Brief Articles

Synthesis and Benzodiazepine Receptor Affinity of Pyrazolo[1,5-*a*]pyrimidine Derivatives. 3. New 6-(3-Thienyl) Series as α1 Selective Ligands

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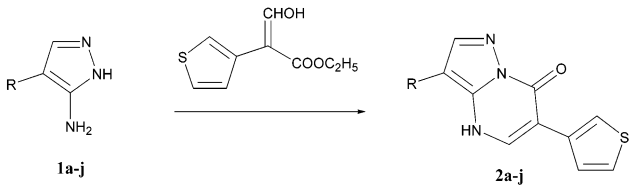
New 3-aryl-6-(3-thienyl)pyrazolo[1,5-*a*]pyrimidin-7-ones (**2a**−**j**) are synthesized and evaluated in vitro on Bz/$GABA_A$ receptors and on recombinant benzodiazepine receptors ($\alpha x\beta 2/3\gamma 2$; x = 1−3, 5) expressed in HEK293 cells. SAR studies on the new compounds are conducted and molecular modeling is accomplished to better investigate requirements leading to subtype selectivity. Some of the synthesized compounds are tested in vivo to explore their pharmacological effect as a consequence of their high $\alpha 1\beta 2\gamma 2$ subtype selectivity observed in vitro.

Recent advances in molecular cloning techniques have led to the characterization of a large number of Bz/$GABA_A$ receptor subtypes, and this rich heterogeneity of $GABA_A$ receptors suggests that different effects of Bz not only are produced in different brain regions but also are mediated by different $GABA_A$ receptor subtypes.[1] In particular, the detailed knowledge of the molecular properties and of the exact anatomical distribution of different $GABA_A$ receptor subtypes seems to be a prerequisite for understanding the physiological Bz/$GABA_A$ actions and for developing drugs acting through individual $GABA_A$ receptor subtypes, thus revising the benzodiazepines pharmacology. Among various Bz/$GABA_A$ receptors, $\alpha 1\beta 2\gamma 2$ is the most widespread subtype and is identified with type I BzR ($\omega 1$) while $\alpha 2\beta 2\gamma 2$, $\alpha 3\beta 2\gamma 2$, and $\alpha 5\beta 2\gamma 2$ ion channels are type II BzR ($\omega 2$). Despite the great variety of compounds that bind to Bz binding sites, only few ligands show high subtype selectivity (e.g., β-CCT, Zolpidem, Zaleplon, and CL 218,872 as α1 selective ligands; SB-205384 as α3 selective ligand; L-655,708 and RY80 as α5 selective ligands), and therefore, the discovery of subtype-selective ligands remains today an interesting challenge. As many authors have suggested[2,3] for defining the differences of Bz/$GABA_A$ receptors, it could be useful to determine the spatial properties of the lipophilic pockets, which in the pharmacophoric models are proposed to be different in the different subtypes. In an attempt to prepare selective ligands for Bz/$GABA_A$ receptor subtypes, we have synthesized new pyrazolo[1,5-*a*]-pyrimidin-7-ones bearing the 3′-thienyl ring at the 6 position. Various 3′-substituted phenyl rings, chosen on the basis of the high affinity values previously observed in the 6-pyrazol-3′(5′)-yl series,[4] were introduced at the 3 position, and other aromatic rings, characterized by different lipophilic and steric properties, were also taken into account. The synthesis of 4,7-dihydro-3,6-arylpyrazolo[1,5-*a*]pyrimidin-7-ones (**2a**−**j**) was performed by a one-step reaction between 3-amino-4-arylpyrazoles and ethyl 2-thien-3′-yl-3-hydroxypropenoate (Scheme 1); the chemical strategy employed for the synthesis was extensively described in our previous paper.[5]

Scheme 1



a R = 3′-$CH_3C_6H_4$ **f** R = benzyl
b R = 3′-$CF_3C_6H_4$ **g** R = 1′-naphthyl
c R = 3′-BrC_6H_4 **h** R = 2′-naphthyl
d R = 3′-FC_6H_4 **i** R = 2′-pyridyl
e R = 4′-FC_6H_4 **j** R = 3′-pyridyl

