A Novel Class of Potent 3-Isoxazolol $GABA_A$ Antagonists: Design, Synthesis, and Pharmacology

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Introduction. 4-Aminobutyric acid (GABA, **1**) (Figure 1) is the major inhibitory neurotransmitter in the central nervous system (CNS) and exerts its effect through activation of the ionotropic $GABA_A$ and $GABA_C$ receptors and the metabotropic $GABA_B$ receptors.[1,2] The GABAergic system, especially $GABA_A$ receptors, has been associated with certain neurological and psychiatric disorders and is a potential target for therapeutic intervention in such disorders.[2,3] To pharmacologically characterize this receptor class, a number of $GABA_A$ ligands bioisosterically derived from GABA, such as the selective agonists muscimol (**2**)[4] and 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol (THIP, **3**),[5,6] have been developed. Gabazine (**4**),[7] now used as a standard antagonist for the $GABA_A$ receptors, represents another structural class of ligand.

The nature of dysfunction of the GABAergic system in schizophrenia is still unclear, but there is growing indirect evidence supporting the hypothesis of $GABA_A$ receptor hyperactivity as being a component of the mechanisms underlying schizophrenic symptoms. On the basis of receptor binding studies, an increase in the density of $GABA_A$ receptors in brains from schizophrenic patients has been observed.[8−10] Furthermore, activation of $GABA_A$ receptors has been reported to produce psychotomimetic effects in normals and to stimulate psychotic symptoms in schizophrenics.[11] In Alzheimer's disease, a reduced central cholinergic neurotransmission appears to be an important factor.[12] Since these acetylcholine neurons are under inhibitory GABAergic control, a stimulation of cholinergic transmission could, in principle, be achieved by blockade of $GABA_A$ receptors.[13] Therapeutic GABAergic approaches in Alzheimer's disease seem to be supported by studies on different ligands for the $GABA_A$ receptor in animal models relevant to learning and memory.[14,15]

These observations suggest that in Alzheimer's disease as well as schizophrenia, $GABA_A$ antagonists or low-efficacy partial agonists may have therapeutic interest. In a previous study we have described 5-(4-piperidyl)-3-isoxazolol (4-PIOL, **5**)[16,17] and, more recently, analogues of 4-PIOL as low-efficacy partial $GABA_A$ agonists showing dominating antagonist profiles.[18] The structure−activity relationships (SARs) of these 4-PIOL analogues and the corresponding analogues of muscimol (**2**) and THIP (**3**) are not straightforward and do not seem to fit into previously described $GABA_A$ agonist pharmacophore models.[18−20]

