Refinement of the Benzodiazepine Receptor Site Topology by Structure−Activity Relationships of New *N*-(Heteroarylmethyl)indol-3-ylglyoxylamides

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N-(Heteroarylmethyl)indol-3-ylglyoxylamides (**1**−**26**) were synthesized and evaluated as ligands of the benzodiazepine receptor (BzR) to probe the hydrogen bonding properties of the so-called S_1 site of the BzR by means of suitable heterocyclic side chains. SARs were developed in light of our hypothesis of binding modes A and B. Pyrrole and furan derivatives adopting mode A (**2**, **8**, **10**, **20**, **22**) turned out to be more potent (K_i values < 35 nM) than their analogues lacking hydrogen bonding heterocyclic side chains. These data suggest that the most potent indoles interact with a hydrogen bond acceptor/donor (HBA/D) group located within the S_1 site of the BzR. Compounds **1**, **2**, **8**, **19**, **20**, and **22**, tested at recombinant rat $\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_2\gamma_2$, and $\alpha_5\beta_3\gamma_2$ BzRs, elicited selectivity for the $\alpha_1\beta_2\gamma_2$ isoform. On the basis of published mutagenesis studies and the present SARs, we speculate that the S_1 HBA/D group might be identified as the hydroxyl of α_1-Tyr209 or of other neighboring amino acids.

Introduction

GABA (γ-aminobutyric acid) is the major inhibitory neurotransmitter in the central nervous system (CNS) and operates through three different receptor types, the ionotropic $GABA_A$ and $GABA_C$ receptors, and the metabotropic $GABA_B$ receptors.[1,2] Besides playing an important role in central transmission processes, these receptors, especially the $GABA_A$ receptors, have increasingly attracted interest as therapeutic targets, due to their involvement with several neurological and psychiatric disorders such as anxiety, sleeplessness, epilepsy, and amnesia.[3] The $GABA_A$ receptor is a ligand-gated chloride ion channel with a heteropentameric structure made up of a total of 21 subunits (α_{1-6}, β_{1-4}, γ_{1-4}, θ, π, ϵ, ρ_{1-3}, and δ) of which 16 have been found in the mammalian CNS.[1,3] The major isoform of $GABA_A$ receptors contains α, β, and γ subunits, with α and β subunits being necessary for the GABA-activated ion-channel function, and γ subunits giving sensitivity to benzodiazepines (Bz). It has been proposed that the stoichiometry of the three subunits in the pentamer is $2\alpha2\beta1\gamma$, with the Bz binding site (BzR) occurring at the interface of the α and γ subunits.[4] The majority of benzodiazepine-sensitive $GABA_A$ receptor subtypes in the brain are $\alpha_1\beta_x\gamma_2$, $\alpha_2\beta_x\gamma_2$, $\alpha_3\beta_x\gamma_2$, and $\alpha_5\beta_x\gamma_2$, while the $\alpha_4\beta_x\gamma_2$ and $\alpha_6\beta_x\gamma_2$ subtypes do not respond to benzodiazepines and are therefore called benzodiazepine-insensitive receptors.[5,6] The β subunit type does not seem to influence the pharmacology of Bz, whereas it has been demonstrated[5−7] that the α subunit is the main determinant of BzR ligand action selectivity (therefore, BzR subtypes take their names from the α subunit). In situ mRNA hybridization, subunit specific immunoprecipitation, and immunoaffinity chromatography have made it possible to determine the distribution of the $GABA_A$ subtypes in the brain. The $\alpha_1\beta_x\gamma_2$ subtypes are the dominant ones, seeing that they are present in both cerebellum and cortex, the $\alpha_2\beta_x\gamma_2$ and $\alpha_3\beta_x\gamma_2$ are moderately abundant and are found mainly in the cortex and hippocampus, whereas the $\alpha_5\beta_x\gamma_2$ receptors are scarce, as they are widely expressed only in the hippocampus. This regional heterogeneity of the $GABA_A$ receptor subtypes in the brain has suggested that the different subtypes might be associated with different physiological effects. Actually, the α_1 containing BzR should be responsible for the sedative action, the α_2 subtype should mediate the anxiolytic activity and the myorelaxation effects, the role of the α_3 subtype remains unclear, or at least, it seems to be partly involved in mediating anxiety behavior, and finally, the α_5 subtype has been associated with cognition processes such as learning and memory.[5−8]

BzR ligands modulate $GABA_A$ receptors increasing or decreasing the action of the inhibitory GABA neurotransmitter, which, by binding to its receptor, determines the opening of the chloride channel. Depending on the type of efficacy, BzR ligands have been classified as agonists (anxiolytic, anticonvulsant, sedative-hypnotic, and myorelaxant agents), antagonists (nil efficacy), and inverse agonists (anxiogenic, somnolytic, proconvulsant, or even convulsant agents).[9,10]