The new compounds were tested for their ability to displace [^{3}H]Ro15-1788 binding from bovine brain membranes. Most of the synthesized compounds exhibited high affinity for the Bz/$GABA_A$ receptor complex (Table 1), and according to our previous hypothesis,[5] they possess the structural features required for binding to the BzR, in conformity with the comprehensive pharmacophore/receptor model proposed by Zhang and coworkers.[6] Namely, the essential anchoring of the ligands to donor site H1 on the receptor protein is caused by the carbonyl group at the 7 position together with the unsubstituted N^1 in a three-centered interaction. Suit-

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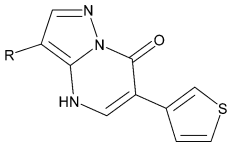
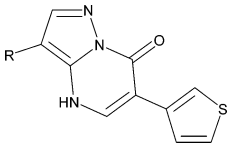
Table 1. Affinity Values of Compounds **2a**−**j** on Homogenate Bovine Brain Membrane



compd	R	% inhibition[a]	K_i [b] (nM)	GR[c]
I[d]	Ph	94 ± 6	111 ± 4	1.71
II[d]	3′-thienyl	100 ± 7	16 ± 1	1.30
III[d]	3′-$OCH_3C_6H_4$	92 ± 1	42 ± 2	1.25
2a	3′-$CH_3C_6H_4$	97 ± 2	26 ± 1	2.00
2b	3′-$CF_3C_6H_4$	96 ± 3	5.8 ± 1.2	2.00
2c	3′-BrC_6H_4	87 ± 1	8.8 ± 0.2	1.72
2d	3′-FC_6H_4	92 ± 1	73 ± 3	1.20
2e	4′-F C_6H_4	99 ± 9	31 ± 2	0.90
2f	benzyl	55 ± 4	*e*	*e*
2g	1′-naphthyl	92 ± 1	40 ± 2	1.02
2h	2′-naphthyl	30 ± 4	*e*	*e*
2i	2′-pyridyl	98 ± 1	3.9 ± 0.5	1.10
2j	3′-pyridyl	100 ± 2	30 ± 2	1.51
diazepam[f]			10	1.5

[a] Percent inhibition value of specific ^{3}[H]RO15-1788 binding at 0.2 nM concentration is the mean ± SEM of five separate experiments, each done in triplicate. [b] K_i values represent the mean ± SEM derived from five independent experiments, conducted in triplicate. [c] GABA ratio values (K_i without GABA/K_i with GABA) are the mean of three separate experiments, performed in triplicate. [d] Data obtained from ref 5, where the synthesis of compounds **I**−**III** is also reported. [e] Not determined. [f] Data obtained from ref 3 for comparison purposes.

Table 2. Affinity Values at α1β2γ2, α2β2γ2, α3β2γ2, α5β3γ2 $GABA_A$/BZ Subtypes



		K_i [a] (nM)			
compd	R	α1	α2	α3	α5
II[b]	3′-thienyl	23 ± 4	200 ± 20	196 ± 20	223 ± 20
III[b]	3′-$OCH_3C_6H_4$	43 ± 4	827 ± 60	*c*	737 ± 51
2b	3′-$CF_3C_6H_4$	10 ± 1	540 ± 50	89 ± 9	207 ± 10
2c	3′-BrC_6H_4	10 ± 3	291 ± 30	111 ± 12	22 ± 2
2g	1′-naphthyl	47 ± 3	>10000	*c*	156 ± 12
2i	2′-pyridyl	7.0 ± 0.8	927 ± 83	*c*	740 ± 51
2j	3′-pyridyl	25 ± 2	315 ± 25	*c*	210 ± 10
Diazepam[d]		14	20	15	11
Zolpidem[d]		26.7	156	383	>10000

[a] K_i values represent the mean ± SEM derived from three independent experiments, conducted in triplicate. [b] Compounds **II** and **III** were previously synthesized; see ref 5 for details. [c] Not determined. [d] These ligands were employed for comparison purposes in this set of assays, and the reported values were obtained from ref 3.

