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AMINOMETHYL-2,6-DIFLUOROPHENOLS AS A NOVEL CLASS OF INCREASED LIPOPHILICITY $GABA_C$ RECEPTOR ANTAGONISTS

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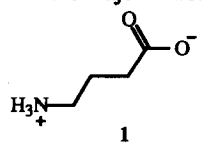
Abstract: 3- and 4-(Aminomethyl)-2,6-difluorophenols were tested for activity against the three major classes of GABA receptors. 4-(Aminomethyl)-2,6-difluorophenol was shown to be a competitive and somewhat selective antagonist at ρ1 $GABA_C$ receptors expressed in *Xenopus* oocytes (K_B = 75.5 μM with a 95% Confidence Interval range of 75.2 μM to 75.8 μM). This is the first in a novel class of increased lipophilicity $GABA_C$ receptor antagonists with little activity at α1β2γ2 $GABA_A$ and $GABA_B$ receptors.

γ-Aminobutyric acid (GABA; **1**) is one of the major inhibitory neurotransmitters in the mammalian



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central nervous system (CNS) and is essential for the overall balance between neuronal excitation and inhibition. GABA hyperpolarizes neurons via a large number of receptor subtypes which are grouped under three major classes of receptors, termed $GABA_A$, $GABA_B$, and $GABA_C$ receptors.[1] These receptors, along with GABA transporters and the enzyme that degrades GABA, namely, GABA aminotransferase (GABA-AT), have been the target for drug design and development with a view to treat a wide variety of neurological disorders. $GABA_A$ and $GABA_C$, which are pharmacologically, biochemically, and physiologically different receptors,[1,2] are Cl^- ion channels and function to produce fast synaptic inhibition. $GABA_A$ receptors are comprised of a mixture of glycoproteins consisting of α1-6, β1-4, γ1-3, δ and ε subunits.[1] Two α-subunits, two β-subunits, and one other subunit, consisting of a γ-, δ-, or ε-subunit, is required for a fully functional $GABA_A$ receptor.[1] In contrast, the $GABA_C$ receptor is made up solely of ρ–subunits, of which two human subtypes (ρ1 and ρ2) have been identified.[3,4] The $GABA_B$ receptors are G-protein coupled receptors that produce slow, long-lasting synaptic inhibition.[5] These receptors modulate intracellular messenger systems, activate K^+-channels, and inactivate voltage-dependent Ca^{2+}-channels.

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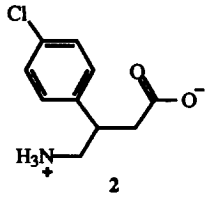
$GABA_A$ receptors are selectively inhibited by the alkaloid, bicuculline, and are modulated by benzodiazepines, steroids, and barbiturates.[1] $GABA_B$ receptors are selectively activated by baclofen (**2**) and inhibited by phaclofen, the phosphonic acid analogue of baclofen.[5] $GABA_C$ receptors are insensitive to the $GABA_A$ receptor antagonist, bicuculline, and the $GABA_B$ receptor agonist, baclofen.[2] $GABA_C$ receptors are selectively activated by *Z*-4-aminobut-2-enoic acid (**3**, *cis*-4-aminocrotonic acid; CACA) and *cis*-2-aminomethylcyclopropanecarboxylic acid (**4**, CAMP).[2] (1,2,5,6-Tetrahydropyridine-4-yl)methylphosphinic acid (**5**, TPMPA) is a potent and selective $GABA_C$ receptor antagonist.[6,7] In the retina, $GABA_C$ receptors have been well characterized, however, little is known about the role of $GABA_C$ receptors in other parts of the CNS. One possible reason for this is the lack of selective $GABA_C$ receptor agonists and antagonists that cross the blood-brain barrier.



