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Evaluation of Difluoromethyl Ketones as Agonists of the γ-Aminobutyric Acid Type B ($GABA_B$) Receptor

Changho Han,[†] Amy E. Salyer,[†] Eun Hoo Kim,[†] Xinyi Jiang,[†] Rachel E. Jarrard,[†] Matthew S. Powers,[‡] Aaron M. Kirchhoff,[‡] Tolani K. Salvador,[§] Julia A. Chester,[‡] Gregory H. Hockerman,*[,†] and David A. Colby*[,†,§]

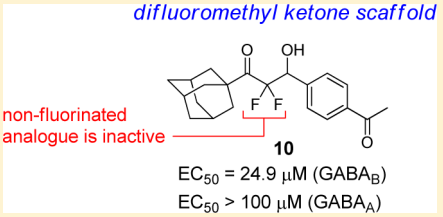
[†]Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, Indiana 47907, United States

[‡]Department of Psychological Sciences, Purdue University, West Lafayette, Indiana 47907, United States

[§]Department of Chemistry, Purdue University, West Lafayette, Indiana 47907, United States

Supporting Information

ABSTRACT: The design, synthesis, biological evaluation, and in vivo studies of difluoromethyl ketones as $GABA_B$ agonists that are not structurally analogous to known $GABA_B$ agonists, such as baclofen or 3-aminopropyl phosphinic acid, are presented. The difluoromethyl ketones were assembled in three synthetic steps using a trifluoroacetate-release aldol reaction. Following evaluation at clinically relevant GABA receptors, we have identified a difluoromethyl ketone that is a potent $GABA_B$ agonist, obtained its X-ray structure, and presented preliminary in vivo data in alcohol-preferring mice. The behavioral studies in mice demonstrated that this compound tended to reduce the acoustic startle response, which is consistent with an anxiolytic profile. Structure–activity investigations determined that replacing the fluorines of the difluoromethyl ketone with hydrogens resulted in an inactive analogue. Resolution of the individual enantiomers of the difluoromethyl ketone provided a compound with full biological activity at concentrations less than an order of magnitude greater than the pharmaceutical, baclofen.



INTRODUCTION

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system, and its primary roles are to regulate the excitation of neurons and the release of other neurotransmitters. The actions of GABA are guided by two major types of GABA receptors, $GABA_A$ and $GABA_B$. $GABA_A$ receptors are ion channels that increase the threshold for excitation in neurons and other cells, and they are physiologically distinct, pentameric assemblies of 19 different subunits.[1] The $GABA_A$ receptor is modulated by the benzodiazepine sedative/hypnotics, such as diazepam, and newer nonbenzodiazepine sedatives, such as zolpidem, zopiclone, and zaleplon.[2] $GABA_B$ receptors are G-protein coupled receptors that can inhibit the release of neurotransmitters such as acetylcholine, noradrenalin, serotonin, glutamic acid, and dopamine.[3,4] They are functional heterodimers of $GABA_{B1}$ and $GABA_{B2}$, and the former contains the GABA-binding site and the latter is the direct activator of G-protein (G_i and G_o types). The $GABA_B$ receptors mediate the action of GABA through modulation of potassium channels,[5] calcium channels,[6,7] and adenylyl cyclase.[8] Unlike targeting the $GABA_A$ receptor, few pharmaceuticals are selective ligands for the $GABA_B$ receptor, except for the muscle relaxant, baclofen. Moreover, all of the other known $GABA_B$ agonists are structural analogues of GABA or baclofen; therefore, structure–activity data at this receptor is quite restricted.[9] However, targeting the $GABA_B$ receptor presents a potential strategy for pharmacological intervention for anxiety and mood disorders and alcohol, cocaine, heroin, and nicotine addiction in addition to its uses in muscle relaxation (e.g., spinal and lower esophageal musculature).[9−11]

Herein, we report the synthesis, structure–activity data and preliminary behavioral studies in mice of difluoromethyl ketone-based $GABA_B$ agonists. These structures are a divergence from existing $GABA_B$ agonists, such as GABA and baclofen, yet retain substantial potency and selectivity for the $GABA_B$ receptor over $GABA_A$ receptors. Structure–activity investigations determined that replacing the fluorines of the difluoromethyl ketone with hydrogens resulted in an inactive analogue. In the preliminary studies in mice, an active compound tended to reduce the acoustic startle response, which is consistent with an anxiolytic drug profile. The acoustic startle response is commonly used in both humans and animals to assess anxiety-related behavior.[12]

Baclofen and 3-aminopropyl phosphinic acid represent the prototypical structures of selective $GABA_B$ agonists (Figure 1).[9,13] Baclofen is a racemic mixture of 3-(*para*-chlorophenyl)-substituted GABA, the majority of its biological activity being displayed by the (*R*)-enantiomer.[13] Baclofen is approved as a muscle relaxant and has demonstrated substantial preclinical and clinical potential in alcohol, cocaine, heroin, and nicotine addiction, panic and post-traumatic stress disorders, and gastroesophageal reflux (GERD).[9] Other baclofen derivatives

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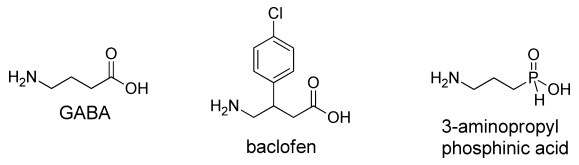


Figure 1. Structures of GABA and $GABA_B$ agonists.

with structural modifications at the 3-position are also $GABA_B$ agonists,[14−16] notably, substituted thiophene and benzofuran are potential surrogates for the *para*-chlorophenyl group.[14] 3-Aminopropyl phosphinic acid is a $GABA_B$ agonist[10] and also served as a scaffold for the discovery of additional $GABA_B$ agonists, partial agonists, and antagonists, usually through the placement of substituents at the 2-position.[9,17,18] For example, the analogue with a (*R*)-2-fluoro substituent on 3-aminopropyl phosphinic acid is a candidate molecule for inhibition of GERD.[18] 3-Aminopropyl phosphinic acid and its derivatives have demonstrated a potential role as muscle relaxants, anxiolytics, and treatments for addiction.[9] Because of the substantial therapeutic possibilities for the $GABA_B$ ligands, other classes of agents such as allosteric modulators have been developed.[9,19,20] Nevertheless, the published structure−activity data for $GABA_B$ agonism is primarily limited to baclofen and 3-aminopropyl phosphinic acid; therefore, we aimed to address this deficiency and concomitantly demonstrate a novel role for difluoromethyl ketones. This class of fluorinated compounds has established their fundamental importance in modulating unique biological activity or serving as a transition-state mimic.