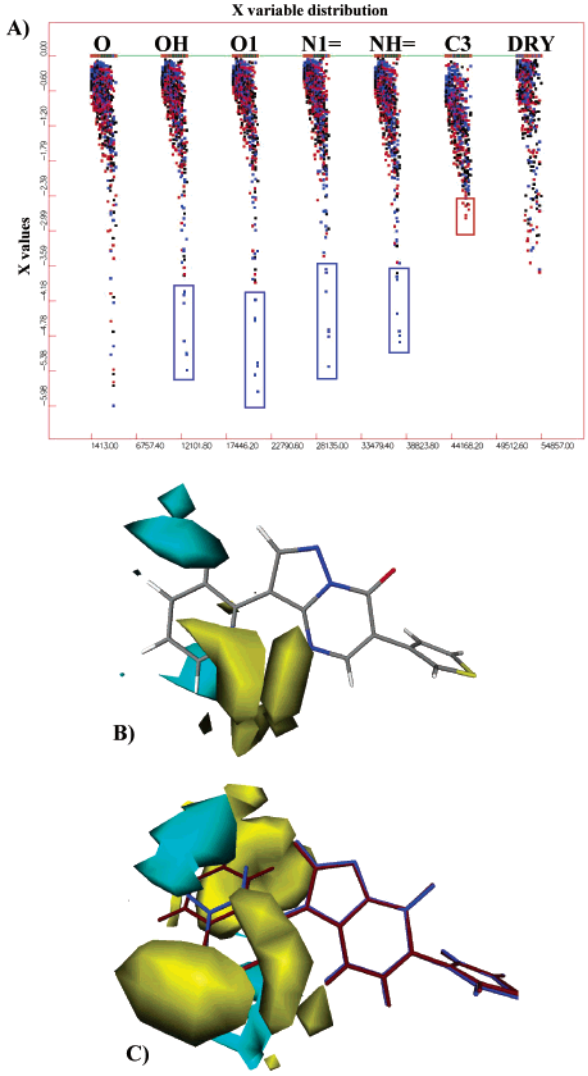


Figure 1. (A) *x* variable distribution for GRID probes used to characterize compounds: **2c** (black), **2g** (red), **2i** (blue). The colored boxes show MIFs (molecular interaction fields) important for **2g** (red), using the C3 probe, and **2i** (blue), wherever a hydrogen-donor probe is used. GRID probes are the following: acceptor (O); donor/acceptor (OH, O1, NH═); donor (N1═); steric (C3); hydrophobic (DRY). (B) CPCA pseudofield plot for N1═ probe. Shown are field differences between compounds **2i** and **2g**. Yellow contours indicate regions where H-bonding interactions are possible for **2i** but not for **2g**. Representation of **2i** is shown. (C) CPCA pseudofield plot for C3 probe. Shown are field differences between compounds **2i** and **2g**. Cyano contours indicate the hydrophobic interaction between the probe and the aromatic ring system of **2g**. Representation of compounds **2i** and **2g** is shown.

able occupation of the lipophilic pockets on the receptor protein by the substituents at the 3 and 6 positions seems to play an important role in modulating affinity and efficacy.

Compounds **2a**−**e** showed a general improvement in the binding affinity in comparison to the corresponding 6-pyrazolyl ligands.[4] The introduction of a 1-naphthyl group (**2g**), unlike the isomer **2h**, appears to be a well-tolerated modification, and this result can support our previously proposed hypothesis[4] that the lipophilic pocket, which accommodates the 3-substituents, seems to be larger than the area where the 6-substituent fits. On the other hand, no Bz receptor recognition was shown by compound **2f**, suggesting the presence of a repulsive region, S, which delimits the depth of the lipophilic pocket where the 3-substituents interact. Compound **2i**, bearing the 2-pyridyl ring at the 3 position, exhibits the highest affinity value of all the synthesized compounds. Following these preliminary binding studies, some of the new ligands (**2b**, **2c**, **2g**, **2i**, **2j**) and compounds **II** and **III**, previously synthesized,[5] which exhibited high affinity values, were tested on recombinant rat αxβ2/3γ2 (x = 1−3, 5) $GABA_A$/Bz receptor subtypes (Table 2).