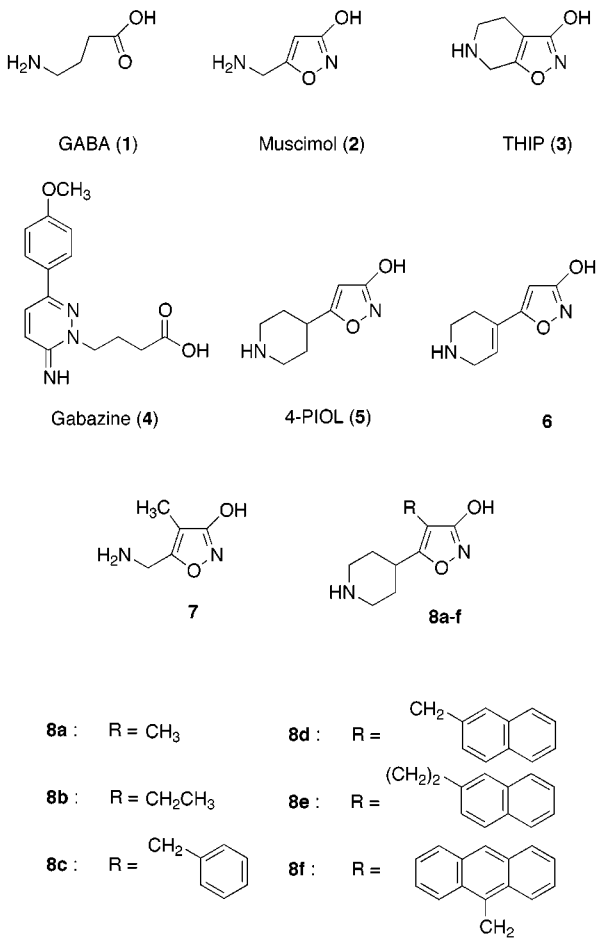

Figure 1. Structures of GABA (**1**), the $GABA_A$ agonists muscimol (**2**), THIP (**3**), and **7**, the $GABA_A$ antagonist gabazine (**4**), the low-efficacy partial $GABA_A$ agonists 4-PIOL (**5**) and **6**, and the new 3-isoxazolols (**8a**−**f**).

Molecular Modeling. Previous attempts to elucidate the structural relationships of muscimol (**2**), THIP (**3**), and 4-PIOL (**5**) in their bioactive conformations at the $GABA_A$ receptor have all been based on the assumption that superimpositions of the amino nitrogens and the 3-isoxazolol rings are required. Compounds **2** and **3** have been superimposed using this assumption,[20] and Buur et al.[19] superimposed **3** and 4-PIOL (**5**) in this manner by assuming that the 3-isoxazolol moiety in the bioactive conformation of **5** adopts an axial position of the piperidine ring. However, several lines of arguments make these superimpositions less probable, and in order to test the superimposition proposed by Buur et al., compound **6** was synthesized and pharmacologically characterized. This compound was found to have an affinity for the $GABA_A$ receptor which is lower than that of **5** by only a factor of 3.[18] Since an axial position of the 3-isoxazolol ring is ruled out in compound **6** due to ring unsaturation, it is not likely that the corresponding ring system in **5** adopts an axial orientation of the piperidine ring when binding to the receptor. Further-

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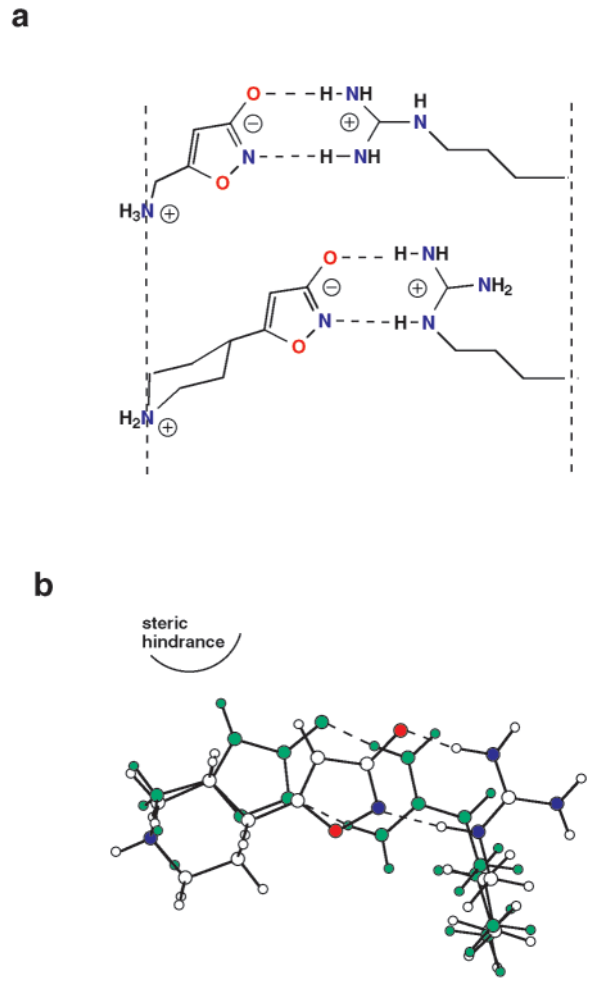