RESULTS AND DISCUSSION

Chemistry. Structural data for the $GABA_B$ receptor is quite scarce, yet site-directed mutagenesis and ligand binding studies have revealed that two key active-site residues, Ser246 and Asp471, have critical interactions with GABA and baclofen (Figure 2).[21] Also, Tyr366 plays a pivotal role in the binding of

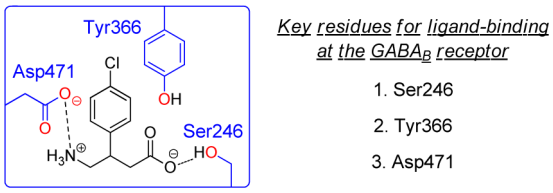
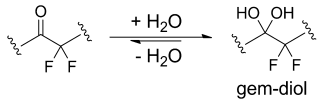


Figure 2. Baclofen-$GABA_B$ receptor binding motif determined from site-directed mutagenesis and ligand-binding studies.

baclofen to the $GABA_B$ receptor. In this model, the interaction between the carboxylate of baclofen and the serine residue of the $GABA_B$ receptor presents a unique opportunity to discover a new potential use for α-fluorinated ketones as agonists at this receptor. This assertion builds upon two literature precedents from investigations with α-fluorinated ketones in medicinal chemistry: (1) their ability to form hemiketals with serine residues in an active site[22,23] and (2) their ability to serve as surrogates for carboxylic acids[24] and phosphinic acids.[25] We envisioned that applying these data would also leverage existing structure−activity data for $GABA_B$ agonists and together enable a manifold of unique ligands to be identified for the $GABA_B$ receptor. Another driving force that enabled this investigation was that we have recently discovered a synthetic method to assemble α,α-difluorinated ketones, or difluoromethyl ketones.[26] Approaches to create fluorinated molecules are rising, and simultaneously, an increasing number of pharmaceutical agents contain fluorine, typically because this atom can enhance drug-like qualities and limit metabolism.[27] Difluoromethyl ketones are prominent examples of such fluorinated compounds because the presence of the electron-withdrawing fluorine atom causes the ketone to revert to a hydrate, or *gem*-diol, in the presence of water (eq 1).[25,28,29] This change



(1)

increases aqueous solubility and imparts a tetrahedral shape, which can further enable activity as a transition state mimic. Thusly, difluoromethyl ketones have displayed biological activity as inhibitors of matrix metalloprotease,[25] esterase,[28] malarial aspartic protease,[29] γ-secretase,[30] and renin.[31] However, a potential role for difluoromethyl ketones as agonists of $GABA_B$ receptors has not been demonstrated.

Recently, we have disclosed a novel method to assemble difluoromethyl ketones by aldol reactions with difluoroenolates generated following the release of trifluoroacetate (Figure 3).[26]

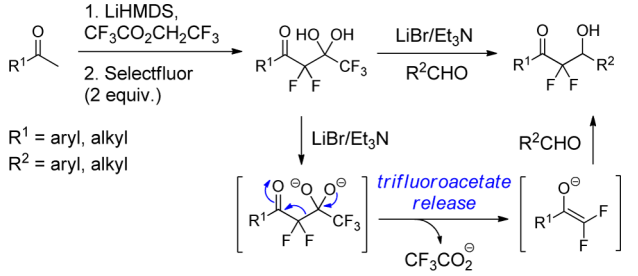


Figure 3. Difluoromethyl ketones from trifluoroacetate-release aldol reaction.

This approach was developed from the literature precedent that hexafluoroacetone hydrate undergoes a rapid fragmentation to release trifluoroacetate after the addition of base.[32] The aldol reaction is rapid (reaction time is 3 min at rt) and promoted under many conditions; LiBr and Et_3N were the optimum conditions for the transformation with aldehydes. Difluoromethyl ketones can be accessed in only three synthetic steps from a precursor methyl ketone. This strategy is high yielding, versatile, and fully compatible with many alkyl and aryl aldehydes and ketones.[26] Also, a complementary approach for difluoromethyl ketones is available by halogenation of the difluoroenolate generated by trifluoroacetate release, followed by copper-mediated coupling.[33] This difluoromethyl ketone scaffold from the aldol process presents the requisite difluoromethyl ketone to propel these investigations as potential agonists as well as a β-hydroxy group that could recapitulate the role of the amino group of GABA (and baclofen).

Using the three-step approach, difluoromethyl ketones **1**−**12** were assembled for evaluation as potential $GABA_B$ ligands (Figure 4).[26] The compounds represent a focused selection of difluoromethyl ketones that were assembled from pairing alkyl, aryl, and α,β-unsaturated aldehydes and ketones. All of the molecules were prepared as racemic mixtures, except for adducts **8** and **9**, which are mixtures of epimers derived from coupling with chiral aldehydes. The difluoromethyl ketone **12** resulted from protonation of the difluoroenolate rather than

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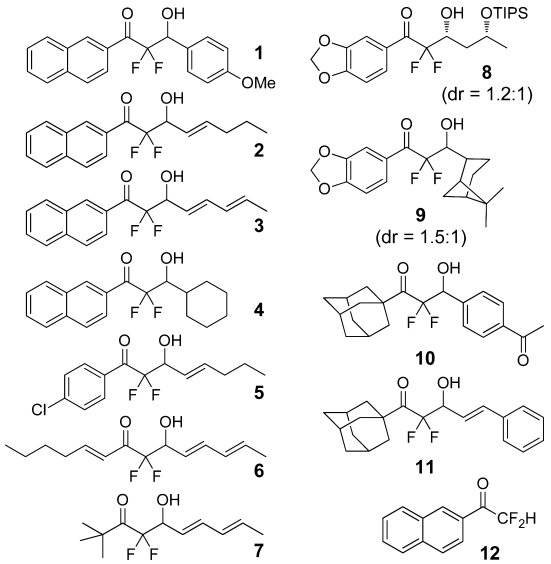


Figure 4. Structures of difluoromethyl ketones **1**–**12**.

reaction with an aldehyde and was prepared to provide additional insight into structure–activity relationships. An X-ray structure of compound **10** was obtained to reveal the conformation of the difluoromethyl ketone backbone (Figure 5). Only Percy and co-workers have reported an X-ray structure

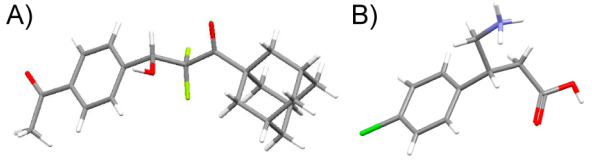


Figure 5. X-ray structures of $GABA_B$ ligands: (A) compound (±)-**10**, (B) baclofen.

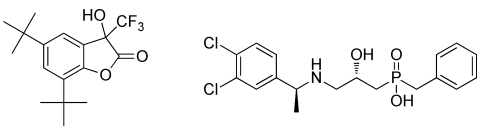
of an analogous acyclic α,α-difluoro-β-hydroxy ketone.[34] The X-ray structure of baclofen is also presented in Figure 5B.[35] The crystalline difluoroketone **10** exists as the ketone, rather than a hydrate, according to the X-ray structure. Difluoroketones are known to convert partially or completely into hydrate in an aqueous environment.[25,28,29,36] Accordingly, the tendency to favor the hydrate-form over the ketone-form in the presence of water is dependent upon the structure of the difluoroketone as well as the relative concentration of water.