Interestingly, all the tested compounds display a marked α1 subtype selectivity with the exception of

Table 3. Muscle Relaxant, Anticonvulsant, and Anxiolytic-like Effects of Compounds **II**, **2b**, and **2i** in Comparison with Diazepam and Zolpidem

		muscle relaxant effect			antianxiety activity, light/dark box		
treatment[a]	mg/kg	*n*	rotarod test, no. of falls in 30s	anticonvulsant activity against PTZ-induced attacks, %	*n*	no. of crosses	time (s) in light
CMC, 1%	10 mL	40	0.2 ± 0.1	20	22	17.9 ± 1.6	86.9 ± 6.5
Diazepam	0.3 po	6	0.5 ± 0.2	83.3*	8	22.0 ± 7.6*	85.0 ±9.3
	1 po	6	0.7±0.2*	100**	6	21.5 ± 7.5	117.5 ± 20.8***
Zolpidem	3	20	0.2±0.1	70			
	10	20	0.8±0.2**	90			
II	10	11	0.0±0.0	36.4	11	19.7 ± 3.2	94.9 ± 8.5
	30	11	0.3 ± 0.1	9.1	13	11.8 ± 1.9	72.3 ± 10.4
2b	10	17	0.1 ± 0.1	29.4	11	17.1 ± 1.9	69.4 ± 5.1
	30	12	0.2 ± 0.1	0	12	12.7 ± 2.2	77.3 ± 10.7
2i	10	21	0.3 ± 0.2	19	10	19.6 ± 1.67	90.6 ± 5.3
	30	10	0.3 ± 0.2	10			
2i + Diazepam	10 1	10	0.5 ± 0.17	90	10	26.2 ± 2.0	120.9± 9.9
2i + Zolpidem	10 10	10	1.1 ± 0.4	90			

[a] Treatment with compounds **II**, **2b**, and **2i** was performed 30 min and Diazepam and Zolpidem 20 min before the test. (∗) $P < 0.05$, (∗∗) $P \leq 0.01$, and (∗∗∗) $P < 0.001$ versus control mice (CMC).

compound **2c**, which exhibits comparable affinity values for both α1β2γ2 and α5β3γ2 subtypes. The α1 selectivity value exhibited by compound **2i** suggests that an H bond could be involved in the interaction between the ligand and the receptor protein, as an optimal condition of binding for length and orientation in comparison with the isomer **2j**. However, it is not to be excluded that the highest α1 selectivity could be simply derived from the reduced flexibility due to intramolecular H bonding between N_4H and the 2′-pyridyl endocyclic nitrogen.

To gain some qualitative information about the structural features responsible for the different selectivity profiles of the investigated ligands, some were selected on the basis of their α1 and α5 receptor subtype affinity and submitted to a GRID/CPCA study[7] (Figure 1). Because of both the small number of the available molecules (seven) and the number of chemical groups used for their description (seven), neither a 3D QSAR analysis nor a traditional GRID/PCA approach seemed suitable for dealing with the selectivity of pyrazolopyrimidine derivatives. On the basis of the qualitative results (Figure 1), it can be supposed that α1 selectivity of compound **2i** arises from the hydrogen bond interaction that could be established by the molecule with an amino acidic residue able to act as hydrogen bond donor.[2,8] Moreover, the lower subtype selectivity, expressed as an α1/α5 ratio value, observed for compound **2g** (α1/α5 =1/3) in comparison with **2i** (α1/α5 =1/100), could be due to the presence in the binding site of either an aromatic amino acidic residue or a nonpolar amino acidic side chain. The latter hypothesis can find experimental confirmation from the evidence that α5Ile215, corresponding to α1Val211, is one of the amino acids responsible for α5 selectivity.[9] With reference to compound **2c**, none of the interaction energies from any of the used probes are favorable for the 3′-bromine derivative, thus indicating that those interactions cannot successfully be used to explain its affinity for both α1β2γ2 and α5β3γ2 subtypes.

Encouraged by the in vitro tests on the BzR recombinant subtypes, compounds **II**, **2b**, and **2i** were chosen for in vivo studies to explore their pharmacological effect as a consequence of the high α1β2γ2 subtype selectivity observed in vitro (Tables 3 and 4). All the tested compounds were ineffective per se at doses of 10 and 30 mg/kg, thus ruling out any agonistic or partial agonistic profiles; therefore, with the aim of evaluating potential antagonistic profiles, compound **2i**, endowed with the highest affinity and α1 selectivity values, was selected for in vivo evaluation together with Diazepam and Zolpidem.