Thus, a better knowledge of the structure of the site of action of the $GABA_A$/BzR complex should aid in the design and synthesis of high affinity subtype-selective ligands, which may lead to the selective treatment of anxiety, sleep disorders, convulsions, and memory deficits with fewer side effects. Unfortunately, our understanding of the $GABA_A$/BzR complex functioning is limited by the lack of high-resolution three-dimensional structural information. An acetylcholine-binding protein (AChBP) homologous to the N-terminal extracellular portion of the nicotinic acetylcholine receptor was crystallized by Breijc et al. in 2001.[11] This nicotinic acetylcholine receptor belongs, with $GABA_A$, glycine, and the 5-HT_3 receptors, to the same gene family, the ligand-gated ion channels (LGICs).[12,13] Thus, $GABA_A$ receptors possess a structural and, to a small extent, sequential homology with the crystallized protein.

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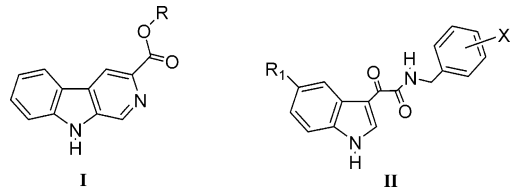
Several models of the extracellular, ligand-binding domain of the $GABA_A$/BzR complex have recently been proposed on the basis of the AChBP structure.[14−17] Despite these advances, no comprehensive models have so far been obtained. Before serious modeling can be performed, more information about the orientation of the residues in the BzR binding site has to be available.[18]

Some help to clarify the manner in which ligands bind to the $GABA_A$/BzR may be offered by the use of the techniques of chemical synthesis, radioligand binding, and receptor mapping. The pharmacophore/topological model proposed by Cook et al.[19] greatly improved our understanding of the structure−affinity relationships of BzR ligands in terms of ligand−receptor interactions. This model consists of several BzR (sub)sites: (i) a hydrogen bond acceptor (A_2), (ii) a hydrogen bond donor (H_1), (iii) a hydrogen bond donor/acceptor (H_2/A_3), (iv) four lipophilic pockets (L_1, L_2, L_3, and L_{Di}), and (v) sterically forbidden sites (S_1, S_2, and S_3) as boundaries of the receptor binding cleft.

Some years ago, we presented a new class of BzR ligands designed as open chain analogues of *β*-carbolines **I**, the *N*-(benzyl)indol-3-ylglyoxylamides **II** (Chart 1).[20] In these indole

Chart 1



derivatives, the effects of the R_1 and X substituents on potency were found to be interdependent. Specifically, the affinity was favored by hydroxy/methoxy or halogens as X substituents, depending on whether the 5-position of the indole nucleus was substituted (R_1 = Cl/NO_2) or not (R_1 = H), respectively. Thus, while affinity in the 5-Cl/NO_2 series was optimized by X = 3′,4′-$(OMe)_2$ (K_i = 11 nM), in the 5-H series, the highest potency was reached with X = 4′-Cl (K_i = 67 nM). The structure−affinity relationships of compounds **II** were subsequently rationalized by assuming that in their interaction with the receptor site, they adopt two alternative binding modes called A and B (Figure 1).[21] The 5-Cl/NO_2 ligands bind to the BzR in

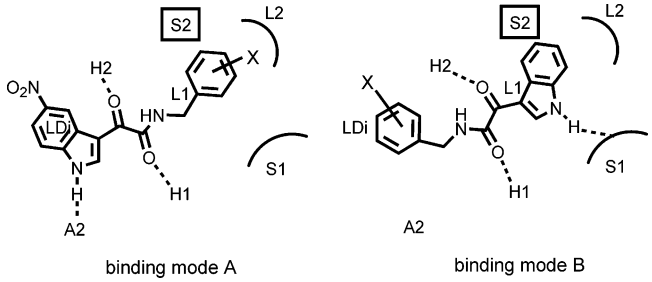
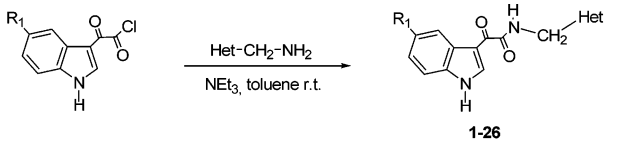


Figure 1. The hypothetical binding modes A and B of indole BzR ligands within the framework of Cook's pharmacophore/topological model.[19]

mode A, by interacting with the A_2 site (through the indole NH), with the H_1 and H_2 sites (through the C═O2 and C═O1), and with the L_1, L_2, and L_{Di} lipophilic regions (filled by the CH_2, the side chain phenyl ring, and the benzene moiety of the indole nucleus, respectively). In this orientation, the ligand affinity is enhanced by hydroxy and/or methoxy X substituents.