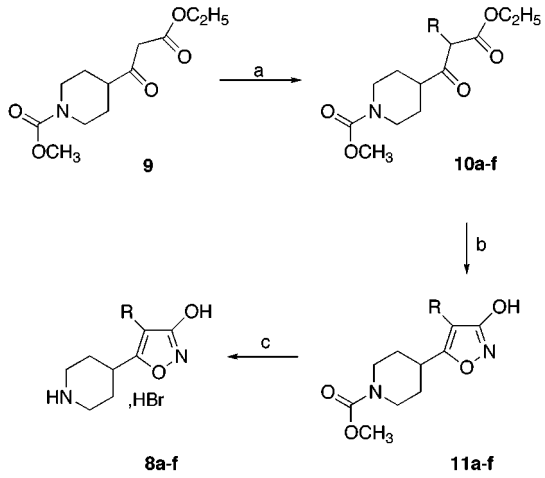
Figure 2. Hypothesis for the binding of muscimol (**2**) and 4-PIOL (**5**) to the $GABA_A$ receptor (a) and a molecular least-squares superimposition of the proposed bioactive conformations of **2** (green) and **5** binding to two different conformations of an arginine residue (b).

more, the replacement of the isoxazol oxygens in **3** and **5** by a sulfur atom has markedly different effects on $GABA_A$ receptor affinity.[18,20] The affinity of the sulfur analogue of **5** is 7 times higher than that of the parent compound,[18] whereas the sulfur analogue of **3** displays more than 300 times lower affinity than **3** itself.[21] These observations strongly indicate that the 3-isoxazolol rings in **3** and **5** do not have identical positions in the receptor binding cavity.

Ab initio quantum chemical calculations on the conformational properties of **2** indicate that in order for **2** to mimic the conformation of **3**, a very high conformational energy (8.9 kcal/mol) is required.[22] Thus, it is highly improbable that the bioactive conformation of **2** corresponds to that of **3**. Furthermore, the very low affinity of 4-methylmuscimol (**7**) (IC_{50} > 100 μM)[23] compared to that of **2** (IC_{50} = 0.006 μM)[6] is most probably due to strong steric repulsions between the 4-methyl group and the receptor. This makes a superimposition of the 3-isoxazolol ring systems of **2** and **3** less likely as the "sterically forbidden" 4-methyl group would then overlap with a methylene group in **3**, which is not compatible with the high $GABA_A$ receptor affinity of **3** (IC_{50} = 0.13 μM).[6]

To overcome the problems of the structural relationships of the bioactive conformations and the receptor

Scheme 1[a]



[a] Reagents: (a) NaOEt, RBr; (b) $NH_2OH{\cdot}HCl$, NaOH, then concd HCl; (c) HBr, AcOH.

binding modes of **2** and **5**, it was envisaged that an arginine residue is a suitable binding partner to the carboxylate group of the endogenous ligand, GABA (**1**), as well as to the 3-isoxazolol anions of **2** and **3**. High-level quantum chemical ab initio calculations show that a bidentate interaction between the arginine side chain and the ligand is compatible with observed SARs.[24] The involvement of an arginine residue in the binding of muscimol has recently been demonstrated by site-directed mutagenesis studies.[25−27]

The flexibility of the arginine side chain makes it feasible to accommodate **2** and **5** in the same binding pocket by assuming that the side chain adopts different conformations in its binding to the two ligands, as schematically illustrated in Figure 2a. A least-squares molecular superimposition of mutually low-energy conformations of **2** and **5** calculated by using the MM3* force field implemented in the MacroModel program[28] is shown in Figure 2b.

In the proposed binding modes of **2** and **5**, the 3-isoxazolol rings do not overlap (Figure 2). This implies that the 4-position in **5** does not correspond to the "forbidden" 4-position in **2**. Thus, in contrast to the muscimol (**2**) case, substitution in the 4-position of **5** may be allowed. Since no 4-substituted 4-PIOLs have so far been reported, the properties at the $GABA_A$ receptor of such compounds are of considerable interest to explore.

We here describe the synthesis and pharmacological evaluation of a series of 4-PIOL (**5**) analogues **8a**−**f** in which the above-mentioned 4-position of the 3-isoxazolol ring was substituted by alkyl groups such as methyl and ethyl, a benzyl group, and more bulky and lipophilic aromatic groups such as naphthylmethyl, naphthylethyl, and anthracylmethyl (Figure 1).