Table 4. Effect of Compounds **II**, **2b**, and **2i** on Mouse's Curiosity and Explorative Capacity in the Hole Board Test in Comparison with Zolpidem[a]

		no. of	no. of counts	
treatment	mg/kg po	mice	holes	plane
CMC, 1%	0.1 mL/ 10 g po	21	52.5 ± 2.7	120.6 ± 6.3
Zolpidem	3	11	45.0 ± 5.4	84.7 ± 7.28**
	10	12	26.3 ± 4.1***	54.4 ± 5.9***
II	10	9	52.7 ± 3.9	112.2 ± 12.2
2b	10	9	60.7 ± 3.4	139.6 ± 12.3
2i	10	14	55.9 ± 4.9	96.6 ± 6.5*
	30	10	50.9 ± 3.7	110.4 ± 8.8
2i + Zolpidem	10 3	4	25.8 ± 3.8∧	68.0 ± 5.1
2i + Zolpidem	10 10	16	9.38 ± 3.2∧∧	28.7 ± 3.9∧∧∧

[a] Substances and CMC were administered po 30 min before the test. Zolpidem, together with **2i**, was administered 20 min before the test. (∗∗∗) $P < 0.001$, (∗∗) $P < 0.01$, and (∗) $P < 0.05$ versus control mice (CMC, 1%). (∧∧∧) $P < 0.001$, (∧∧) $P < 0.01$, and (∧) $P < 0.05$ versus Zolpidem (3 or 10 mg/kg) treated mice.

As shown in Table 3, compound **2i** (10 mg/kg) failed to antagonize the anxiolytic effect of Diazepam (1 mg/kg) in the light/dark box test and does not seem to significantly influence the motor impairment observed in the rotarod test, produced by Diazepam (1 mg/kg) or by Zolpidem (10 mg/kg). This evidence is consistent with the interpretation of some authors that anxiolytic and the myorelaxant actions are probably mediated by α2 and α3 Bz/$GABA_A$ subtypes, while binding sites containing the α1 isoforms do not appear to share these effects.[10−12] In fact, the pharmacological effects mediated via α1 $GABA_A$ receptors are expected to be sedative, amnesic, ataxic, and in part anticonvulsant.[1,12] In addition, compound **2i** (10 mg/kg), when administered with Diazepam (1 mg/kg) or with Zolpidem (10 mg/kg), is unable to antagonize the protective effect of both

Diazepam and Zolpidem against convulsions, chemically induced in mice by PTZ.

This result at first glance seems to be in disagreement with the potential antagonist profile of **2i**. However, it is known that "convulsion" is complex; several lines of evidence support the general notion that the anticonvulsant effect exerted by classical Bz groups involves a very complicated neuronal network and that even different convulsions (MES- and PTZ-induced) are treated with different drugs. Recent studies propose that only tonic convulsions are mediated via the α1 Bz/$GABA_A$ receptors, while myoclonic jerks may be due to the other subtypes.[12]

In continuing our pharmacological investigation, compound **2i** was used in association with Zolpidem (3 and 10 mg/kg) in the hole board test and, surprisingly, a synergic sedative effect was shown by the treated mice. The possibility that this effect can be imputed to Zolpidem or its influence on the metabolism of compound **2i** seems to be rather remote, since this hypothesis would hold true only in the hole board test and not in the others mentioned above.

Recent physiological studies on the GABA system and interneuron connections highlight the existence of a wide variety of GABAergic interneurons, which are involved in the regulation of other neuronal networks or in its self-regulation (GABA to GABA); their stoichiometry is mainly expressed by α1 subtype.[13−15] Although speculative, it can be suggested that **2i** could interfere with the "fail-safe" mechanism that ensures that pyramidal neurons do not fire excessively[13] and that this compound might cause a loss of "fine-tuning" of GABA activity and consequently an increase of the maximal effect, thus producing a strong ataxic effect as observed in Zolpidem treated mice.

Supporting Information Available: Experimental section, including chemistry, binding studies, molecular modeling, and pharmacological methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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