Binding mode B requires R_1 = H (substituents bulkier than a hydrogen would be repelled by the sterically forbidden region

Scheme 1



R_1 = H, NO_2

Het = 1*H*-pyrrol-2-yl, 1-methyl-1*H*-pyrrol-2-yl, indol-2-yl, fur-2-yl, 5-methylfur-2-yl, thien-2-yl, pyrid-2-yl, pyrid-3-yl, pyrid-4-yl, 1*H*-pyrrol-3-yl, fur-3-yl, thien-3-yl, imidazol-2-yl.

S_2) and is favored by electron-attracting X groups. This binding mode relies on the following interactions: C═O2 and C═O1 are hydrogen-bound to the H_1 and H_2 sites; the L_1, L_2, and L_{Di} lipophilic regions are occupied by the pyrrole and benzene moieties of the indole nucleus, and the side chain phenyl ring, respectively; a hydrogen bond is donated by the indole NH to a heteroatom belonging to the S_1 site.

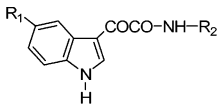
To verify our hypothesis of a hydrogen bond function located on the surface of the S_1 site, and with the aim of exploiting this interaction to improve the affinity of indolylglyoxylamides, we prepared and carried out biological assays on a series of *N*-(heteroarylmethyl)indol-3-ylglyoxylamides **1**−**26**. The *N*-(pyrrol-2-ylmethyl)indol-3-ylglyoxylamides **1** and **2** formally derive from benzylamides **II** by replacement of the side phenyl ring with a pyrrole moiety capable of donating a hydrogen bond. The N-methylated analogues **3** and **4** were designed as negative controls to verify the effect of the hydrogen bonding properties of **1** and **2** on the affinity. Moreover, to assess whether the hydrogen bond acceptor group on S_1 could also act as a hydrogen bond donor, the *N*-(fur-2-ylmethyl)indol-3-ylglyoxylamides **7**−**10**, featuring an acceptor oxygen atom, were prepared and tested. To gain information about the location of the hypothesized hydrogen bond acceptor/donor group (HBA/D) at the S_1 site, the 3-pyrrolylmethyl **19**, **20**, the 3-furylmethyl **21**, **22**, the 2-pyridylmethyl **13**, **14**, the 3-pyridylmethyl **15**, **16**, and the 4-pyridylmethyl **17**, **18** derivatives were also investigated. Finally, to probe the dimensions and the physicochemical properties of the S_1 site environment, the 2-indolylmethyl **5**, **6**, the 2-thienylmethyl **11**, **12**, the 3-thienylmethyl **23**, **24**, and the 2-imidazolylmethyl **25**, **26** derivatives were also synthesized and biologically evaluated.

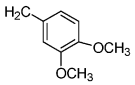
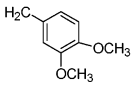
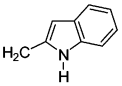
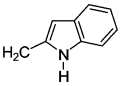
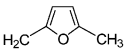
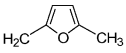
Chemistry. The general synthetic procedure used in the preparation of compounds **1**−**26** involved the acylation of indole and 5-nitroindole with oxalyl chloride, in accordance with a published procedure.[22] The indolylglyoxylyl chlorides obtained were allowed to react in mild conditions with the appropriate amine in the presence of triethylamine in toluene solution (Scheme 1). The amines employed for the synthesis of compounds **1**−**26** were commercially available or obtained in accordance with published procedures (see Experimental Section), except for the unknown 3-aminomethylpyrrole, which was prepared by slightly modifying the literature procedure for the obtainment of 2-aminomethylpyrrole,[23] involving the reduction of pyrrole-3-carbaldehyde oxime with sodium in ethanol solution.

All target products were purified by recrystallization from the appropriate solvent, and their structures were confirmed by IR, ^{1}H NMR, and elemental analysis. The physical and spectral data of all the newly synthesized compounds **1**−**26** are reported in the Supporting Information.