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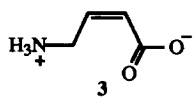

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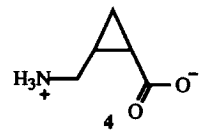

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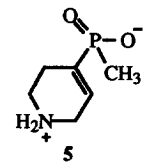

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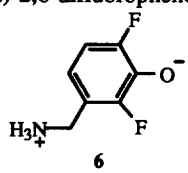
3- and 4-(Aminomethyl)-2,6-difluorophenol (**6** and **7**, respectively) were designed to be increased



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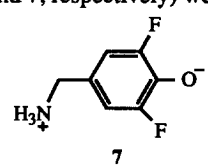

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lipophilicity bioisosteres of GABA and were shown to be competitive inhibitors of GABA-AT.[8] Because of the relatively low pK_a of the difluorophenol group[9] (calculated to be 6.52 in **6** and 6.98 in **7**[10]), these compounds favor the zwitterionic form at neutral pH and mimic the structure of GABA. However, because of the benzene ring and fluorine substitution, these compounds are much more lipophilic than GABA and **5**; the log D (pH 7.0) for GABA is -3.1, for **5** is -3.7, and for **6** or **7** is -1.7.[10]

In this paper, we report the pharmacological evaluation of **6** and **7** on $GABA_A$ $GABA_B$, and $GABA_C$ receptors. The effects of **6** and **7** were measured using two-electrode voltage clamp techniques in *Xenopus* oocytes,[11] expressing human $\alpha1\beta2\gamma2$ $GABA_A$ receptor and $\rho1$ $GABA_C$ receptor subtypes,[12] while [^{3}H]GABA binding on rat brain membranes[13,14] was used to test for $GABA_B$ receptor affinity.[15,16] The human $\alpha1\beta2\gamma2$ $GABA_A$ receptor subtype is one of the major subtypes constituting up to 20% of all $GABA_A$ receptors in human brain.[1] Thus, this receptor subtype is a good representative for measuring the effects of compounds on $GABA_A$ receptors.

GABA (100 μM and 1 μM; **Figure 1**) activates inward currents in oocytes expressing $\rho1$ $GABA_C$ receptors clamped at –60 mV. Compound **7** (100 μM) does not activate currents on its own, but inhibits the current produced by 1 μM GABA at $\rho1$ $GABA_C$ receptors expressed in oocytes (**Figure 1**). Dose-response curves in the presence of increasing concentrations of **7**[17] show that it is a moderately potent and competitive inhibitor of $\rho1$ $GABA_C$ receptors expressed in oocytes ($\log K_B = -4.122 \pm 0.023$; $K_B = 75.5$ μM with a 95% Confidence Interval range of 75.2 μM to 75.8 μM; **Figure 2**). In contrast, **6** has no effect in activating currents on its own nor does it inhibit the current produced by 1 μM GABA at $\rho1$ $GABA_C$ receptors expressed in oocytes (**Figure 1**).

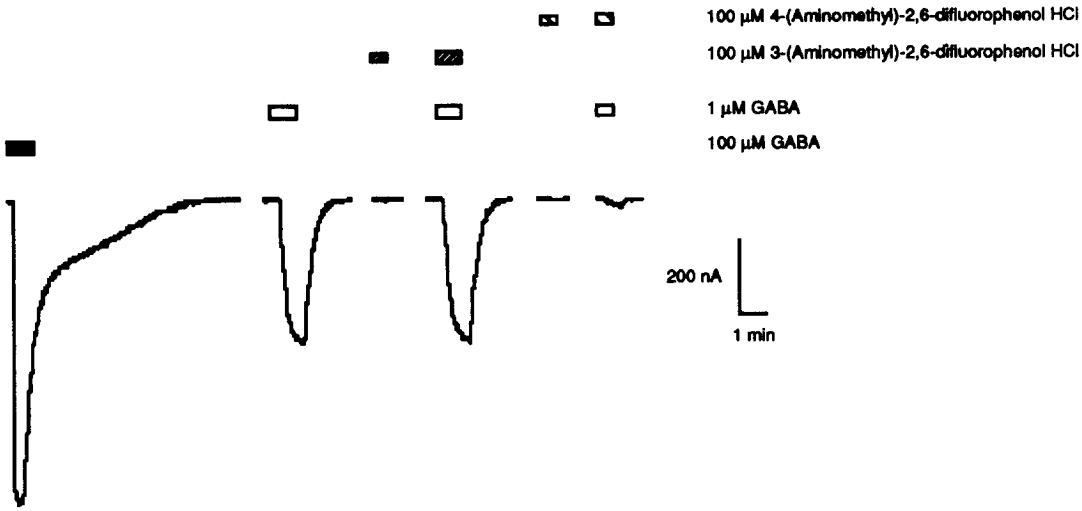

Figure 1. GABA (100 μM; duration indicated by solid bar) activated a maximal inward current in oocytes expressing ρ1 $GABA_C$ receptors clamped at –60 mV. GABA (1 μM) activated the receptor (duration indicated by open bar) by 50%. 3-(Aminomethyl)-2,6-difluorophenol (**6**; 100 μM) did not activate the receptor (duration indicated by heavily hatched bar). When co-applied with GABA (1 μM), **6** (100 μM) did not inhibit the current produced by GABA. 4-(Aminomethyl)-2,6-difluorophenol (**7**; 100 μM) did not activate the receptor (duration indicated by lightly hatched bar), but when co-applied with GABA (1 μM), **7** (100 μM) inhibited the current produced by GABA by 95%.