Pharmacology. Biological evaluation of difluoromethyl ketones **1**–**12**, baclofen, and GABA was performed to measure potency and selectivity at $GABA_B$ and $GABA_A$ receptors (Table 1). $GABA_B$ agonist activity was measured using a cell line expressing both subunits of the human $GABA_B$ receptor, along with the reporter gene, CRE-luciferase (CRE-Luc). The introduced $GABA_B$ receptor utilizes the endogenous signaling pathway of the host cells, and activation of $GABA_B$ receptors leads to inhibition of cAMP production. In this assay, forskolin, a small-molecule activator of adenylyl cyclase (AC), was used to stimulate cAMP production, and subsequently, inhibition of forskolin-stimulated accumulation of cAMP was measured. The $GABA_B$ allosteric activator, *rac*-BHFF,[20] was synthesized according to the published protocol[37] and examined as an additional positive control (Figure 6). Dose–response curves and EC_{50} values were obtained for compounds displaying substantial activity at 100 μM. The naphthyl aldol products **1**–

Table 1. $GABA_B$ and $GABA_A$ Assay Data for Difluoromethyl Ketones

compd	$GABA_B$ EC_{50} (μM)[a]	$GABA_A$ EC_{50} (μM)[a]
baclofen	1.7 ± 0.10	>100
rac-BHFF	1.9 ± 0.90	>100
GABA	0.53 ± 0.33	2.30 ± 0.59
1	61.8 ± 3.01	>100
2	66.9 ± 1.19	>100
3	99.3 ± 3.78	>100
4	53.5 ± 1.76	>100
5	>100	nd
6	>100	nd
7	>100	nd
8	>100	nd
9	nd[b]	nd
10	24.9 ± 1.30	>100
11	40.0 ± 3.59	>100
12	>100	nd

[a]Values are given with standard error. [b]Cytotoxic in assay. nd is not determined.



rac-BHFF CGP55845

Figure 6. Other ligands of the $GABA_B$ receptor.

4 and adamantyl analogues **10** and **11** display significant activity in the $GABA_B$ assay, and GABA, *rac*-BHFF, and baclofen served as positive controls. Analysis of these data validated our hypothesis that difluoromethyl ketones can serve as $GABA_B$ agonists. The EC_{50} values calculated from this assay of $GABA_B$ receptor activity for the positive controls (i.e., GABA and baclofen) are comparable to the prior reported literature.[16,18,21,38] The most potent agent is the difluoromethyl ketone **10** (EC_{50} = 24.9 μM), bearing an adamantyl group adjacent to the α,α-difluoroketone and a *para*-acetyl phenyl group flanking the secondary alcohol. The other adamantyl derivative **11** is less active (EC_{50} = 40.0 μM) than **10**, yet more potent than all of the naphthyl-derived analogues **1**–**4**. Analysis of structure–activity data emphasizes the importance of a naphthyl or an adamantyl group adjoining the α,α-difluoroketone, but the functionality neighboring the hydroxy group can tolerate a much wider range of structures. However, the simple naphthyl difluoroketone **12** is inactive, which indicates that importance of the hydroxy group. Additional pharmacological evaluation of **10** revealed that its effects can be reversed with (2*S*)-3-[[(1*S*)-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl](phenylmethyl)phosphinic acid (CGP55845), a $GABA_B$ antagonist (Figure 6).[39] Thus, the inhibition of forskolin-stimulated AC activity is, indeed, mediated by the $GABA_B$ receptor. Compound **10** is not cytotoxic in an MTS cell viability assay (unlike compound **9**, for example). The dose–response curves for compound **10**, GABA, and baclofen depict the relative potencies as well as the slope of the curves (Figure 7). Even though GABA and baclofen are more potent (EC_{50} = 0.53 and 1.7 μM, respectively) than the difluoromethyl ketone **10**, the dose–response curve of compound **10** is steeper than both of the other agonists. These differences in slope merited further investigation to deduce potential binding modes as well

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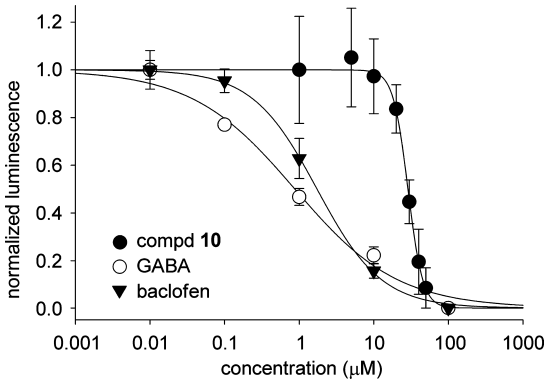

Figure 7. Dose–response curves for baclofen, GABA, and compound **10** at the $GABA_B$ receptor.

as the relative effects of each enantiomer of the racemic compound **10** (see below).

All of the active compounds were also screened for agonist activity at the $GABA_A$ receptor ($\alpha_1\beta_2\gamma_2$) because GABA also activates $GABA_A$ receptors in vivo. This assay is an electrophysiological assay, utilizing the whole-cell voltage-clamp method, because the $GABA_A$ receptor is a ligand-gated chloride channel. Specifically, HEK 293 cells were transiently transfected with α_1, β_2, and γ_2 subunits of the rat $GABA_A$ receptor, and compounds were applied to cells clamped at −60 mV. Of the compounds tested, only GABA displays activity at the $GABA_A$ receptor, with a potency similar to that previously reported (EC_{50} = 2.30 μM). Thus, the pharmacological evaluation strategy produces data for potency and efficacy in $GABA_B$ and $GABA_A$ as well as $GABA_B$ vs $GABA_A$ selectivity. These data validate that the difluoromethyl ketones (i.e., **1**–**4**, **10**, and **11**) are, indeed, agonists of the $GABA_B$ receptor and selective for $GABA_B$ over $GABA_A$ receptors (see Table 1). Electrophysiology results for compounds **1**–**3** and GABA in HEK cells are presented in Figure 8. They depict the magnitude of the response at $GABA_A$ receptors from multiple applications of 100 μM GABA as well as its absence following sequential treatment with 100 μM of the difluoromethyl ketones.

