Letters

Synthesis, in Vitro Affinity, and Efficacy of a Bis 8-Ethynyl-4*H*-imidazo[1,5*a*]-[1,4]benzodiazepine Analogue, the First Bivalent α5 Subtype Selective BzR/GABA$_A$ Antagonist

Xiaoyan Li,[†] Hui Cao,[†] Chunchun Zhang,[†] Roman Furtmueller,[‡] Karoline Fuchs,[‡] Sigismund Huck,[‡] Werner Sieghart,[‡] Jeffrey Deschamps,[§] and James M. Cook[†,*]

Department of Chemistry, University of Wisconsin−Milwaukee, Milwaukee, Wisconsin 53201, Brain Research Institute, Medical University, Vienna, A-1090 Vienna, Austria, and Laboratory for the Structure of Matter, Code 6030, Naval Research Laboratory, 4555 Overlook Avenue, SW, Washington, D.C. 20375

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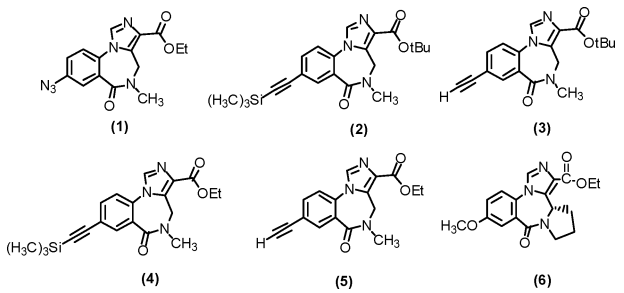
Abstract: The synthesis and in vitro affinity of the α5β3γ2 (α5) subtype selective BzR/GABA$_A$ antagonist **7** is described. This ligand is selective for α5 subtypes in vitro and is a potent antagonist of the effects of diazepam only at α5β3γ2 subtypes (oocytes). Ligands such as **7** will be important in the determination of which physiological function(s) are subserved by this GABA$_A$ α5 subtype.

The γ-aminobutyric acid (GABA$_A$) receptors are heteroligomeric membrane-bound protein complexes that are composed of several subunits. The inhibitory effects of GABA mediated by these receptors can be modulated by a number of pharmacological agents that selectively bind to allosteric sites at these receptors.[1−4] GABA$_A$ receptors are pentameric assemblies of proteins derived from a family of subunits (6α, 4β, 4 γ, 1δ, 1π, 1θ, 1ϵ, and 3ρ)[4,5] which form a chloride ion channel. The most common form of the GABA$_A$ receptor contains α, β, and γ subunits[4−6] and recombinant receptors containing these subunits mimic the biological, electrophysiological, and pharmacological properties of native GABA$_A$ receptors. The benzodiazepine binding site occurs at the interface of the α and γ subunits;[7] furthermore, the α subunit is the key determinant of benzodiazepine binding and efficacy. However, it is clear the gamma subunit is also required for benzodiazepine binding.[4,6] Receptors containing the α5 subunit are of minor abundance (5%) in the whole brain, but are expressed to a significant extent in the hippocampus, where they comprise 15−20% of the diazepam-sensitive GABA$_A$ receptor population and are predominantly coassembled with the β3 and γ2 subunits.[6,8] Both in situ hybridization and immuno-histochemical studies indicate that the hippocampus is relatively enriched in α5-containing GABA$_A$ receptors compared to other brain areas.[9,10]

Scheme 1



Interest in BzR/GABA$_A$ α5 subtypes has been stimulated recently by the report of Möhler et al.[11] on α5 knockin mice. In brief, this group has provided strong evidence that hippocampal extrasynaptic α5 GABA$_A$ receptors play a critical role in associative learning.[11]

