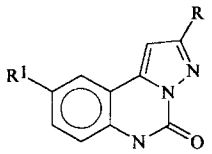
At first sight, the data listed in Table 1 show that the pyrazolobenzoxazines of series **1** and **2**, although devoid of both the pharmacophoric descriptors d and a_1, seem to bind to the BZR. Series **1** and **2** compounds are 1-deaza analogs of 1,2,4-triazolo[1,5-*c*][1,3]benzoxazin-5-ones[11] and isosters of pyrazolo[1,5-*c*]quinazolines[5] some of which displayed nanomolar BZR affinity (see Chart 1). The affinities of pyrazolo-benzoxazines of series **1** and **2** are in the micromolar range, except for the 4-chlorophenyl derivative **1d**, which in accordance with previous data [5, 11] is completely inactive.



Pyrazolo-benzoxazines



Triazolo-benzoxazines



Pyrazolo-quinazolines

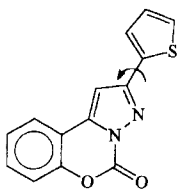
Chart 1

The GR values of the most active compounds **2b** and **2e** are those of partial inverse agonists like their 1-aza analogs triazolobenzoxazines[11]. Comparison of the BZR affinity of the esters of series **2** indicates that the smaller the ester group the better the binding activity[5]. In contrast with previous findings[5, 11] the presence of the 9-chlorosubstituent in these novel pyrazolobenzoxazines increases the affinity. In fact, the 9-chloro substituted **1c, 1f, 2b,** and **2e** displayed higher BZR affinity than their corresponding 9-unsubstituted analogs **1b, 1e, 2a,** and **2d**, respectively.

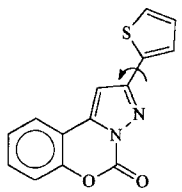
The SAR on the pyrazolobenzoxazines are thus similar to those of their 1-aza analog triazolobenzoxazines[11] and isoster pyrazoloquinazolines[5], which bind to the BZR according to the schematic representation shown in Figure 1. It follows that the pyrazolobenzoxazines also bind to the BZR in a similar way. This seems to suggest that both proton donor d and proton acceptor a_1 may be absent, being optional binding sites only affecting the potency of a BZR ligand.

However, a better observation of the binding data reported in Table 1 shows that the esters of series **2** are more active than the 2-aryl derivatives of series **1** and that there is a difference in binding activity among the 2-aryl derivatives of series **1**: the 2-thienyl **1e** and **1f** are the most active within them.

The higher BZR affinity of **1e**, **1f**, and series **2** compounds may be explained either by the existence in the BZR, corresponding to the L_2 lipophilic area, of the small hydrophilic pocket proposed by Crippen[12], which would accommodate the sulfur atom of the thienyl moiety or the carbonyl oxygen of the esters, or by the preferred *anti*-conformation of these same heteroatoms at the C-2 in the biologically active form[2]. In fact, the 2-thienyl substituent and the 2-carbethoxy group can exist in either two low-energy conformations, *syn* or *anti* (see Figures 2 and 3).

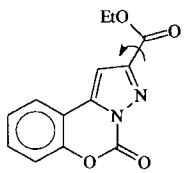


syn-conformation

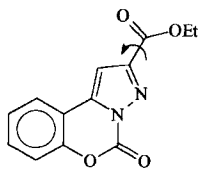


anti-conformation

Figure 2. The *syn*- and *anti*-conformations of compound **1e**.



syn-conformation



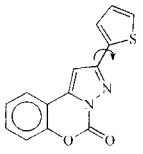
anti-conformation

Figure 3. *Syn*- and *anti*-conformations of compound **2a**.

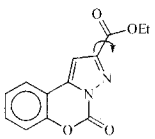
In the bioactive *anti*-conformation the sulfur atom of the thienyl ring of **1e** and **1f** and the carbonyl oxygen of the esters of series **2** can act as a_1 extranuclear proton acceptor which would explain their higher BZR affinity with respect to the other series **1** compounds. However, calculations of the heats

of formation of the *syn*- and *anti*-conformations[13] of **1e** and **2a** (see Table 2) reveal that the difference in the heat of formation between the two rotamers is insignificant (about 0.5 kcal/mol). As a consequence both *syn*- and *anti*-conformers may bind to the BZR, with the bioactive *anti*-conformer having enhanced affinity.