Binding Studies. The binding affinity of each newly synthesized indole derivative at the BzR in bovine brain membranes was determined by competition experiments against the radiolabeled antagonist [^{3}H]flumazenil[24] and was expressed as the K_i value only for those compounds inhibiting radioligand binding

Table 1. Inhibition of [^{3}H]Flumazenil Specific Binding to Bovine Cortical Membranes by Indolylglyoxylamides **1**−**26**



n°	R_1	R_2	K_i (nM)[a]	n°	R_1	R_2	K_i (nM)[a]
IIa[b]	H		120.0 ± 11	**13**	H		6000.0 ± 432
IIb[b]	NO_2		117.0 ± 12	**14**	NO_2		439.0± 37
IIc[b]	H		94.0 ± 10	**15**	H		105.0 ± 9
IId[b]	NO_2		11.0 ± 3	**16**	NO_2		483.0 ± 35
1	H		531.0 ± 48	**17**	H		426.0 ± 42
2	NO_2		33.0 ± 3	**18**	NO_2		770.0 ± 62
3	H		4590.0 ± 376	**19**	H		204.0 ± 17
4	NO_2		134.5 ± 13	**20**	NO_2		21.0 ± 4
5	H		1134.0± 97	**21**	H		447.0 ± 41
6	NO_2		>10000	**22**	NO_2		22.0 ± 3
7	H		324.0± 30	**23**	H		212.0 ± 15
8	NO_2		12.6 ± 2	**24**	NO_2		148.0 ± 12
9	H		249.0 ± 23	**25**	H		1745.0 ± 142
10	NO_2		15.6 ± 3	**26**	NO_2		>10000
11	H		285.0 ± 18	**diazepam**			10.0 ± 0.9
12	NO_2		128.0 ± 11	**flumazenil**			0.90 ± 0.05

[a] K_i values are means ± SEM of three determinations. [b] Data taken from ref 21.

by more than 80% at a fixed concentration of 10 μM (Table 1). For comparison purposes, the binding data of benzylindol-3-ylglyoxylamides **IIa**−**d** are also included in Table 1.[21]

The subtype selectivity of the most active compounds (**1**, **2**, **8**, **19**, **20**, and **22**) was tested by assaying their ability to displace [^{3}H]flumazenil in membranes from HEK293 cells expressing rat $\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_2\gamma_2$, and $\alpha_5\beta_3\gamma_2$ $GABA_A$/Bz receptor subtypes (Table 2).[21]

Results and Discussion

As shown by the data reported in Table 1, the 5-NO_2 pyrrole derivative **2**, which is supposed to interact with the BzR in mode A, is 3.5-fold more potent than benzylamide **IIb**, thus suggesting that the pyrrole NH could donate a hydrogen bond to an acceptor group located at the S_1 site of the BzR. This hypothesis is supported by the detrimental effect resulting from methylation of the pyrrole nitrogen, as compound **4** is 4-fold less potent than **2** and therefore equipotent to the benzylamide derivative **IIb**.

Figure 2 shows a low resolution model of interaction between the pyrrole derivative **2** and the BzR, where the putative hydrogen bonding function of the S_1 site is represented as the side chain of a hydroxyl-bearing amino acid.

The 5-H derivative **1** (hypothesized to adopt binding mode B at the BzR) could in principle interact with the A_2 acceptor site through the pyrrole NH. However, the affinity value of **1** (K_i = 531 nM) similar to, even if slightly lower than, that of the benzylamide **IIa** (K_i = 120 nM), which bears a side phenyl ring with no hydrogen bond ability, leads to suppose that the pyrrole moiety of **1** is not likely engaged in any hydrogen bond with the BzR. Furthermore, the pyrrole moiety, which substitutes the more lipophilic side chain phenyl ring of **IIa**, establishes a

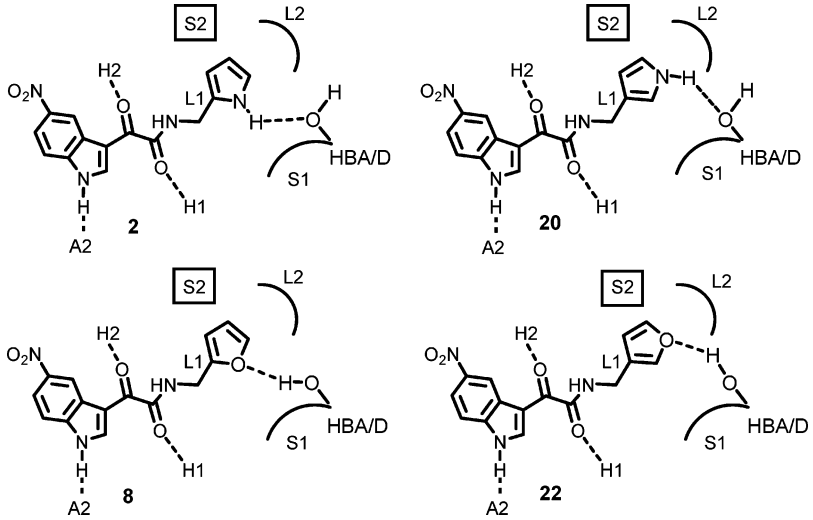


Figure 2. Hypothetical binding mode A at the BzR of compounds **2** and **20**, **8** and **22**, assumed to form a hydrogen bond with the HBA/D function located at the S_1 site.