Previously, a series of α5 subtype selective ligands [(**2**, RY-023), (**3**, RY-024), (**4**, RY-079), and (**5**, RY-080)] based on the structure of (**1,** Ro 15−4513) have been reported from this laboratory,[12−16] as well as several ligands by McKernan, Atack et al. (see **6**).[17,18] These ligands are BzR inverse agonists in vivo, and a number have been shown to enhance cognition.[17−21] One of these ligands was shown by Bailey et al.[19] to be important in the acquisition of fear conditioning and provided further evidence for the involvement of hippocampal GABA$_A$/benzodiazepine receptors in learning and anxiety.[19] This was supported by the work of DeLorey et al.[20] in a memory model with a ligand closely related to α5 subtype selective inverse agonists **3** and **4**. While the physiological and pharmacological roles of specific GABA$_A$ receptor subtypes have not been fully evinced, the synthesis of subtype selective ligands should prove useful tools with which to clarify this. The ligands **2**−**6** depicted in Scheme 1 were 40−70-fold more selective for α5 subtypes; however, better selectivity remains a goal of paramount importance. To enhance subtype selectivity, the bivalent form[22−24] of **5**,[13,14] that is 1,3-bis(8-ethynyl-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo-[1,5*a*][1,4]benzodiazepine-3-carboxy)propyl diester, (**7**, XLi093) was designed. The synthesis, α5 subtype selectivity and antagonist properties of this ligand form the basis of this letter.

Chemistry. In brief, isatoic anhydride (**8**) and sarcosine (**9**) were heated in DMSO, followed by bromination to provide the bromide **11** (via **10**), as illustrated in Scheme 2. The conversion of bromide **11** into the imidazobenzodiazepine **12**, followed the classic work of Fryer et al. of the Roche group.[25−27] This bromide was converted into **4** by a Heck-type coupling reaction,[13,14] and the silyl group was removed in high yield on treatment with TBAF/H$_2$O/THF.[13,14] Hydrolysis of the ester function of **5** provided the acid **13** in excellent yield and this material was subjected to a standard CDI-mediated coupling reaction to furnish bivalent ligand **7** in 73.4% yield.

* To whom correspondence should be addressed. E-mail: capncook@csd.uwm.edu.
[†] University of Wisconsin−Milwaukee.
[‡] Brain Research Institute.
[§] Naval Research Laboratory.

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Scheme 2

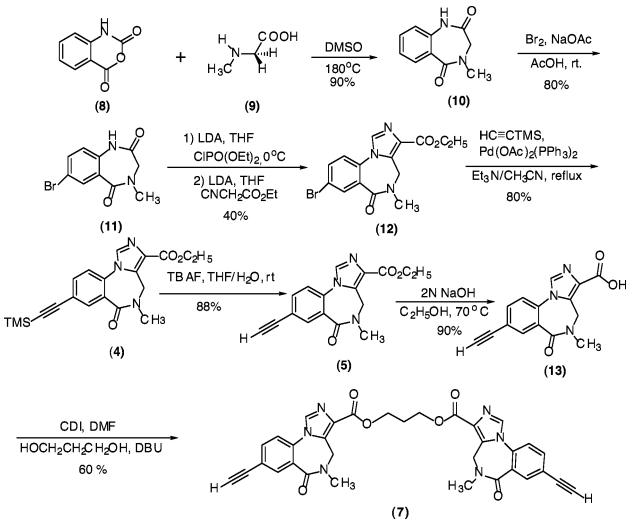

Table 1. In Vitro Binding Affinities at $GABA_A$/BzR Subtypes[a]

ligand	α1	α2	α3	α4	α5	α6
1	33	2.6	25	N/A	0.3	3.8
2	197	143	255	7.8	2.61	58.6
3	26.9	26.3	18.7	N/A	0.4	5.1
4	121	142	198	159	5.0	114
5	28.4	21.4	25.8	53	0.49	28.8
6	48.5	27.4	24.5	N/A	0.45	83
7	>1000	>1000	858	1550	15	>2000

[a] Values Reported are in nM. [a] K_i values represent the mean of two determinations which differed by less than 10%. Data were generated using Ltk^- cell membranes expressing human $\alpha x\beta 3\gamma 2$ receptors. 1.8 nM [^{3}H]Ro15−1788 and 8 nM [^{3}H]Ro15−4513 (for cells expressing $\alpha 4\beta 3\gamma 2$ and $\alpha 6\beta 3\gamma 2$) were used as radioligands.