Table 2. Heats of formation of compounds **1e** and **2a** in their *syn*- and *anti*-conformations[a].



1e



2a

Compd	Heat of formation (kcal/mol)
1e (*syn*)	72.63
1e (*anti*)	73.12
2a (*syn*)	–42.08
2a (*anti*)	–42.69

[a] The heats of formation were calculated using the AM1 method of the MOPAC 6.0 software package.

Thus, Crippen's small hydrophilic pocket at the L_2 level and the preferred *anti*-conformation may both account for the BZR affinities of **1e**, **1f**, and **2a–e**.

In conclusion, the synthesis, the binding activity and the SAR studies of the novel pyrazolobenzoxazines revealed that the contemporary absence of the proton donor d and proton acceptor a_1, although both optional pharmacophoric descriptors, strongly decreases BZR binding. When the proton donor d is absent the heteroatom acceptor a_1 is necessary either in the tricyclic core, as in triazolo-benzoxazines[11], or in the appended substituent at the C-2 to obtain sub-micromolar BZR affinity.

Acknowledgments

Valuable assistance in animal handling was provided by M.G. Giovannini.

Experimental Section

A) 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 analyzer 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, dd = double doublet, ddd = double doublet of doublets, t = triplet, q = quartet, m = multiplet, br = broad, and ar = aromatic protons. The physical data of the newly reported compounds are listed in Tables 3 and 4.

Table 3. Physical data for the intermediate compounds.

Compd	R	R^1	mp (°C)	solv[a]	yield (%)
3b[b]	2-FC_6H_4	H	163–167	A	99
3c	2-FC_6H_4	Cl	155–159	B	61
3d[c]	4-ClC_6H_4	H	149–150	C	70
3e[d]	2-thienyl	H	160–164	A	88
3f	2-thienyl	Cl	185–187	D	65
4b	2-FC_6H_4	H	193–196	A	90
4c	2-FC_6H_4	Cl	171–173	C	80
4d[e]	4-ClC_6H_4	H	179–180	C	65
4e	2-thienyl	H	162–164	C	70
4f	2-thienyl	Cl	152–154	C	40
5b	2-FC_6H_4	H	170–173	E	10
5c	2-FC_6H_4	Cl	201–204	F	13
5d	4-ClC_6H_4	H	184–186	E	14
5e	2-thienyl	H	120–122	G	15
6f	2-thienyl	Cl	220–222	A	82
8	COOEt	Cl	oil		75
10	COOEt	Cl	172–174	H	83
11[f]	COOH	H	240–242	I	86
12[g]	COOH	Cl	308 dec	I	90
13	$COOCHMe_2$	H	160–161	E	61
14	COOMe	H	160–163	J	70
15	COOMe	Cl	206–208	A	90

[a] Purification solvents: A = ethyl acetate. B = cyclohexane/ethyl acetate. C = ethanol. D = glacial acetic acid. E = diethyl ether. F = chloroform. G = diethyl ether/petroleum ether. H = methanol. I = ethanol/water. J = benzene/petroleum ether. [b] Ref. [14]. [c] Ref. [15] mp 151°C. [d] Ref. [16] mp not reported. [e] Ref. [17] mp not reported. [f] Ref. [20] mp 234–235°C. [g] Ref. [20] mp 307°C dec.

Table 4. Physical data for pyrazolo[1,5-*c*][1,3]benzoxazines.

Compd	R	R^1	mp (°C)	solv[a]	yield (%)
1b	2-FC_6H_4	H	185–186	A	45
1c	2-FC_6H_4	Cl	265–267	A	64
1d[b]	4-ClC_6H_4	H	257–260	A	63
1e	2-thienyl	H	180–183	A	23
1f	2-thienyl	Cl	252–254	B	64
2b	COOEt	Cl	269–271	A	48
2c	$COOCHMe_2$	H	177–179	A	64
2d	COOMe	H	200–202	C	75
2e	COOMe	Cl	310–312	A	61

[a] Purification solvents: A = dry column chromatography, eluting system tetrahydrofuran. B = toluene. C = ethanol. [b] Ref. [18] mp 257–259°C.