Chemistry. The analogues of **5** were all synthesized using the same route as outlined in Scheme 1 starting from the β-oxo ester **9**, which was synthesized by a modification of the reported method.[16] Alkylation of **9** was performed using the appropriate alkyl halide in the presence of sodium ethoxide to give the compounds **10a**−**f**. Cyclization of the alkylated β-oxo esters with hydroxylamine at −30 °C followed by heating with concentrated hydrochloric acid at 80 °C gave the 3-isox-

Table 1. Receptor Binding and in Vitro Electrophysiological Data

compd	[^{3}H]muscimol binding[a] K_i (μM)[c]	electrophysiology[b] IC_{50} (μM)[c]
gabazine (**4**)	0.074 (0.059; 0.094)	0.24 (0.22; 0.25)
4-PIOL (**5**)	9.1 (8.2; 10)	110 (76; 170)
8a	10 (10; 11)	26 (22; 31)
8b	6.3 (5.1; 7.6)	10.3 (7.7; 13)
8c	3.8 (3.3; 4.5)	4.0 (3.7; 4.3)
8d	0.049 (0.043; 0.057)	0.37 (0.31; 0.44)
8e	0.49 (0.43; 0.54)	0.89 (0.83; 0.95)
8f	5.9 (5.0; 7.0)	1.3[d] (1.2; 1.5)

[a] Standard receptor binding on rat brain synaptic membranes, $n = 3$. [b] Whole-cell patch clamp recordings from cerebral cortical neurons cultered for 7−9 days, $n = 6-17$. [c] Mean and SEM were calculated assuming a logarithmic distribution of the IC_{50} value.[29] Hence, numbers in parentheses (min; max) indicate ±SEM according to a logarithmic distribution of IC_{50}. [d] Because of slow onset of antagonism, the parameters for **8d** were calculated using the response magnitude after 5-s application.

azolols **11a**−**f**, which were deprotected by treatment with hydrogen bromide in glacial acetic acid to give the target compounds **8a**−**f**.

In Vitro Pharmacology. The affinities of the compounds **8a**-**f** for $GABA_A$ and $GABA_B$ receptor sites or GABA uptake sites in rat brain membrane preparations, using either [^{3}H]muscimol or [^{3}H]GABA as the radioactive ligand, were determined using a modified version of the methods described previously.[18] At test concentrations of 100 μM for **8a**−**c** and, due to solubility problems, of 10 μM for **8d**−**f**, none of the compounds showed a detectable affinity for $GABA_B$ receptors or GABA uptake sites. Like **5**, all of the tested compounds did show affinity for $GABA_A$ receptor sites, and binding affinities (K_i values) of compounds **8a**−**f** for $GABA_A$ receptor sites are listed in Table 1.

Introduction of alkyl groups such as methyl and ethyl as well as a benzyl group in the 4-position of the 3-isoxazolol ring of **5** is tolerated, compounds **8a**−**c** showing affinity for the $GABA_A$ receptor sites comparable to that of **5**. Remarkably, introduction of the more bulky 2-naphthylmethyl group in the same position to afford **8d** not only was tolerated but led to a 70-fold increase in binding affinity as compared to the benzyl analogue **8c**. Extension of the linker joining the 2-naphthylmethyl group and the 3-isoxazolol ring to give compound **8e** resulted in a 10-fold reduction in affinity for the $GABA_A$ receptor relative to **8d**. Further reduction in $GABA_A$ receptor affinity was found for compound **8f**, where the 2-naphthylmethyl group of **8d** has been replaced by a 9-anthracylmethyl group.