Following the identification of the active compounds, structure–activity investigations were performed on **10** to elucidate the role of difluorination and the secondary alcohol as

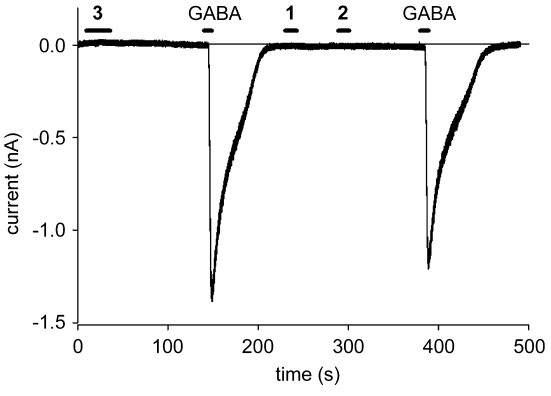

Figure 8. Example trace from electrophysiological experiments testing the agonist activity of compounds **1**–**3** and GABA at the $GABA_A$ receptor. Solid bars represent the time during which the indicated compounds (100 μM) were applied to $GABA_A$ receptor-expressing cells under voltage clamp.

well as the effects of chirality. The pharmaceutical baclofen is available as a racemic mixture, but it is well-known that (*R*)-(−)-baclofen is the major active component.[13] The trifluoroacetate-release aldol reaction provides racemic mixtures of products; therefore, the separation of the difluoromethyl ketone (±)-**10** was conducted to resolve each of its individual enantiomers for subsequent pharmacological evaluation. Using the secondary alcohol of (±)-**10** as a handle, chiral esters derived from naproxen, histidine, and proline were prepared, and fortunately, the *N*-Boc proline adduct **13** allowed complete separation by preparative TLC (Figure 9). Accordingly, mixture (±)-**10** was esterified with (*S*)-Boc-proline using CDI, and the intermediates were cleanly separated by preparative TLC. Saponification of each compound with KOH in methanol provided (+)-enantiomer **10** ($[\alpha]^{23}_D$ +36.0 (*c* 0.667, $CHCl_3$)) and (−)-enantiomer **10** ($[\alpha]^{23}_D$ −35.8 (*c* 0.558, $CHCl_3$)). Two derivatives of compound (±)-**10** were also synthesized to obtain the structure–activity data at the secondary alcohol and difluoromethyl ketone. Methylation of **10** with CH_3I in the presence of Ag_2O afforded the methyl ether **14**. The preparation of the nonfluorinated analogue of **10** was accomplished by the aldol reaction of 1-adamantyl methyl ketone with 4-acetylbenzaldehyde to give the adduct **15** in 68% yield. The structure **15** represents the nonfluorinated counterpart **10** and will be used to understand the pharmacophoric function of the difluoromethyl ketone because both fluorines are replaced with hydrogen atoms.

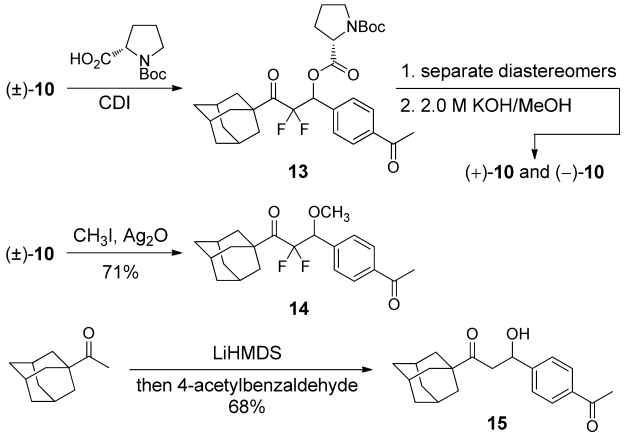

Figure 9. Separation of enantiomers of compound (±)-**10** and synthesis of methyl ether **14** and nonfluorinated analogue **15**.

Pharmacological evaluation of the (+)-enantiomer **10**, (−)-enantiomer **10**, methyl ether **14**, and nonfluorinated analogue **15** along with (±)-baclofen and (−)-baclofen provided additional structure–activity data (Table 2). The (+)-enantiomer **10** was twice as potent at $GABA_B$ (EC_{50} = 15.9 μM) as the (−)-enantiomer **10** (EC_{50} = 37.8 μM) and provides excellent agreement with the observed activity for the racemic mixture. The methylated compound **14** and the nonfluorinated analogue **15** are both inactive at the $GABA_B$ receptor at 100 μM. The absence of biological activity displayed by **14** and **15** demonstrates the importance of the β-hydroxy substituent as well as the fluorines adjacent to the methyl ketone, respectively. The latter structure–activity relationship validates the utility of the difluoromethyl ketone and supports that this group is pivotal for activity at the $GABA_B$ receptor, because by simply exchanging the two fluorine atoms on **10** with hydrogen atoms,

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Table 2. Biological Activity for Resolved Enantiomers of Compound 10 and Derivatives 14 and 15[a].

compd	$GABA_B$ EC_{50} (μM)[b]	Hill slope[c]	$GABA_A$ EC_{50} (μM)[b]
GABA	0.53 ± 0.33	0.54 ± 0.11	2.30 ± 0.59
rae-BHFF	1.9 ± 0.90	0.92 ± 0.30	>100
(±)-baclofen	1.7 ± 0.10	0.98 ± 0.05	>100
(−)-baclofen	1.0 ± 0.40	nd	nd
(±)-**10**	24.9 ± 1.30	3.8 ± 0.60	>100
(+)-**10**	15.9 ± 1.84	2.2 ± 0.39	nd
(−)-**10**	37.8 ± 0.78	9.53 ± 1.66	nd
(±)-**14**	>100	nd	nd
(±)-**15**	>100	nd	nd

[a]Hill slopes are calculated at $GABA_B$. [b]Values are given with standard error. [c]Calculated for $GABA_B$. nd is not determined.

the activity is lost. The dose−response curves of the individual enantiomers and racemic mixture are as expected (Figure 10).

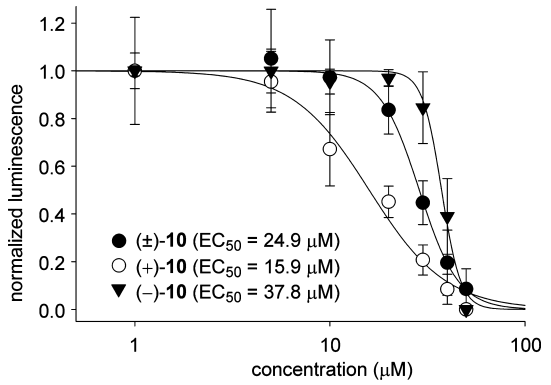

Figure 10. Dose−response curves for (+)-**10**, (−)-**10**, and (±)-**10** at the $GABA_B$ receptor.

The more potent enantiomer, (+)-**10**, presents a dose−response with a less steep curve (Hill slope = 2.2) than its enantiomer (Hill slope = 9.53) and the racemic mixture (Hill slope = 3.8), accordingly. This difference in the Hill slopes for the enantiomers may be potentially interpreted as a difference in the binding modes for the individual enantiomers or may reflect additional allosteric activation. Similar to the racemate, both enantiomers of compound **10** are not cytotoxic in an MTS cell viability assay. Overall, the (+)-enantiomer **10** is a $GABA_B$ agonist that displays its potency within 10-fold of the pharmaceutical agent, baclofen, and is a key structure for additional development. Although the determination of the absolute stereochemistry of (+)-enantiomer **10** would provide additional structural data, the promising nature of the racemate (±)-**10** as a $GABA_B$ agonist, as well as its three-step preparation, impelled a subsequent in vivo investigation.