The calculations of low-energy conformations and their corresponding energies (heat of formation) were carried out using the semiempirical quantum mechanical AM1 method of MOPAC 6.0 software package[13] running on an IBM RISC 6000 3CT workstation. The conformational minimization protocol BFGS, up to a gradient value <10 (GNORM = 10), and minimization protocol EF (Eigenvector Following), up to a gradient value < 1 (GNORM = 1), were applied.

General Procedure for the Preparation of 1-Aryl-3-(2-hydroxyaryl)-2-propen-1-ones **3b–f**[14–16]

A solution of potassium hydroxide (12 g) in water (10 mL) was added to a solution of the suitable salicylaldehyde (81 mmol) and acetophenone (50 mmol) in absolute ethanol. The resulting red solution was stirred at room temperature for 1 h. The mixture was diluted with ice and water (300 mL) and acidified with 6N hydrochloric acid. The yellow solid was collected, washed with water and recrystallized. Compound **3c** displayed the following spectral data: ^{1}H NMR ($CDCl_3$): 6.93 (d, 1H, ar, J = 8.7 Hz), 7.18–7.35 (m, 4H, 3H ar + H-2), 7.53–7.64 (m, 2H, ar), 7.82–7.91 (m, 1H, ar), 8.12 (d, 1H, H-3, J = 16.0 Hz). IR = 3400, 1650.

General Procedure for the Preparation of 4,5-Dihydro-3-aryl-5-(2-hydroxyaryl)pyrazoles **4b–f**[17]

Hydrazine hydrate (55%, 0.6 mL, 8.92 mmol)) was added to a suspension of **3b–f** (8.92 mmol) in ethanol (50 mL). The mixture was refluxed for 1 h. The solid was collected, washed with diethyl ether and recrystallized. Compound **4c** displayed the following spectral data: ^{1}H NMR ($CDCl_3$): 3.21 (dd, 1H, H-4, J = 17.0, 14.3 Hz), 3.55 (ddd, 1H, H-4, J = 17.0, 10.2, 3.0 Hz), 4.89 (dd, 1H, H-5, J = 14.3, 10.2 Hz), 6.85 (d, 1H, ar, J = 8.7 Hz), 7.02–7.23 (m, 4H, ar), 7.33–7.44 (m, 1H, ar), 7.81 (t, 1H, ar, J = 7.6 Hz). IR: 3340.

General Procedure for the Preparation of 3-Aryl-5-(2-hydroxyaryl)pyrazoles **5b–e**

A solution of lead tetraacetate (6.46 mmol) in anhydrous dichloromethane (60 mL) was added dropwise and under stirring to a solution of **4b–e** (5.87 mmol) in anhydrous dichloromethane (300 mL). The mixture was stirred at room temperature for 2 h. The solid was filtered off and the solution was washed once with 1.5 M hydrochloric acid (200 mL), with water (3 × 200 mL), and dried over Na_2SO_4. The solvent was evaporated at reduced pressure to yield an oil which was dissolved in ethanol (15 mL). Concentrated hydrochloric acid (0.18 mL) was added to the ethanolic solution. The solution was heated at 60 °C for 15 min. and then concentrated at half volume at reduced pressure. Addition of chloroform (150 mL) gave a solution which was washed with water (3 × 100 mL) and dried over Na_2SO_4. Evaporation at reduced pressure of the solvent yielded a residue which was purified by column chromatography, using the chloroform/ethyl acetate (9:1 *v/v*) system as eluent and then recrystallized. Compound **5c** displayed the following spectral data: ^{1}H NMR ($CDCl_3$): 6.96–7.01 (m, 2H, ar), 7.16–7.43 (m, 4H, ar), 7.60 (d, 1H, ar, J = 2.5 Hz), 7.70–7.77 (m, 2H, ar), 10.75 (br s, 1H, OH or NH). IR: 3440.