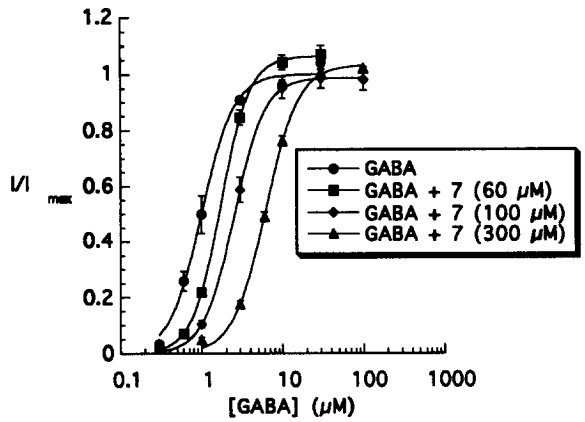

Figure 2. Dose-response curves of GABA and GABA in the presence of 60 μM, 100 μM and 300 μM **7** with human ρ1 $GABA_C$ receptor. log K_B = -4.122 ± 0.023; K_B = 75.5 μM with a 95% Confidence Interval range of 75.2 μM to 75.8 μM, the apparent binding constant for the antagonist **7**.[17] Data are the mean ± S.E.M (n = 3-6 oocytes).

GABA (100 μM and 10 μM; **Figure 3**) activates inward currents in oocytes expressing α1β2γ2 $GABA_A$ receptors clamped at –60 mV. Neither **6** nor **7** (100 μM) activates currents at α1β2γ2 $GABA_A$ receptors expressed in oocytes (**Figure 3**). However, **7** weakly inhibits the current produced by 10 μM GABA by 35%, where 10 μM GABA produces 25% of the maximal current produced by a saturating dose of GABA (**Figure 3**). Furthermore, **6** and **7** (up to 1 mM, n = 2) do not significantly displace [^{3}H]GABA bound to $GABA_B$ receptors in rat brain membranes (i. e., IC_{50} > 1 mM, n = 2). Thus these compounds do not have any significant affinity for the GABA binding site of $GABA_B$ receptors and, therefore, show neither agonistic nor antagonistic activity with $GABA_B$ receptors. It should be noted that these experiments cannot exclude the possibility that **6** and **7** might bind to $GABA_B$ receptors at sites separate from the GABA site.

Compound **7** is a somewhat selective antagonist for ρ1 $GABA_C$ receptors expressed in oocytes. It is approximately 150-times more potent as an antagonist at $GABA_C$ receptors than as an inhibitor of GABA-AT. It has weak inhibitory actions at $GABA_A$ receptors and little, if any, effect at $GABA_B$ receptors. Compound **7**, though, is approximately 35 times weaker as a $GABA_C$ receptor antagonist than the previously reported $GABA_C$ receptor antagonist **5**. However, the increased lipophilicity of **7** compared to that of GABA and **5** makes this compound more effective in crossing the blood-brain barrier and thus more accessible to the brain.