To determine if compound **10** has either $GABA_A$-potentiating or $GABA_A$ antagonizing activity, we applied 1 μM GABA alone or simultaneously with 100 μM (±)-**10** to cells under voltage clamp expressing the $GABA_A$ receptors as in Figure 8. As shown in parts A and B of Figure 11, compound **10** did not change the current amplitude elicited by 1 μM GABA and thus does not potentiate $GABA_A$ receptor responses to GABA. We also applied 100 μM compound **10** simultaneously with 100 μM GABA, a near-saturating concentration in this assay. We found that compound **10** did not affect the amplitude of the Cl^- current elicited by 100 μM GABA to stimulate Cl^-. Combined with the data in Table 1,

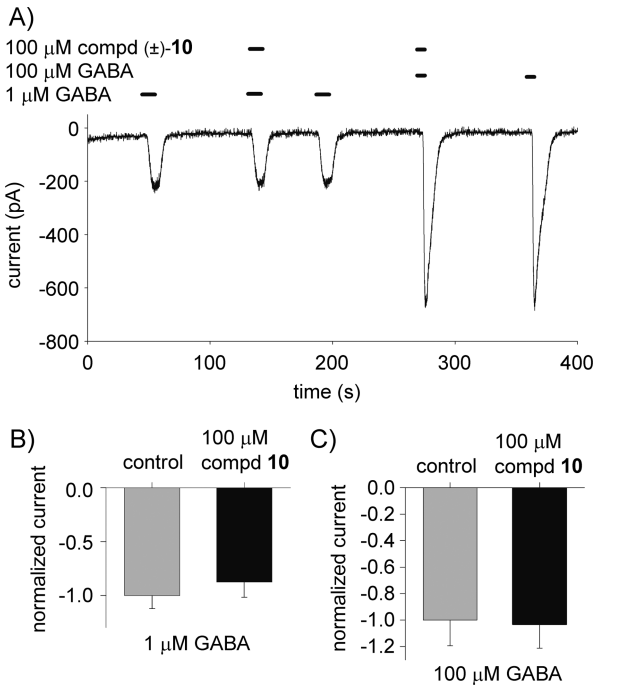

Figure 11. Compound (±)-**10** does not potentiate or antagonize the activity of GABA at the $GABA_A$ receptor. (A) Representative trace of an experiment showing stimulation of Cl^- current by 1 and 100 μM GABA in the absence or presence of 100 μM (±)-**10** recorded from a cell transfected with the $GABA_A$ receptor subunits as depicted in Figure 8. Solid bars represent the time during which the indicated compounds were applied to the cell. (B) Normalized current amplitude (mean ± SE; N = 5) stimulated by 1 μM GABA in the absence (gray bar) or presence (black bar) of 100 μM (±)-**10**. (C) Normalized current amplitude (mean ± SE; N = 5) stimulated by 100 μM GABA in the absence (gray bar) or presence (black bar) of 100 μM (±)-**10**.

these data show that compound **10** has no discernible effect on the gating of the $GABA_A$ receptor Cl^- channel.

Behavioral Pharmacology. Rodent startle models, including plain acoustic startle and fear-potentiated startle, have predictive validity for identifying potential candidates for treating clinical anxiety disorders.[12,40] Anxiety disorders are highly comorbid with addictions such as alcohol dependence.[41,42] In two experiments, we used a mouse line selectively bred for high-alcohol preference (HAP2) to assess the potential anxiolytic effects of compound (±)-**10** and *rac*-BHFF in reference to (±)-baclofen (Figure 12). HAP2 mice have a genetic predisposition toward high-alcohol preference, are a genetic animal model of alcohol dependence, and also display greater anxiety-related behavior, as shown by greater plain acoustic startle[43] and fear-potentiated startle.[44] In experiment 1, mice were pretreated by intraperitoneal (ip) dosing with either (±)-baclofen (7.5 mg/kg) or vehicle 25 min before the start of the test session. The dose for (±)-baclofen was obtained from prior studies.[45] Figure 12A shows anxiolytic effects of (±)-baclofen in HAP2 mice. ANOVA indicated a Treatment × Trial Type (noise-alone, light + noise) interaction [$F(1,10)$ = 26.3, $p < 0.001$]. Follow up analyses of Treatment within each Trial Type showed that (±)-baclofen significantly reduced the startle response on both noise-alone [$F(1,12)$ = 8.1, p = 0.015] and light + noise [$F(1,12)$ = 26.3, $p < 0.001$] trials. Analyses of Trial Type within each treatment group [(±)-baclofen, vehicle] indicated a significant difference between noise-alone and light

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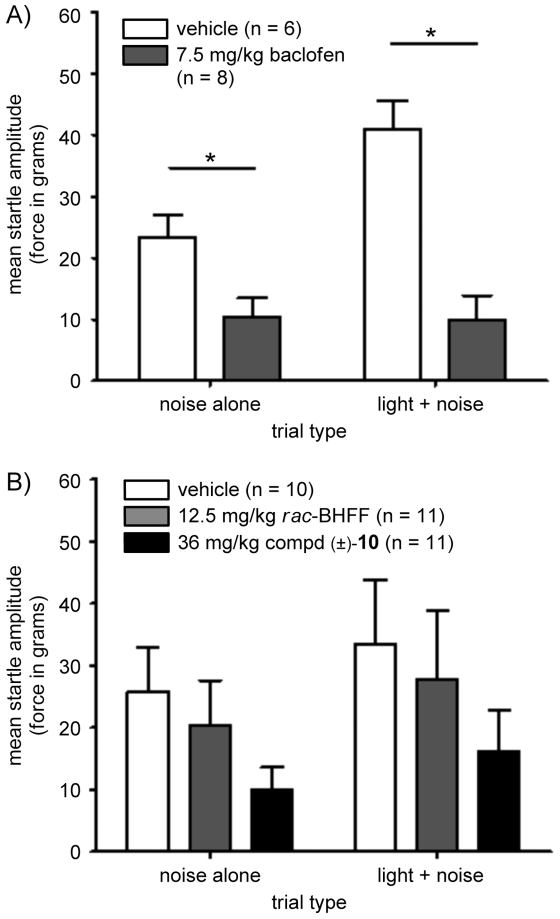

Figure 12. Mean (± SEM) startle amplitude measured as force in grams in male and female HAP2 mice. Effects of drug treatments on startle response amplitude during both the noise-alone and light + noise trials are shown (see Experimental Section). (A) Mice were treated by ip injection with vehicle or (±)-baclofen (7.5 mg/kg). (B) Mice treated by ip injection with vehicle, *rac*-BHFF (12.5 mg/kg), or compound (±)-**10** (36 mg/kg). Initial analyses indicated no interactions with sex, so data are reported collapsed across this factor. * $p < 0.05$.