Table 2. Inhibition of [^{3}H]Flumazenil Specific Binding on Rat $\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_2\gamma_2$, and $\alpha_5\beta_3\gamma_2$ $GABA_A$/Bz Receptor Subtypes by Selected Compounds[a]

	K_i (nM)[b] or % inhibition (10 μM)[c]		
no.	$\alpha_1\beta_2\gamma_2$	$\alpha_2\beta_2\gamma_2$	$\alpha_5\beta_3\gamma_2$
1	271 ± 15	47% ± 5	1500 ± 143
2	73.5 ± 6	585 ± 43	5% ± 1
8	20 ± 3	155 ± 12	28% ± 3
19	272 ± 18	46% ± 4	681 ± 53
20	9.2 ± 0.8	154 ± 13	1600 ± 152
22	56.6 ± 4	236 ± 19	9% ± 2
zolpidem	50 ± 3	765 ± 63	35% ± 3

[a] The ability of the compounds to displace [^{3}H]flumazenil was measured in membranes from HEK293 cells expressing the $\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_2\gamma_2$, and $\alpha_5\beta_3\gamma_2$ subtypes, as described in the Experimental Section. [b] K_i values are means ± SEM of three determinations. [c] Percentage inhibition values of specific [^{3}H]flumazenil binding at 10 μM concentration are means ± SEM of three determinations.

less effective lipophilic interaction with the receptor binding site. The much lower potency of **3** (K_i = 4590 nM) with respect to **1** should therefore be due to a poor steric complementarity of this compound with the L_{Di} region.

Compounds **5** and **6**, which contain an indole moiety in the side chain, were designed to maintain the hydrogen bond donor ability, while increasing lipophilicity. The poor affinity displayed by these compounds appears to stem from their excessive overall bulk compared with the corresponding pyrrole derivatives **1** and **2**. Taken together, these data suggest that the BzR binding cleft possesses precise dimensions and cannot accommodate bulky groups into the L_2 and L_{Di} lipophilic regions simultaneously, as earlier proposed by Cook et al.[25]

As mentioned, the 2-furylmethyl derivatives **7**−**10** were designed to probe the hydrogen bond donor ability of the S_1 site. The K_i values of the 5-NO_2 furans **8** and **10** were similar to that exhibited by the corresponding 5-NO_2 pyrrole **2**, thus suggesting that a bifunctional hydrogen bond acceptor/donor (HBA/D) group may exist at the S_1 site.

The shift of the methylene spacer from position 2 to 3 of the pyrrole or furan ring gave compounds **19**, **20**, and **21**, **22**, which possess a similar affinity to that of their 2-substituted analogues. We speculate that the side chains of the 5-NO_2 compounds **2**, **8**, **20**, and **22** possess the right geometry, in the binding orientation A, to interact properly with the HBA/D group at the S_1 site. Figure 2 illustrates our hypothesis of how 5-NO_2 pyrrole and furan derivatives **2** and **20**, **8** and **22** might be oriented within the BzR binding cleft.

The similar affinity displayed by thiophene derivatives **11**, **12**, **23**, and **24** with respect to that of benzylamides **IIa** and **IIb** can be explained by considering that thiophene and benzene are similar in several physicochemical properties, such as size, lipophilicity (logP values are 1.89 and 2.13, respectively), and incapacity to form hydrogen bonds.

The introduction of 2-pyridyl, 3-pyridyl, and 4-pyridyl rings into the glyoxylamide side chain gave compounds **13**/**14**, **15**/**16**, and **17**/**18**. The 5-H derivatives **13**, **15**, and **17** showed a potency no better than that of the benzylamide **IIa**, thus confirming the hypothesis of binding mode B in which the ligands do not form any hydrogen bond with the A_2 acceptor site. Analogously to our hypothesis for the 5-NO_2 furan derivatives **8**, **10**, and **22**, the interaction at the BzR of the 5-NO_2 pyridine derivatives **14**, **16**, and **18** should in theory be favored by a hydrogen bond formed between the ligand pyridine nitrogen and the receptor HBA/D function. However, the pyridine derivatives **14**, **16**, and **18** turned out to be significantly less potent than the furan analogues **8**, **10**, and **22**. These findings might be attributed to the different hydrogen bonding geometric properties of nitrogen and oxygen as acceptors in aromatic heterocycles. Nobeli and co-workers investigated the directionality and relative strengths of hydrogen bonds between C−OH and aromatic rings containing nitrogen and/or oxygen heteroatoms, using crystal structure data and theoretical calculations.[26] These authors showed that the hydrogen bonds in the C−OH···O(furan) system are more widely scattered around the aromatic ring plane compared with the C−OH···N(pyridine) system. More specifically, the interaction energy is destabilized up to 5.0 kJ/mol upon deviations of the C−OH hydrogen from the pyridine plane by 25−35° or from the furan plane by 50°. In the quoted article, Nobeli's group concluded that "substituting oxygen for nitrogen heterocycles will generally have a marked effect on the binding of a protein ligand".[26] In light of the above-mentioned studies, given that the HBA/D group at the S_1 site might correspond to an OH placed out of the plane of the ligand side chain heterocycle, only the furan oxygen, but not the pyridine nitrogen, in addition to the pyrrole NH, would form a significantly attractive hydrogen bond with the receptor protein. These concepts are schematized in Figure 3 by means of three-dimensional models of ligand moieties illustrating the putative HBA/D function. The model discussed so far might also explain