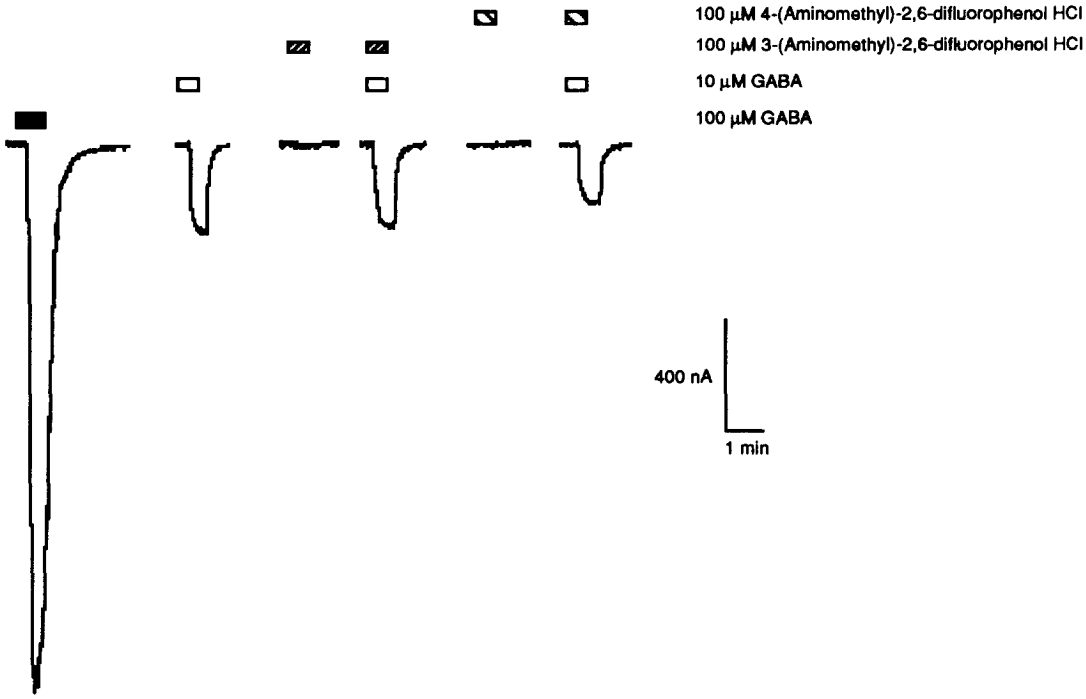

Figure 3: GABA (100 μM; duration indicated by solid bar) activates a maximal inward current in oocytes expressing α1β2γ2 $GABA_A$ receptors clamped at –60 mV. GABA (10 μM) activates the receptor (duration indicated by open bar) by 25%, whereas 3-(aminomethyl)-2,6-difluorophenol (**6**; 100 μM) did not activate the receptor (duration indicated by heavily hatched bar). When co-applied with GABA (10 μM), **6** (100 μM) does not inhibit the current produced by GABA. 4-(Aminomethyl)-2,6-difluorophenol (**7**; 100 μM) does not activate the receptor (duration indicated by heavily hatched bar), but when co-applied with GABA (10 μM), **7** (100 μM) inhibits the current produced by GABA by 35%.

In conclusion, **7** is a novel $GABA_C$ receptor antagonist with increased lipophilicity. This supports the notion that the 2,6-difluorophenol moiety is a bioisostere for a carboxylic acid group. The increased lipophilicity of this moiety should facilitate crossing of the blood-brain barrier, which is essential for characterizing the role that $GABA_C$ receptors play in vivo and for drug development in general.

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10. The pK_a and log D (7.0) values were calculated using the program from Advanced Chemistry Development, Inc., Toronto, Canada.
11. *Xenopus laevis* were anaesthetized with 0.17% ethyl 3-aminobenzoate and a lobe of the ovaries was removed. The lobe was rinsed with OR2 (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2.6H_2O$, 5 mM HEPES, pH 7.5) and treated with collagenase A (2 mg/mL in OR2, Bohringer Manheim) for 2 h. Released oocytes were then rinsed in frog Ringer solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2.6H_2O$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.5) supplemented with 2.5 mM pyruvate, 0.5 mM theophylline, and 50 μg/mL of gentamycin, and stage V - VI oocytes were collected.
12. Method followed (a) Chebib, M.; Vandenberg, R. J.; Froestl, W.; Johnston, G. A. R. *Eur. J. Pharmacol.* **1997**, *329*, 223. (b) Chebib, M.; Vandenberg, R. J.; Johnston, G. A. R. *Br. J. Pharmacol.* **1997**, *8*, 1551. (c) Chebib, M.; Mewett K. N.; Johnston, G. A. R. *Eur. J. Pharmacol.* **1998**, *357*, 227. In brief, human α1, β2, and γ2 subcloned in pcDM8 were linearized using the restriction enzyme, NOT1. Human ρ1 cDNA subcloned in pcDNA1.1 was linearized using the restriction enzyme XbaI. Capped RNAs were synthesized from linearized plasmid containing ρ1, α1, β2, and γ2 cDNAs using the "Mmessage Mmachine" kit from Ambion Inc. (Austin, Texas, USA). ρ1 cRNA (10 ng/50 nL), 10 ng/50 nL of a 1:1:1 mixture of α1, β2, and γ2 cRNAs was injected into defolliculated Stage V - VI *Xenopus* oocytes. Oocytes were stored at 16 °C, and 2 to 8 days later receptor activity was measured by two electrode voltage clamp recordings using a Geneclamp 500 amplifier (Axon Instruments Inc., Foster City, CA., USA), a MacLab 2e recorder (AD Instruments, Sydney, NSW, Australia), and Chart version 3.5 program. Oocytes were voltage clamped at -60 mV while and continuously superfused with frog Ringer solution. For receptor activation measurements the indicated concentrations of drug were added to the buffer solution.