+ noise trials in the vehicle-treated group [$F(1,5) = 16.5$, $p = 0.01$; light + noise > noise-alone, indicating fear-potentiated startle] but not in the (±)-baclofen-treated group ($p = 0.6$). In experiment 2, mice were pretreated with either vehicle, *rac*-BHFF (12.5 mg/kg ip), or compound (±)-**10** (36 mg/kg ip) 25 min before the start of the test session. The dose for *rac*-BHFF was obtained from prior studies,[20] and a relative dose for the novel agent (±)-**10** was selected using the pharmacological data (see Table 1) as well as its larger relative molecular weight to baclofen. Figure 12B shows that both *rac*-BHFF and compound (±)-**10** reduced the startle response on both trial types, with a greater reduction in the compound (±)-**10** group, suggesting anxiolytic-type effects from these compounds. However, ANOVA indicated a main effect of Trial Type [$F(1,29) = 7.8$, $p < 0.01$; light + noise > noise-alone] but no treatment effect ($p = 0.2$) or interaction. It should also be noted that a reduction in startle magnitude could also be related to muscle-relaxant effects of these drugs, a possibility that will require further testing. These in vivo data provide preliminary evidence of bioavailability and biological activity for the difluoromethyl ketone (±)-**10** in a relevant rodent model. Additional studies are planned to validate the distribution of the difluoromethyl ketone into the CNS because the pharmacokinetics of difluoromethyl ketones have not been widely explored. Two reports support that fluorinated ketones can display favorable pharmacokinetic profiles.[31,46] Specifically, difluoromethyl ketones developed as renin inhibitors have lowered blood pressure in normotensive monkeys following oral administration.[31] Second, fluorinated ketones developed as phospholipase A_2 inhibitors have reduced symptom onset in the CNS disease, experimental autoimmune encephalitis, following intravenous and intraperitoneal dosing.[46] The latter study demonstrates that a trifluoromethyl ketone, which is likely to exist as a hydrate, is able to access the CNS.

CONCLUSIONS

The design, synthesis, biological evaluation, and in vivo studies of difluoromethyl ketones as $GABA_B$ agonists that are not structurally analogous to any known $GABA_B$ agonists are presented. This class of molecules provides a path to extend the structure–activity data for $GABA_B$ agonists beyond the confines of the existing molecules designed from baclofen or 3-aminopropyl phosphinic acid. Biological evaluation with a unique set of GABA receptor subtypes has enabled investigation at clinically relevant receptors. Moreover, these results were directly translated to an animal model to assess drug effects on the acoustic startle response. During the course of this work, we have identified the difluoromethyl ketone (±)-**10** as a potent $GABA_B$ agonist, obtained its X-ray structure, and presented preliminary in vivo data in mice that suggest it has biological activity. Resolution of this racemic mixture provided individual enantiomers, one of which was determined to be highly active and only 10-fold less potent in receptor binding than the pharmaceutical agent, baclofen. The implications of these studies are that difluoromethyl ketones can serve as potent agonists of the $GABA_B$ receptor with high selectivity over the $GABA_A$ receptor. The in vivo data suggest this compound warrants further study as a potential anxiolytic drug. An additional investigation of the difluoromethyl ketone was conducted by synthesizing the nonfluorinated counterpart of the active **10**, i.e. **15**, did not display any activity at the $GABA_B$ receptor. These structure–activity data demonstrate the pivotal nature of the difluoromethyl ketone for activity at the $GABA_B$ receptor. Additional studies to ascertain the exact stereochemical configuration of the chiral secondary alcohol in the most potent analogue are underway. We have previously reported that the assignment of absolute stereochemistry of α-fluorinated alcohols is more complex than their nonfluorinated counterparts because the usual Mosher's ester analysis provides an ambiguous assignment.[26] Difluoromethyl ketones can display good selectivity profiles, and we now demonstrate their selectivity for $GABA_B$ over $GABA_A$ receptors. Similarly, Diederich and co-workers have developed difluoromethyl ketones with selectivity for malarial asparatic proteases over the human cathepsins D and E.[29] However, these data must be placed into context that difluoromethyl ketones are known to bind to proteases,[25,29] esterase,[28] secretase,[30] and other targets;[31,36] therefore, these novel $GABA_B$ agonists must also be evaluated at other targets to further determine their potential use in vivo. We will also need to replicate the behavioral studies in order to increase our power to detect a significant anxiolytic effect of compound (±)-**10** and *rac*-BHFF. Increasing the dosages of these two test compounds may also enhance the anxiolytic effects in mice. The studies detailed here open a new avenue to extend the structure–activity relation-

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ships for potent agonist activity at the $GABA_B$ receptor, and this novel pharmacophore provides numerous opportunities for drug design in the modulation of muscle contractility and the treatment of anxiety and addiction-related behavior. This work also highlights the powerful synthetic approach of using trifluoroacetate-release to assemble fluorinated structures for medicinal chemistry.

EXPERIMENTAL SECTION

Chemistry. The synthesis of compounds **1**−**12** was conducted according to the literature procedure.[26] *rac*-BHFF was obtained using the reported synthesis.[37] Preparation and characterization data for the new compounds, (−)-**10**, (+)-**10**, **12**, and **13** are listed below. HPLC analysis was conducted to establish a level of ≥95% purity, and additionally, combustion analysis is provided for (+)-**10** and **12**.

Separation of Compounds (−)-10 and (+)-10. To a solution of (*S*)-Boc-proline (231 mg, 1.08 mmol) in CH_2Cl_2 (1 mL), CDI (174 mg, 1.08 mmol) was added and stirred for 5 min at rt. Then, the reaction mixture was transferred to another vial containing 3-(4-acetylphenyl)-1-(adamantan-1-yl)-2,2-difluoro-3-hydroxypropan-1-one **10**[26] (78 mg, 0.22 mmol) and stirred at 70 °C overnight. Next, the reaction mixture was cooled to rt and quenched with saturated aqueous NH_4Cl (2 mL) at the same temperature, and the resultant mixture was extracted with CH_2Cl_2 (6 mL × 5). The organics were dried over Na_2SO_4 and concentrated under reduced pressure. Preparatory TLC (SiO_2, 4:2:2:1.5 hexanes:pentanes:petroleum ether:-EtOAc) afforded the two pure diastereomers that were individually treated with a solution of 2 M KOH (1 mL) in MeOH (2 mL). After stirring for 2 h at rt, the reaction mixtures were quenched with saturated aqueous NH_4Cl (6 mL) and extracted with CH_2Cl_2 (6 mL × 5). The organics were dried over Na_2SO_4 and concentrated under reduced pressure. SiO_2 flash chromatography (3:7 EtOAc:hexanes) afforded (+)-**10** in 14% yield (10.9 mg) as a solid. SiO_2 flash chromatography (3:7 EtOAc:hexanes) afforded (−)-**10** in 14% yield (10.5 mg) as a solid.