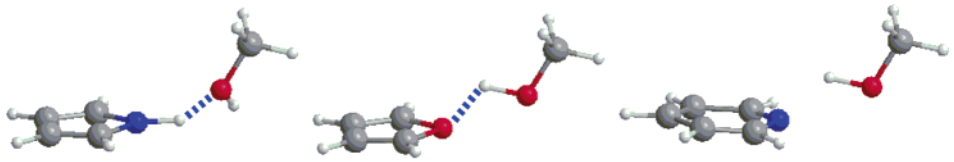


Figure 3. Three-dimensional models illustrating our hypothesis of a hydrogen bond between ligands featuring a pyrrole or a furan moiety (but not pyridine) in the side chain and a hydroxyl-bearing amino acid located in the vicinity of the S_1 site of the BzR binding cleft.

the high potency of the 3′,4′-dimethoxyphenyl-bearing 5-NO_2 derivative **IId**, as the result of a hydrogen bond formed between the 3′-OMe and the HBA/D function.

An additional point supporting the existence of an HBA/D group at the S_1 site is the markedly higher potency of the 5-NO_2 ligands compared with the 5-H counterparts, whenever the glyoxylamide side chain forms a hydrogen bond with the BzR in binding mode A (compare the K_i values of the couples **IId**/**IIc**, **8**/**7**, **10**/**9**, **20**/**19**, and **22**/**21**). In contrast, the couples of 5-NO_2/5-H derivatives which are not engaged in such a hydrogen bond do not display the affinity gap favoring the 5-NO_2 ligand (compare the K_i values of the couples **IIb**/**IIa**, **12**/**11**, and **24**/**23**).

The inactivity of imidazole derivatives **25** and **26**, which feature both hydrogen bond accepting and donating functions in the heteroaryl ring, might be ascribed to their high intrinsic hydrophilicity (the logP of imidazole is −0.08) as well as their propensity to be partly protonated at physiological pH (the pK_b of imidazole is 7.0).

A number of compounds (**1**, **2**, **8**, **19**, **20**, and **22**) were tested for their ability to displace [^{3}H]flumazenil from recombinant rat $\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_2\gamma_2$, and $\alpha_5\beta_3\gamma_2$ $GABA_A$/Bz receptor subtypes (Table 2). All of the ligands had enhanced affinities for the $\alpha_1\beta_2\gamma_2$ isoform compared with the $\alpha_2\beta_2\gamma_2$ and $\alpha_5\beta_3\gamma_2$ subtypes. It should be noted that the 5-H derivatives **1** and **19**, hypothesized to bind according to mode B, showed a high $\alpha_1\beta_2\gamma_2$ selectivity over the $\alpha_2\beta_2\gamma_2$ subtype, but a lower selectivity over the $\alpha_5\beta_3\gamma_2$ subtype; on the other hand, the 5-NO_2 derivatives **2**, **8**, **20**, and **22**, hypothesized to adopt binding mode A, displayed a very good $\alpha_1\beta_2\gamma_2$ selectivity over the $\alpha_5\beta_3\gamma_2$ subtype, but only a moderate selectivity over the $\alpha_2\beta_2\gamma_2$ subtype. It has been suggested that the basic topology of the BzR subtypes is highly conserved with the exception of the L_2 and L_{Di} lipophilic pockets, whose different dimensions might play a role in determining selectivity profiles.[27] In particular, it is hypothesized that the L_{Di} and L_2 regions are wider in the $\alpha_1\beta_2\gamma_2$ and $\alpha_5\beta_2\gamma_2$ binding sites, respectively, with respect to the other subtypes. Consequently, full occupation of L_{Di} or L_2 may account for $\alpha_1\beta_2\gamma_2$ and $\alpha_5\beta_2\gamma_2$ selectivity, respectively.[27] Finally, simultaneous occupation of L_{Di} and L_2 may promote α_2 selectivity.[28] This is consistent with the general $\alpha_1\beta_2\gamma_2$ selectivity profile displayed by our *N*-(heteroarylmethyl)indol-3-ylglyoxylamides filling the L_{Di} region in either of the two possible binding modes A and B. Additionally, the moderate affinity at the $\alpha_2\beta_2\gamma_2$ subtype shown by the 5-NO_2 derivatives **2**, **8**, **20**, and **22** might be related to simultaneous occupation of the L_{Di} and L_2 regions in binding mode A, whereas the 5-H derivatives **1** and **19**, which adopt binding mode B, display a significant affinity at the $\alpha_5\beta_3\gamma_2$ subtype thanks to a strong interaction of the bulky indole ring with the L_2 pocket.