General Procedure for the Preparation of 2-Arylpyrazolo[1,5-c][1,3]benzoxazin-5-ones **1b–e**[18]

Triphosgene (0.55 mmol) and triethylamine (2.76 mmol) were successively added to a solution of **5b–e** (1.38 mmol) in tetrahydrofuran (10 mL). The mixture was stirred at room temperature. The reaction was monitored by TLC, and subsequent amounts of triphosgene and triethylamine were added until the disappearance of the starting pyrazole. Elimination of the triethylamine hydrochloride and evaporation at reduced pressure of the solvent yielded a residue which was worked up with diethyl ether, collected, and recrystallized.

2-(2-Fluorophenyl)pyrazolo[1,5-*c*][1,3]benzoxazin-5-one (**1b**): ^{1}H NMR ($CDCl_3$): 7.15–7.60 (m, 7H, ar), 7.87 (d, 1H, ar, J = 7.9 Hz), 8.32 (t, 1H, ar, J = 7.9 Hz). IR: 1790.

2-(2-Fluorophenyl)-9-chloropyrazolo[1,5-*c*][1,3]benzoxazin-5-one (**1c**): ^{1}H NMR ($CDCl_3$): 7.16–7.54 (m, 6H, ar), 7.83 (d, 1H, ar, J = 2.2 Hz), 8.31 (t, 1H, ar, J = 7.6 Hz). IR: 1850.

2-(4-Chlorophenyl)pyrazolo[1,5-*c*][1,3]benzoxazin-5-one (**1d**)[18]: ^{1}H NMR ($CDCl_3$): 7.20 (s, 1H, ar), 7.39–7.49 (m, 4H, ar), 7.53–7.61 (m, 1H, ar), 7.81 (d, 1H, ar, J = 7.9 Hz), 7.96 (d, 2H, ar, J = 8.4 Hz).

2-(2-Thienyl)pyrazolo[1,5-*c*][1,3]benzoxazin-5-one (**1e**): ^{1}H NMR ($CDCl_3$): 7.05 (s, 1H, ar), 7.14 (dd, 1H, ar, J = 5.0, 3.7 Hz), 7.40–7.46 (m, 3H, ar), 7.52–7.66 (m, 2H, ar), 7.81 (d, 1H, ar, J = 7.9 Hz). IR: 1780.

5,10b-Dihydro-2-(2-thienyl)-9-chloro-1H-pyrazolo[1,5-c][1,3]benzoxazin-5-one (**6f**)

Triphosgene (0.213 g, 0.72 mmol) and triethylamine (0.49 mL, 3.6 mmol) were added to a solution of **4f** (0.476 g, 1.8 mmol) in anhydrous tetrahydrofuran (40 mL). The mixture was stirred at room temperature for 1 h. Elimination of the solid (triethylamine hydrochloride) and evaporation of the solvent at reduced pressure yielded a residue which was treated with diethyl ether (2 mL), filtered and recrystallized. ^{1}H NMR (DMSO-d_6): 3.57 (dd, 1H, H-1, J = 16.5, 13.2 Hz), 3.97 (dd, 1H, H-1, J = 16.5, 10.3 Hz), 5.54 (dd, 1H, H-10b, J = 13.2, 10.3 Hz), 7.22–7.27 (m, 2H, ar), 7.46–7.56 (m, 3H, ar), 7.84 (d, 1H, ar, J = 5.0 Hz). IR: 1740.

2-(2-Thienyl)-9-chloropyrazolo[1,5-c][1,3]benzoxazin-5-one (**1f**)

A solution of tetrachloro-1,2-benzoquinone (0.319 g, 1.3 mmol) in anhydrous toluene (10 mL) was added dropwise to a hot solution of **6f** (0.395 g, 1.3 mmol) in anhydrous toluene (25 mL). The solution was refluxed for 3 h. Upon cooling, an orange precipitate was obtained which when recrystallized yielded **1f** as white crystals. ^{1}H NMR (DMSO-d_6): 7.22–7.27 (m, 1H, ar), 7.54–7.76 (m, 5H, ar), 8.23 (d, 1H, ar, J = 2.5 Hz). IR: 1810.