(+)-3-(4-Acetylphenyl)-1-(adamantan-1-yl)-2,2-difluoro-3-hydroxypropan-1-one (10). $[\alpha]^{23}_D$ +36.0 (*c* 0.667, $CHCl_3$). Anal. Calcd for $C_{21}H_{24}F_2O_3$: C, 69.60; H, 6.67. Found: C, 66.45; H, 6.51. Other characterization data is identical to the data reported for compound **10**.[26]

(−)-3-(4-Acetylphenyl)-1-(adamantan-1-yl)-2,2-difluoro-3-hydroxypropan-1-one (10). $[\alpha]^{23}_D$ −35.8 (*c* 0.558, $CHCl_3$). Other characterization data is identical to the data reported for compound **10**.[26]

3-(4-Acetylphenyl)-1-(adamantan-1-yl)-2,2-difluoro-3-methoxypropan-1-one (14). A solution of **10** (9.8 mg, 0.028 mmol) and CH_3I (35 μL, 0.56 mmol) in CH_2Cl_2 (0.5 mL) was treated with Ag_2O (42 mg, 0.18 mmol). After 6 h, CH_3I (50 μL, 0.8 mmol) was added to reaction. After 16 h, additional CH_3I, (100 μL, 1.6 mmol) was added. After 20 h, reaction was diluted with MeOH (20 mL) and filtered through Celite. The residue was concentrated under reduced pressure, suspended in CH_2Cl_2 (5 mL), and then washed with water (5 mL). The aqueous layer was separated and extracted with CH_2Cl_2 (2 × 5 mL). Combined organics were concentrated under reduced pressure to give **14** as a colorless solid (7.2 mg) in 71% yield: mp 85−87 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.98 (d, *J* = 8.3 Hz, 2H), 7.52 (d, *J* = 8.1 Hz, 2H), 4.86 (dd, *J* = 19.8, 5.2 Hz, 1H), 3.28 (s, 3H), 2.62 (s, 3H), 2.04 (br s, 3H), 1.94 (m, 6H), 1.72 (m, 6H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 204.4 (dd, J_{CF} = 29.8, 22.2 Hz, 1C), 197.8, 138.5, 137.5, 129.0 (2), 128.2 (2), 116.5 (dd, J_{CF} = 269, 253 Hz, 1C), 81.1 (dd, J_{CF} = 30.1, 21.2 Hz, 1C), 58.0, 46.6, 36.7 (3), 36.4 (3), 27.6 (3), 26.7. ^{19}F NMR (282 MHz, $CDCl_3$) δ −102.5 (dd, J_{FF} = 278, J_{HF} = 4.3 Hz, 1F), −123.4 (dd, J_{FF} = 278, J_{HF} = 19.9 Hz, 1F). HRMS (APCI) *m*/*z* calcd for $C_{22}H_{26}O_3F_2$ $(M+H)^+$ 377.1928, found 377.1930. Anal. Calcd for $C_{22}H_{26}O_3F_2 \cdot 0.5CH_2Cl_2$: C, 64.51; H, 6.50. Found C, 67.02; H, 6.45.

3-(4-Acetylphenyl)-1-(-adamantan-1-yl)-3-hydroxypropan-1-one (15). To a −78 °C solution of *n*-BuLi (0.14 mL, 2.5 M in hexanes) in THF (3 mL) was added hexamethyldisilazane (71 μL, 0.337 mmol). After 20 min at −78 °C, a solution of 1-adamantyl methyl ketone (50.0 mg, 0.280 mmol) in THF (1 mL) was added dropwise, and the reaction mixture was stirred for 40 min at −78 °C. Next, a solution of 4-acetylbenzaldehyde (50.0 mg, 0.337 mmol) in THF (1 mL) was added dropwise, and the reaction mixture was stirred for 20 min at −78 °C. Then the reaction mixture was quenched with saturated aqueous NH_4Cl (10 mL) at −78 °C. The mixture was warmed to rt and extracted with CH_2Cl_2 (3 × 10 mL). The combined organics were dried over Na_2SO_4 and concentration under reduced pressure. SiO_2 flash chromatography (8:2 hexanes/EtOAc) afforded the product **15** as a colorless oil in 68% yield (62.5 mg). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.94 (d, *J* = 8.4 Hz, 2H), 7.45 (d, *J* = 8.2 Hz, 2H), 5.16 (dt, *J* = 8.6, 2.7 Hz, 1H), 3.78 (d, *J* = 3.0 Hz, 1H), 2.86 (dd, *J* = 18.0, 3.3 Hz, 1H), 2.80 (dd, *J* = 18.0, 8.8 Hz, 1H), 2.59 (s, 3H), 2.03 (br s, 3H), 1.78−1.70 (m, 9H), 1.67−1.64 (m, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 216.4, 197.8, 148.4, 136.3, 128.6 (2), 125.7 (2), 69.5, 46.6, 44.7, 37.9 (3), 36.3 (3), 27.7 (3), 26.6. IR (film) ν_{max} 3468, 2905, 2850, 1682, 1268 cm^{-1}. HRMS (EI) *m*/*z* calcd for $C_{21}H_{26}O_3$ $(M)^+$ 326.1882, found 326.1876.

Cell Culture for $GABA_{BR}$/Cre-Luc Cells. $GABA_{BR}$/Cre-Luc cells were used between passages 2−26 and grown in media containing DMEM F-12 (Invitrogen, Carlsbad, CA), 5.8 mM $NaHCO_3$ (pH 7.0−7.2), 100 units/mL penicillin, 100 μg/mL streptomycin, 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA), 2 μg/mL puromycin (Sigma, St. Louis, MO), and 200 μg/mL G418 (Sigma) (growth medium).

Construction of HEK293 Cells Stably Expressing Human $GABA_{B1}$ and $GABA_{B2}$ Receptor cDNAs, and the Creluc Reporter ($GABA_{BR}$/Cre-Luc Cells). The human $GABA_{B1}$ receptor cDNA (GenBank BC050532) and the human $GABA_{B2}$ receptor cDNA (GenBank BC035071) were subcloned into the first and second multiple cloning site of the pIRES plasmid vector (ClonTech, Mountain View, CA), respectively. This vector (GABA-B1/B2/pIRES) allows both proteins to be translated from a single mRNA, and its integrity was verified by DNA sequencing. GABA-B1/B2/pIRES was linearized with BstBI between the Neo^R and Amp^R regions. Linearized GABA-B1/B2/pIRES was then transfected into HEK293/Cre-luc cells[47] in growth medium without G418, using calcium phosphate transfection.[48] Twenty-four hours post transfection, selection medium (growth medium with 400 μg/mL G418) was added to plates, and the cells were incubated at 37 °C in 5% CO_2. After several days in selection medium, colonies were picked and amplified in selection medium. G418-resistant clonal cell lines were screened for the expression of the $GABA_B$ receptor subunits by rtPCR, Western blot, and by an assay to detect baclofen inhibition of forskolin-stimulated cAMP accumulation using the Cre-Luc reporter system (see below). For the rtPCR assay, total RNA was isolated from several clones using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and cDNA was prepared using Superscript II reverse transcriptase and Taq DNA polymerase (Invitrogen, Carlsbad, CA). The primers used to amplify the $GABA_{B1}$ receptor cDNA were: Forward- GGCCCGGGGCCCCTTTTGCC; Reverse- GGATCA-CACTTGCTGTCGTGG. The primers used to amplify the $GABA_{B2}$ receptor cDNA were: Forward- GGAGCAGGTGCACACG-GAAGCC; Reverse- GGAATACAACTTGACCCGTGACC. After amplification with Taq polymerase, DNA products of the predicted size (268 bp for $GABA_{B1}$ receptor and 338 bp for $GABA_{B2}$ receptor) were observed upon agarose gel electrophoresis and staining with ethidium bromide. Western blotting was performed using an antibody against the $GABA_{B1}$ receptor (Santa Cruz Biochemicals, Santa Cruz, CA) and an antibody against the $GABA_{B2}$ receptor (Alomone Laboratories, Jerusalem, Israel). Detection with the appropriate secondary antibodies revealed specific bands of ∼80 kD, corresponding to the $GABA_{B1}$ receptor, and ∼130 kD, corresponding to the $GABA_{B2}$ receptor. Once the stable $GABA_{BR}$/Cre-Luc cell line was confirmed, a *z*-score of 0.61 was determined for the inhibition of adenylyl cyclase assay using 10 μM forskolin for the high control, and 10 μM forskolin (Tocris, Bristol, UK) + 10 μM (±)-baclofen (Sigma, St. Louis, MO) for the low control.