Using whole-cell patch-clamp recordings from cultured cerebral cortical neurons, performed as described previously,[18] the functional properties of compounds **8a**−**f** in the absence or presence of the specific $GABA_A$ receptor agonist isoguvacine[5,6] (20 μM) were studied. In a previous study, using cerebral cortical neurons, we have characterized **5** as a partial agonist at $GABA_A$ receptors.[18] In the present study, all of the compounds tested were capable of inhibiting the current induced by isoguvacine in a dose-dependent manner, as illustrated in Figure 3, showing a good correlation to the obtained binding affinities (Table 1). As shown in the $GABA_A$ receptor binding assay, the 2-naphthylmethyl analogue **8d** was the most active member of this series showing an antagonist potency comparable with that

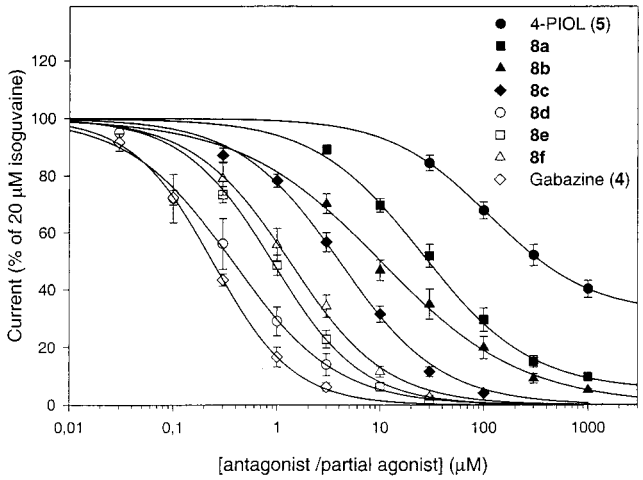


Figure 3. Effect of the partial agonists or antagonists on the response to 20 μM isoguvacine using whole-cell patch-clamp recordings from cultured cerebral cortical neurons. 20 μM isoguvacine and varying concentrations of antagonists/partial agonist were applied simultaneously to the cells. The response of 20 μM isoguvacine alone has been set as 100%, and the other responses are expressed as a fraction of this. The response to isoguvacine is progressively reduced with increasing concentrations of the partial agonist or antagonist. The numbers of cells tested in this way with each compound varied from $n = 6$ to $n = 17$.

of the standard $GABA_A$ antagonist gabazine (**4**). Interestingly, only compounds **8a**,**b** retained some ability to induce currents themselves at a concentration of 1 mM (not shown), while compounds **8c**−**f** showed no detectable agonist effect at test concentrations of 100 μM (**8c**) or 30 μM (**8d**−**f**).

The present results indicate that substitution in the 4-position of the 3-isoxazolol ring of **5** is indeed allowed in contrast to substitution in the corresponding position in **2** and support the hypothesis mentioned above concerning the binding modes of **2** and **5** illustrated in Figure 2, where the 4-positions of the 3-isoxazolol rings of **2** and **5** are placed differently. A tendency for larger substituents to show higher affinity for the $GABA_A$ receptor can be seen for the methyl, ethyl, and benzyl analogues (compounds **8a**−**c**). Extension of the aromatic system from a phenyl to a naphthyl group to give **8d** markedly increases the affinity, with a corresponding increase in potency. The naphthylmethyl group in compound **8d,** seems to be optimal, at least in this series, as regards the sterical and/or conformational requirements for interaction with the antagonist conformation of the $GABA_A$ receptor. Although the affinity of **8f** is 100-fold lower than that of **8d**, it still has affinity for the receptor, which suggests a large cavity at the 4-PIOL recognition site of the $GABA_A$ receptor, capable of accommodating not only alkyl substituents but also aromatic systems of larger size in this type of compounds. Occupancy of this suggested $GABA_A$ receptor cavity by an appropriately sized group may contribute to the stabilization of the antagonist conformation or, alternatively, destabilization of the agonist conformation of the $GABA_A$ receptor.

In conclusion, the modest $GABA_A$ receptor affinity of **5** could be markedly enhanced by introducing large aromatic substituents into the 4-position of the 3-isoxazolol ring of **5**, supporting the hypothesis concerning the binding mode of **5** and muscimol (**2**). Furthermore

the structural modifications led to a change in the pharmacological profile of the compounds from low-efficacy partial $GABA_A$ receptor agonist activity of **5** to potent and selective antagonist effect of **8d** as the key compound. This and related compounds could be useful tools for studies of the $GABA_A$ receptor mechanisms, and further studies in this area are in progress.

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Supporting Information Available: Experimental details. This material is avaible free of charge via the Internet at http://pubs.acs.org.

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