Ethyl 4-(2-hydroxy-5-chlorophenyl)-2,4-dioxobutanoate (**8**)

Compound **8** was obtained from 2-hydroxy-5-chloroacetophenone (2.89 g, 17 mmol) and diethyl oxalate (0.96 mL, 7.15 mmol) following the procedure described to obtain **7**[19]. ^{1}H NMR ($CDCl_3$): 1.35 (t, 3H, CH_3, J = 7.0 Hz), 2.96 (d, 1H, H-3, J = 16.8 Hz), 3.36 (d, 1H, H-3, J = 16.8 Hz), 4.37 (q, 2H, CH_2, J = 7.0 Hz), 6.95 (d, 1H, ar, J = 8.8 Hz), 7.46 (dd, 1H, ar, J = 8.8, 2.6 Hz), 7.87 (d, 1H, ar, J = 2.6 Hz).

Ethyl 5-(2-hydroxy-5-chlorophenyl)pyrazole-3-carboxylate (**10**)

The title compound was obtained from **8** (3.78 g, 14 mmol) and hydrazine hydrate (55%, 0.77 mL, 14 mmol) following the procedure described to obtain **9**[4, 19]. ^{1}H NMR ($CDCl_3$): 1.44 (t, 3H, CH_3, J = 7.0 Hz), 4.45 (q, 2H, CH_2, J = 7.0 Hz), 6.98 (d, 1H, ar, J = 8.8 Hz), 7.16–7.22 (m, 2H, ar), 7.54 (d, 1H, ar, J = 2.4 Hz), 10.34 (br s, 1H, OH or NH), 11.15 (br s, 1H, NH or OH).

General Procedure for the Preparation of 5-(2-Hydroxyaryl)pyrazole-3-carboxylic Acids **11** *and* **12**[20]

A solution of 10% sodium hydroxide (8 mL) and the suitable ester **9** or **10** (2.1 mmol) in ethanol (25 mL) was refluxed for 30 min. The cooled solution was diluted with water (25 mL) and acidified with 6M hydrochloric acid to yield a precipitate which was collected, washed with water and recrystallized.

5-(2-Hydroxyphenyl)pyrazole-3-carboxylic Acid (**11**): ^{1}H NMR (DMSO-d_6): 6.84–6.98 (m, 2H, ar), 7.15–7.24 (m, 2H, ar), 7.76 (d, 1H, ar, J = 7.4 Hz), 10.33 (s, 1H, OH or NH), 13.38 (br s, 1H, NH or OH).

5-(2-Hydroxy-5-chlorophenyl)pyrazole-3-carboxylic Acid (**12**): ^{1}H NMR (DMSO-d_6): 6.98 (d, 1H, ar, J = 8.8 Hz), 7.22 (d, 1H, ar, J = 8.8 Hz), 7.32 (s, 1H, ar), 7.83 (s, 1H, ar), 10.56 (s, 1H, OH or NH), 13.50 (br s, 1H, NH or OH).

General Procedure for the Preparation of 5-(2-Hydroxyaryl)pyrazole-3-carboxylic Esters **13–15**

Concentrated sulfuric acid (0.9 mL) was added to a solution of the appropriate acid **11** or **12** (1.8 mmol) in the suitable alcohol (100 mL). The mixture was refluxed and the reaction monitored by TLC was heated until the starting material disappeared. The solvent was then evaporated at reduced pressure to one third of the starting volume. The concentrated solution was diluted with ice and water (50 mL) and extracted with ethyl acetate (2 × 50 mL). The organic extracts were washed twice with a solution of 0.5% sodium hydrogen carbonate (50 mL each time), with water (2 × 50 mL) and

then dried over Na_2SO_4. Evaporation at reduced pressure of the solvent afforded an oily residue which solidified upon treatment with diethyl ether.

Isopropyl 5-(2-Hydroxyphenyl)pyrazole-3-carboxylate (**13**): ^{1}H NMR (DMSO-d_6): 1.31 (s, 3H, CH_3), 1.35 (s, 3H, CH_3), 5.15 (t, 1H, CH, J = 6.2 Hz), 6.66–7.00 (m, 2H, ar), 7.17–7.25 (m, 2H, ar), 7.75 (d, 1H, ar), J = 7.33 Hz), 10.31 (s, 1H, OH or NH).