13. Rat synaptic membranes were prepared from the whole brain of Sprague-Dawley male rats (about 300 g) as described by Zukin et al.[14] with some modifications. In brief, the rat whole brain was homogenized in 10 volumes of ice-cold buffer containing 0.25 M sucrose, 10 mM Tris, 0.1 mM AEBSF, and 20 μg/mL of bacitracin, pH 7.4. The homogenate was centrifuged at 1000 x *g*, and the supernatant was then centrifuged at 20,000 x *g* for 20 min. The pellet was resuspended (by vortex) in ice-cold distilled water containing 0.1 mM AEBSF and 20 μg/mL bacitracin (pH set to 7.0) and was centrifuged at 8,000 x *g* for 20 min. The supernatant and the upper layer of the pellet was centrifuged at 33,000 x *g* for 20 min. The pellet was resuspended in 50 mM Tris, pH 7.4, containing 0.1 mM AEBSF and 20 μg/mL bacitracin, centrifuged at 33,000 x *g* for 20 min, the pellet was snap frozen in MeOH/Dry Ice and then stored overnight at -70 °C. The frozen pellet was thawed and washed 6 times in 50 mM Tris, pH 7.4, by centrifugation at 8,000 x *g*. The resulting pellet was resuspended in TCI buffer (50 mM Tris, 2.5 mM $CaCl_2$ and 40 μM isoguvacine, pH 7.4), snap frozen in MeOH/Dry Ice, and stored at -70 °C. Protein concentration was determined according to Bradford using the protein assay kit from Bio-Rad with bovine gamma globulin as a standard.
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15. The [^{3}H]GABA competition assay, modified from Olpe et al.,[16] was performed in 200 μL TCI buffer containing 20 nM [^{3}H]GABA (3.29 TBq/mmol), test compound (0-1 mM dissolved in DMSO), and 80 μg of synaptic membrane protein (diluted in TCI buffer and preincubated for 15 min at room temperature). After being incubated for 12 min at room temperature, the assays were terminated by rapid filtration through a glass fiber filter (Printed filtermat B filters, Wallac), which had been presoaked in 0.3% polyethyleneimine (Sigma), using a TOMTEC cell harvester (Orange, CT). The filters were washed with 5 mL of 50 mM Tris (pH 7.4) at 4 °C and dried at 55 °C for 30 min. MeltiLex B/HS scintillator sheet was melted onto the filter, and radioactivity was determined in a Microbeta scintillation counter (Wallac). Nonspecific binding was determined in the presence of 100 μM SKF 97541 (Tocris, MO, USA).
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17. Current (I) as a function of agonist concentration ([A]) was fitted by least squares to $I = I_{max}[A]^{nH}/(EC_{50}{}^{nH}+[A]^{nH})$, where I_{max} is the maximum current, EC_{50} is the effective dose that activates 50% of the maximum current, and n_H is the Hill coefficient. EC_{50} values are expressed as the mean ± S.E.M. (n = 3-6 oocytes) and are determined by fitting data from individual oocytes using Kaleidagraph 3.0 (1993). K_B values are the apparent binding constants for the antagonists and were determined using Schild plot analysis. -logK_B values were determined using the following equation: $\log\{(A)/(A^*)-1\} = m.\log[Ant]-\log K_B$, where A is the EC_{50} of GABA in the presence of a known antagonist concentration, A^* is the EC_{50} of GABA in the absence of the antagonist, [Ant] (EC_{50} = 1.01 ± 0.09 μM) is the concentration of the antagonist, and 'm' is the slope of the curve. For simple competitive antagonism, 'm' is 1. -logEC_{50} values were determined by fitting data to the above function using Kaleidagraph 3.0 (1993). The concentrations of **7** to determine the K_B were 60 μM, 100 M, and 300 μM.