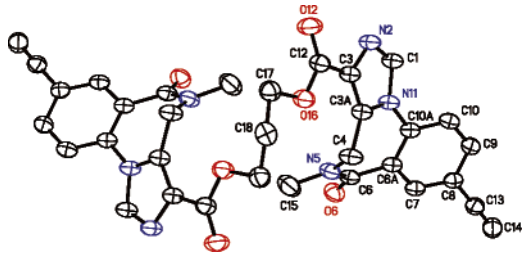

Figure 1. Crystal structure of bivalent ligand **7**.

The binding affinity of **7** in vitro was determined on $\alpha 1-6\beta 3\gamma 2$ LTK cells and is illustrated in Table 1. This ligand **7** bound to $\alpha 5\beta 3\gamma 2$ subtypes with a K_i of 15 nM, but exhibited little or no affinity at other BzR/$GABA_A$ subtypes. Although the potency at all receptor subtypes was decreased by incorporation of the pharmacophore of **5** into the bivalent structure, the loss of affinity at α1, α2, α4 and α6 subtypes was profound. Presumably, since the second unit of **7** lies in the extracellular domain of the BzR, the enriched selectivity is entropic. This was the most α5 subtype selective ligand reported, to date. Depicted in Figure 1 is the ORTEP drawing of the crystal structure of **7** (see Supporting Information for coordinates). The J values ($J = 5.38$) calculated from the dihedral angles were in excellent agreement with those from the solution determined NMR spectrum ($J = 6.39$); hence support for the conformation as shown (Figure 1) derives from the NMR data. This conformation of **7** was then placed in the BzR/$GABA_A$ α5 pharmacophore receptor model previously reported[28−30] from this laboratory; the fit was excellent (Figure 2).

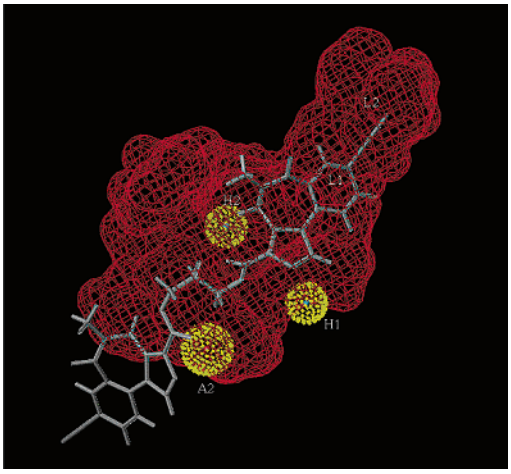

Figure 2. Compound **7** aligned in the included volume of the pharmacophore/receptor model for the $\alpha 5\beta 3\gamma 2$ subtype.

On the basis of the binding affinity of **7** and the fit at the α5 subtype, a study of the efficacy of **7** was then pursued in oocytes.

Effects of 7 on Chloride Currents in $GABA_A$ Receptors.[31,32] Effects of **7** on $GABA_A$ receptors were characterized using Xenopus oocytes expressing the $GABA_A$ receptor subunits α1 to α6 in combination with $\beta 3$ and $\gamma 2$ subunits. Using the two-electrode voltage clamp method, currents in the μA range were measured for all subunit combinations in response to application of a saturating concentration of GABA (10 mM). Two-electrode voltage clamp experiments were performed to test whether **7** triggered chloride currents, modulated GABA-induced currents or antagonized the effects of benzodiazepines in oocytes that express $GABA_A$ receptors.