Methyl 5-(2-Hydroxyphenyl)pyrazole-3-carboxylate (**14**): ^{1}H NMR (DMSO-d_6): 3.85 (s, 3H, CH_3), 6.86–7.01 (m, 2H, ar), 7.18–7.25 (m, 2H, ar), 7.74 (d, 1H, ar, J = 7.7 Hz), 10.30 (br s, 1H, OH or NH), 13.50 (br s, 1H, NH or OH). IR: 3350, 3280, 1725.

Methyl 5-(2-Hydroxy-5-chlorophenyl)pyrazole-3-carboxylate (**15**): ^{1}H NMR (DMSO-d_6): 3.85 (s, 3H, CH_3), 7.00 (d, 1H, ar, J = 8.4 Hz), 7.22–7.35 (m, 2H, ar), 7.84 (d, 1H, ar, J = 2.1 Hz), 10.6 (br s, 1H, OH or NH).

General Procedure for the Preparation of 5-Oxopyrazolo[1,5-c][1,3]benzoxazin-2-carboxylic Esters **2b–e**

The title compounds were obtained from **10, 13–15** (1.38 mmol), triphosgene (0.55 mmol) and triethylamine (2.76 mmol) following the procedure described to obtain **1b–e**.

Ethyl 5-Oxo-9-chloropyrazolo[1,5-*c*][1,3]benzoxazin-2-carboxylate (**2b**): ^{1}H NMR ($CDCl_3$): 1.45 (t, 3H, CH_3, J = 7.1 Hz), 4.49 (q, 2H, CH_2 J = 7.1 Hz), 7.33–7.42 (m, 2H, ar), 7.53 (dd, 1H, ar, J = 8.9, 2.3 Hz), 7.78 (d, 1H, ar, J = 2.3 Hz).

Isopropyl 5-Oxopyrazolo[1,5-*c*][1,3]benzoxazin-2-carboxylate (**2c**): ^{1}H NMR (DMSO-d_6): 1.36 (s, 3H, CH_3), 1.39 (s, 3H, CH_3), 5.24–5.27 (m, 1H, CH), 7.49–7.65 (m, 3H, ar), 7.69 (s, 1H, ar), 8.19 (d, 1H, ar, J = 7.6 Hz).

Methyl 5-Oxopyrazolo[1,5-*c*][1,3]benzoxazin-2-carboxylate (**2d**): ^{1}H NMR (DMSO-d_6): 3.94 (s, 3H, CH_3), 7.45–7.70 (m, 3H, ar), 7.73 (s, 1H, ar), 8.18 (d, 1H, ar, J = 7.7 Hz). IR: 3140, 1810, 1720.

Methyl 5-Oxo-9-chloropyrazolo[1,5-*c*][1,3]benzoxazin-2-carboxylate (**2e**): ^{1}H NMR (DMSO-d_6): 3.94 (s, 3H, CH_3), 7.57–7.71 (m, 2H, ar), 7.80 (s, 1H, ar), 8.38 (d, 1H, ar, J = 2.1 Hz).

B) Biochemistry

Crude synaptic membranes were prepared from cerebral cortices of male Sprague-Dawley rats (170–250 g) according to Zukin et al.[21]. Tissue was homogenized in 15 vol of ice-cold 0.32M sucrose, containing 20 µg/mL 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 0.05M Tris-HCl buffer, at pH 7.4, containing 0.01% (*v/v*) Triton X-100, incubated at 37 °C for 60 min, and centrifuged at 48000*g* for 20 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 ligand concentration in a total 0.5 mL vol. Bound radioactivity was separated by rapid filtration through Whatman GF/B filters using a Brandel cell harvester. Nonspecific binding was determined in the presence of 10 µM diazepam. The IC_{50} values were calculated from displacement curves based on four to six scalar concentrations of the test compounds in triplicate, using the ALLFIT computer program[22], and converted to K_i values by application of the Cheng-Prusoff equation[23]. The GABA ratios[24] of the compounds with the lowest K_i values were calculated by measuring, in the same experiment, the IC_{50} value of each compound in the absence and presence of 0.1 mM GABA. A stock 1 mM solution of the test compounds was prepared in 50% ethanol. Subsequent dilutions were accomplished in buffer. Ethanol up to a final 5% concentration was seen to affect [^{3}H]flunitrazepam binding negligibly (≤ 3%).