On the basis of mutagenesis studies, Buhr and Amin,[29,30] independently postulated that the phenyl moiety of the side chain of α_1-Tyr209 is important for the binding of agonist, antagonist, and inverse-agonist BzR ligands. Thus, this α_1-Tyr209 phenyl ring should interact favorably with a common feature of these ligands, and, consequently, should be located in a region close to the L_1 lipophilic area, which is the only common region of interaction for all BzR ligands in Cook's pharmacophore/topological model.[31] As S_1 is part of the boundaries of the L_1 region, we tentatively hypothesize that the hydroxyl moiety of α_1-Tyr209 might represent the HBA/D group. The role of other amino acid-bearing side chains with hydrogen bond donor/acceptor capabilities, located in the proximity of α_1-Tyr209, cannot be ruled out until accurate three-dimensional information is obtained about the BzR binding site. In this connection, it has been demonstrated that Ser204[32] and Thr206[29] influence affinities for benzodiazepine binding site ligands.

The differences in affinity between the benzylamide **IIb** and the ligands which are thought to form a hydrogen bond with the putative receptor hydroxy group (compounds **2**, **8**, **10**, **20**, and **22**) vary from 3.5-fold to 7.7-fold. Such differences seem to be consistent with the energies associated with "neutral−neutral" hydrogen bonds which stabilize the ligand−receptor affinity up to a maximum of 15-fold, compared with the much stronger contributions to the affinity (up to 3000-fold) that come from "charge-reinforced" hydrogen bonds.[33]

In conclusion, the binding affinity data of the novel *N*-(heteroarylmethyl)indol-3-ylglyoxylamides **1**−**26** supported the existence of a hydrogen bond acceptor/donor (HBA/D) group located within the S_1 site of the BzR. The affinity results at the $GABA_A$/BzR receptor subtypes, performed on the more significant compounds of the series (**1**, **2**, **8**, **19**, **20**, and **22**, Table 2), indicate for these ligands a general $\alpha_1\beta_2\gamma_2$ selectivity over the other subtypes. Interestingly, those compounds adopting binding mode A, such as **2**, **8**, **20**, and **22**, also display a significant affinity at the $\alpha_2\beta_2\gamma_2$ subtype, probably because they simultaneously occupy both L_{Di} and L_2 regions, whereas ligands binding according to mode B (**1** and **19**), show some affinity at the $\alpha_5\beta_3\gamma_2$ subtype, thanks to the full occupation of the L_2 region by the bulky indole moiety.

Experimental Section

Chemistry. Melting points were determined using a Reichert Köfler hot-stage apparatus and are uncorrected. Boiling points were determined using a Büchi Melting Point B-540. IR spectra were recorded with a Pye Unicam Infracord Model PU956 in Nujol mulls. Routine ^{1}H NMR spectra were determined on a Varian Gemini 200 spectrometer using DMSO-d_6 as the solvent. Evaporation was performed in vacuo (rotary evaporator). Anhydrous sodium sulfate was always used as the drying agent. Analytical TLC was carried out on Merck 0.2 mm precoated silica gel (60 F-254) aluminum sheets, with visualization by irradiation with a UV lamp. Elemental analyses were performed by our Analytical Laboratory and agreed with theoretical values to within ±0.4%.

2-Aminomethylfurane, 2-aminomethyl-5-methylfurane, 2-aminomethylthiophene, 2-aminomethylpyridine, 3-aminomethylpyridine, 4-aminomethylpyridine are commercially available products. Indol-3-ylglyoxylyl chloride,[22] 5-nitroindol-3-ylglyoxylyl chloride,[22] 2-aminomethyl-1*H*-pyrrole,[23] 2-aminomethyl-1-methyl-1*H*-pyrrole,[34] 2-aminomethylindole,[35] 3-aminomethylfuran,[34] 3-aminomethylthiophene,[34] 2-aminomethylimidazole,[36] and pyrrole-3-carboxaldehyde[37] were prepared in accordance with reported procedures.