Bivalent ligand **7** at concentrations up to 1 μM did not trigger chloride currents in any of the tested subtypes of the $GABA_A$ receptor. This dimer, however, at micromolar concentrations modulated GABA-induced currents in an α subtype specific manner. To test for agonistic or inverse agonistic effects of **7**, the compound was coapplied with a concentration of GABA that induced approximately 3% of the maximum current amplitude. At concentrations of **7** up to 100 nM, this ligand caused no significant modulation of GABA-induced currents in any subtype of $GABA_A$ receptors tested. At a concentration of 1 μM, **7** caused a significant increase in GABA-induced currents in the $GABA_A$ receptor subtypes $\alpha 4\beta 3\gamma 2$ [+37.6 ± 2.6% (SEM), $p < 0.0001$ in four oocytes] and $\alpha 6\beta 3\gamma 2$ [+ 17.0 ± 2.5% (SEM), $p = 0.0014$ in four oocytes]. Furthermore, 1 μM of **7** caused a reduction in GABA induced currents in the subtype $\alpha 5\beta 3\gamma 2$ [−4.0 ± 1.5% (SEM), $p = 0.0075$ in six oocytes]. However, 1 μM of **7** caused no significant modulation of GABA-induced currents in the $GABA_A$ receptor subtypes $\alpha 1\beta 3\gamma 2$, $\alpha 2\beta 3\gamma 2$, and $\alpha 3\beta 3\gamma 2$.

Ligand **7** shifted the dose response curve for diazepam in a subtype specific manner. To test for benzodiazepine antagonistic effects of **7**, dose−response experiments for the stimulation of GABA-induced currents by diazepam in the absence or presence of 1 μM **7** were performed. Similar conditions for different subtypes were provided by using a concentration of GABA that induced approximately 3% of the maximum current amplitude in

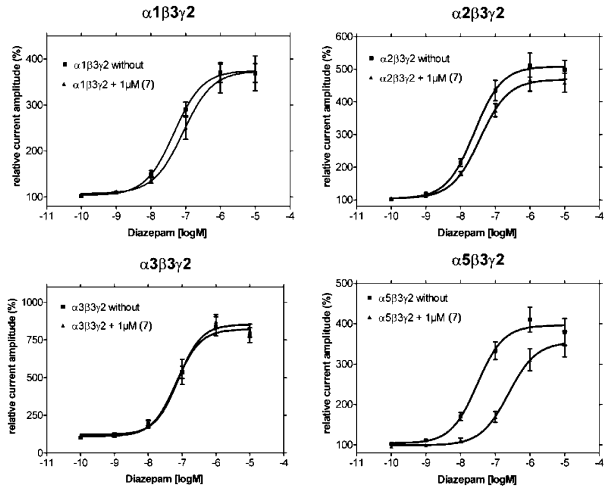

Figure 3.

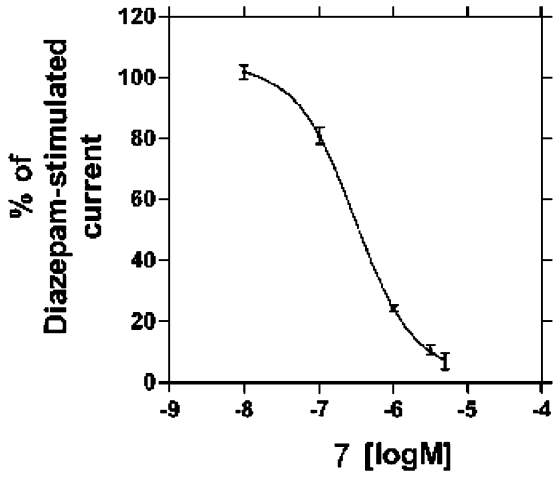

Figure 4.

the respective cell. In oocytes expressing $GABA_A$ receptors of subunit combination $\alpha5\beta3\gamma2$, the presence of 1 μM **7** shifted the dose−response curve for the stimulation of GABA-induced currents by diazepam to the right (Figure 3). Even high concentrations of diazepam could not fully overcome this inhibition since maximum current stimulations in the presence of **7** reached only approximately 85% of current stimulations that were seen in the absence of **7**.