References

[1] W. Zhang, K.F. Koehler, P. Zhang, J.M. Cook, *Drug Des. Discovery* **1995**, *12*, 193–248.

[2] P. Zhang, W. Zhang, R. Liu, B. Harris, P. Skolnick, J.M. Cook, *J. Med. Chem.* **1995**, *38*, 1679–1688.

[3] H.O. Villar, M.F. Davies, G.H. Loew, P.A. Maguire, *Life Sci.* **1991**, *48*, 593–602.

[4] V. Colotta, D. Catarzi, F. Varano, F. Melani, G. Filacchioni, L. Cecchi, A. Galli, C. Costagli, *Farmaco* **1996**, *51*, 223–229.

[5] V. Colotta, D. Catarzi, F. Varano, G. Filacchioni, L. Cecchi, A. Galli, C. Costagli, *J. Med. Chem.* **1996**, *39*, 2915–2921.

[6] V. Colotta, L. Cecchi, G. Filacchioni, F. Melani, G. Palazzino, C. Martini, G. Giannaccini, A. Lucacchini, *J. Med. Chem.* **1988**, *31*, 1–3.

[7] V. Colotta, L. Cecchi, F. Melani, G. Filacchioni, C. Martini, G. Giannaccini, A. Lucacchini, *J. Med. Chem.* **1990**, *33*, 2646–2651.

[8] V. Colotta, L. Cecchi, F. Melani, G. Filacchioni, C. Martini, S. Gelli, A. Lucacchini, *J. Pharm. Sci.* **1991**, *80*, 276–279.

[9] V. Colotta, L. Cecchi, F. Melani, G. Filacchioni, C. Martini, S. Gelli, A. Lucacchini, *Farmaco* **1991**, *46*, 1139–1156.

[10] D. Catarzi, L. Cecchi, V. Colotta, G. Filacchioni, C. Martini, L. Giusti, A. Lucacchini, *Farmaco* **1994**, *49*, 89–92.

[11] D. Catarzi, L. Cecchi, V. Colotta, G. Filacchioni, F. Varano, C. Martini, L. Giusti, A. Lucacchini, *J. Med. Chem.* **1995**, *38*, 2196–2201.

[12] A.K. Ghose, G.M. Crippen, *Mol. Pharmacol.* **1990**, *37*, 725–734.

[13] M.J.S. Dewar, E.G. Zoebisch, E.F. Healy, J.J.P Stewart, *J. Amer. Chem. Soc.* **1985**, *107*, 3902–3909.

[14] J. Sallai, M. Gabor, F. Kallay, *Acta Pharm. Hung.*, **1976**, *46*, 49–56. *Chem. Abstr.* **1976**, *85*, 57378p.

[15] C.G. Le Fevre, R.J.W. Le Fevre, *J. Chem. Soc.* **1932**, *134*, 1988–1993.

[16] S.S. Misra, *Monatsh. Chem.* **1973**, *104*, 11–14.

[17] G.A.M. Nawwar, *J. Chem. Res. (S)* **1991**, 344–345.

[18] A.M. Huff, C.F. Beam, *J. Heterocyclic Chem.* **1986**, *23*, 1343–1346.

[19] J. Schmutz, R. Hirt, *Helv. Chim. Acta* **1953**, *36*, 132–138.

[20] V.A. Zagorevskii, D.A. Zykov, *Zhur. Obschchei. Khim.* (English Translation) **1960**, *30*, 3547–3551.

[21] S.R. Zukin, S.H. Yong, *Proc. Natl. Acad. Sci. USA* **1974**, *71*, 4802–4807.

[22] A. De Lean, P.J. Munson, D. Rodbard, *Am. J. Physiol.* **1978**, *235*, E97–102.

[23] Y-C. Cheng, W.H. Prusoff, *Biochem. Pharmacol.* **1973**, *22*, 3099–3108.

[24] H. Mohler, J.G. Richards, *Nature* **1981**, *294*, 763–765.

Received: September 16, 1996 [FP150]