3-Aminomethyl-1*H*-pyrrole. A solution of 1*H*-pyrrole-3-carboxaldehyde[37] (0.810 g, 8.5 mmol), hydroxylamine hydrochloride

(0.945 g, 13.6 mmol), and K_2CO_3 (0.764 g, 5.5 mmol) in 25.0 mL of water was refluxed for 30 min. After cooling, the white crystals that precipitated were collected by filtration and recrystallized from water to give 0.751 g of pure 1*H*-pyrrole-3-carboxaldehyde oxime: yield 94%; mp 150−152 °C (lit. ref 38 mp 149 °C). The oxime obtained was dissolved in 13.0 mL of absolute ethanol, and the solution was maintained in a nitrogen atmosphere. Metallic sodium (2.17 g, 94.0 mmol) was then added, and the mixture was refluxed with stirring until all the sodium had reacted (about 1 h). After cooling, the suspension was treated with 10.0 mL of water and concentrated under reduced pressure, and the residue was extracted with diethyl ether. After drying over Na_2SO_4, evaporation of the solvent gave analytically pure 3-aminomethyl-1*H*-pyrrole as a colorless oil (yield 64%; bp 154 °C). IR ν cm^{-1}: 3350, 3280, 1600, 1580, 1440, 780. ^1H NMR (DMSO-d_6): δ 3.20 (bs, 2H, NH_2); 3.53 (s, 2H, CH_2); 5.96−5.98 (m, 1H, 4-H); 6.45−6.63 (m, 2H, 2-H, 5-H); 10.45 (bs, 1H, NH). Anal. ($C_5H_8N_2$), C, H, N.

General Procedure for the Synthesis of *N*-[(5-Substituted indol-3-yl)glyoxyl]amide Derivatives 1−26. Triethylamine (3.0 mmol) was added dropwise to a stirred suspension, cooled at 0 °C, of 5-substituted indolylglyoxylyl chloride[22] (2.5 mmol) and the appropriate amine (2.75 mmol) in 50 mL of dry toluene. The reaction mixture was left to warm to room temperature, stirred for 24−36 h (TLC analysis), and then filtered. The precipitate collected was triturated with a saturated $NaHCO_3$ aqueous solution, washed with water, and collected again to give a first portion of crude product. The toluene solution was evaporated to dryness, and the residue was treated with saturated $NaHCO_3$ aqueous solution, washed with water, and collected, to yield an additional amount of crude product. The quantities of amide derivatives obtained from the initial insoluble precipitate or from the toluene solution were variable, depending upon the solubility of the various compounds. All products **1−26** were finally purified by recrystallization from the appropriate solvent. Yields, recrystallization solvents, melting points, and analytical and spectral data are reported in the Supporting Information.

Radioligand Binding Studies. [^{3}H]Flumazenil (specific activity 70.8 Ci/mmol) was obtained from NEN Life Sciences Products. All other chemicals were of reagent grade and were obtained from commercial suppliers.

Bovine cerebral cortex membranes were prepared in accordance with ref 39. The membrane preparations were subjected to a freeze−thaw cycle, washed by suspension and centrifugation in 50 mM Tris-citrate buffer pH 7.4 (T1), and then used in the binding assay. Protein concentration was assayed by the method of Lowry et al.[40] [^{3}H]Flumazenil binding studies were performed as previously reported.[21]

HEK293 cells stably expressing rat $GABA_A$ receptor subtypes ($\alpha_1\beta_2\gamma_2$, $\alpha_3\beta_2\gamma_2$, $\alpha_5\beta_3\gamma_2$) were maintained, as previously described[41] in DMEM/Nut Mix F-12 with Glut-I (GIBCO), supplemented with 10% fetal bovine serum, L-glutamine (2 mM), penicillin (100 units/mL), and streptomycin (100 μg/mL) in a humidified atmosphere of 5% CO_2/95% air at 37 °C. Cells were harvested and then centrifuged at 500*g*. The crude membranes were prepared after homogenization in 10 mM potassium phosphate, pH 7.4, and differential centrifugation at 48 000*g* for 30 min at 4 °C. The pellets were washed twice in this manner before final resuspension in 10 mM potassium phosphate, pH 7.4, containing 100 mM potassium chloride.[41]

[^{3}H]Flumazenil binding assays to transfected cells membranes were carried out as previously described.[41] In brief, the cell line membranes were incubated in a volume of 500 μL which contained [^{3}H]flumazenil at a concentration of 1−2 nM and the test compound in the range 10^{-9}−10^{-5} M. Nonspecific binding was defined by 10^{-5} M diazepam. Assays were incubated to equilibrium for 1 h at 4°C.

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Supporting Information Available: Physical (Table 1), spectral (Table 2), and analytical data of **1−26**. This material is available free of charge via the Internet at http://pubs.acs.org.

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