In oocytes expressing $GABA_A$ receptors of the subtypes $\alpha1\beta3\gamma2$, $\alpha2\beta3\gamma2$, or $\alpha3\beta3\gamma2$, 1 μM **7** caused only marginal shifts of dose response curves for the stimulation of GABA-induced currents by diazepam (Figure 3).

Dose−response curves for diazepam with and without 1 μM **7** in oocytes expressing different subunit combinations of $GABA_A$ receptors, subtype combinations are indicated in headings and legends. cRNA-injected Xenopus oocytes were held at −60 mV under two-electrode voltage clamp. Increasing concentrations of diazepam with or without 1 μM of **7** were superfused together with a GABA concentration eliciting app. 3% of the maximal current amplitude. Diazepam and **7** were preapplied for 30 s before the addition of GABA, which was coapplied with the drugs until a peak response was observed. Data were normalized for each curve assuming 100% for the response in the absence of diazepam and **7**. Drugs were made up and diluted as stock solutions in DMSO. Final concentrations of DMSO perfusing the oocyte were 0.1%. Values are presented as mean ± SD of at least four oocytes from at least two batches.

Current stimulation by diazepam in $GABA_A$ receptors of subunit combination $\alpha5\beta3\gamma2$ was dose dependently and fully inhibited by dimer **7** (Figure 4). Hill slopes for this inhibition were 1.09 ± 0.075 (mean ± SD of Hill slopes from four separately fitted experiments).

Inhibition of diazepam stimulation of GABA-induced currents by **7** in $GABA_A$ receptors of subtype $\alpha5\beta3\gamma2$. cRNA-injected Xenopus oocytes were held at −60 mV under a two-electrode voltage clamp. Increasing concentrations of **7** were superfused together with 30 nM diazepam and a GABA concentration that elicited alone 3% of the maximal current amplitude. Data were normalized for each curve. The currents measured in the presence or absence of 30 nM diazepam were taken as 100% or 0%, respectively. Values are presented as mean ± SD of four oocytes from three batches.

Discussion. Receptor binding studies indicated that the bivalent ligand **7** bound almost exclusively to the α5 subtype of $GABA_A$ receptors. The effect of this compound on various $GABA_A$ receptors expressed in Xenopus oocytes was then investigated by electrophysiological studies. Analysis of the data indicated that bivalent ligand **7** up to a concentration of 1 μM did not trigger chloride currents in any one of the tested $GABA_A$ receptor subtypes. At 1 μM, **7** did not modulate GABA induced chloride flux in $\alpha1\beta3\gamma2$, $\alpha2\beta3\gamma2$, $\alpha3\beta3\gamma2$ but stimulated GABA-induced currents in $\alpha4\beta3\gamma2$ and $\alpha6\beta3\gamma2$ and slightly inhibited currents in $\alpha5\beta3\gamma2$. At 1 μM, **7** only marginally influenced diazepam stimulation of GABA-induced current in $\alpha1\beta3\gamma2$, $\alpha2\beta3\gamma2$, and $\alpha3\beta3\gamma2$, but shifted the diazepam dose response curve to the right in $\alpha5\beta3\gamma2$ receptors. The slight reduction in the maximal diazepam stimulation in the presence of 1 μM **7** might have been due to the previously described reduction in current stimulation at high diazepam concentration[31] or might indicate interaction of **7** via two different binding sites. Importantly, bivalent **7** was able to dose dependently and completely inhibit diazepam stimulated currents in $\alpha5\beta3\gamma2$ receptors. It is a quite selective benzodiazepine receptor site antagonist at α5 receptors. As noted, ligands such as **7** will be important in the determination of which physiological function(s) are subserved by this $GABA_A$ $\alpha5\beta3\gamma2$ subtype.

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Supporting Information Available: Crystal structure and refinement data for **7**; experimental methods for the synthesis as well as the efficacy of **7** in oocytes; experimental methods for the electrophysiological studies; methods for computer modeling. This material is available free of charge via the Internet at http://pubs.acs.org.

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