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Adenylyl Cyclase Inhibition Assay. $GABA_{BR}$/Cre-Luc cells were plated at approximately 65000 cells per well in black-walled 96-well tissue culture plates (VWR, West Chester, PA) and incubated overnight at 37 °C and 5% CO_2. Treatments were diluted to 2× the working concentration in Optimem I reduced-serum medium (Invitrogen, Carlsbad, CA) supplemented with 0.02% ascorbic acid (Sigma, St. Louis, MO). Cell culture medium was decanted from cells and equal amounts of the Optimem buffer and 2× treatments were added to cells. Forskolin (10 μM) was added to cells to stimulate cAMP accumulation, and vehicle or 10 μM (±)-baclofen were added along with forskolin to establish the maximum cAMP accumulation, and the portion of the response that could be inhibited by activation of the $GABA_B$ receptor, respectively. A basal control (no forskolin or baclofen) was included with each assay. The indicated concentrations of test compounds, (±)-baclofen, *R*-(−)-baclofen (Tocris, Briston, UK), or *S*-(+)-baclofen (Sigma, St. Louis, MO) or γ-aminobutyric acid (GABA) (Sigma, St. Louis, MO) were added to cells along with 10 μM of forskolin. Reversal of $GABA_B$ receptor-mediated inhibition of adenylyl cyclase activity was demonstrated with the $GABA_B$ receptor antagonist CGP 55845 (10 μM) (Tocris, Bristol, UK). Each condition was performed in triplicate. Cells were incubated with the indicated treatments at 37 °C and 5% CO_2 for 3 h. After the incubation period, the treatments were decanted, and assay plates were allowed to cool to room temperature for 10 min. While protecting the samples from light, 30 μL of phosphate buffered saline (Sigma, St. Louis, MO) and 30 μL Steady Lite Plus (PerkinElmer, Waltham, MA) were added to each well, and the plates were shaken at 500 rpm for 5 min. Luminescence was quantified with a Victor light luminescence reader (PerkinElmer, Waltham, MA).

Cytotoxicity Assay. $GABA_{BR}$/Cre-Luc Cells were plated at approximately 65000 cells per well in black-walled tissue culture plates (VWR, West Chester, PA), incubated overnight at 37 °C and 5% CO_2. Treatments were diluted to 2× the working concentration in Optimem I reduced-serum medium (Invitrogen, Carlsbad, CA). Cell culture medium was decanted from cells, and equal amounts of the Optimem and 2× treatments were added to cells. Each condition was performed in triplicate. Cells were incubated at 37 °C and 5% CO_2 for 3 h with the indicated treatments, and then 20 μL of CellTiter 96 Aqueous One Solution Reagent (Promega, Madison, WI) was added to all of the wells. The plates were protected from light and incubated for 1.5 h at 37 °C and 5% CO_2, and then absorption at 490 nm was quantified with a Synergy 4 plate reader (BioTek, Winooski, VT).

$GABA_A$ Assay. tsA201 cells, derived from human embryonic kidney (HEK) cell line, were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, Grand Island, NY) supplemented with 10% v/v FBS, 100 units/mL penicillin G, and 100 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO) and were maintained at 37 °C in 95% air/5% CO_2. tsA201 cells about 50−70% confluent in a 35 mm culture dish were transfected with the rat $GABA_A$ receptor subunits α_1, β_2, and γ_{2s} (in the plasmid vector pCDNA3.1; Invitrogen, Carlsbad, CA) using the calcium phosphate method.[4] Rat $GABA_A$ receptor subunit plasmid cDNAs were transfected in a 1:1:5:1 ratio for α_1, β_2, γ_{2s}, and GFP, respectively. The cell medium was replaced with fresh cell medium about 5−7 h after transient transfections. After another 20−24 h, transfected cells were trypsinized and plated in 35 mm dishes for electrophysiology recordings the next day.

Voltage clamp experiments were performed at rt using the standard whole-cell configuration. Current responses were recorded with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) and filtered at 1 kHz (six-pole Bessel filter, −3 dB). Electrodes were pulled from borosilicate glass (VWR, West Chester, PA) and fire-polished to resistances of 2−5 MΩ when filled with intracellular solution containing 147 mM *N*-methyl-D-glucamine hydrochloride, 5 mM CsCl, 5 mM Na_2ATP, 5 mM HEPES, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 1.1 mM EGTA at pH 7.2 and an osmolality of 310 mOsm. The extracellular recording solution contained 145 mM NaCl, 3 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM D-glucose, 10 mM HEPES at pH 7.4, and an osmolality of 315 mOsm. The standard holding potential for the cells was −60 mV, and a gap-free recording protocol was used. Whole-cell currents were recorded at a sampling rate of 1 kHz. The current response in the presence of 100 μM GABA allowed for comparing the activity of the synthesized compounds at this $GABA_A$ receptor type.

In Vivo Experiments. *Subjects.* HAP2 mice were used as subjects. For experiment 1 [(±)-baclofen], mice (7 males, 7 females) were between 133 and 142 days old. These mice were part of a larger experimental design and also had previously been exposed to foot shock/acoustic startle testing (same parameters used here), testing for prepulse inhibition of the acoustic startle response, and two injections (separated by 5−7 days) of either vehicle, 5.0 or 7.5 mg/kg (±)-baclofen. Following a washout period of 15 days, mice were counterbalanced into treatment groups so that previous exposure to (±)-baclofen or vehicle was distributed across treatment groups for experiment 1 (i.e., vehicle vs 7.5 mg/kg baclofen); baclofen's startle reducing effect shown in Figure 12 was similar regardless of prior drug history. For experiment 2 (*rac*-BHFF/compound (±)-**10**), experimentally naïve mice (16 males, 16 females) were between 316 and 332 days old at the time of testing. Mice were housed in polycarbonate cages (29.2 cm × 19.0 cm × 12.7 cm) with aspen wood shavings in groups of 2−4 per cage. Ambient room temperature was maintained at 21 ± 2 °C. Mice had free access to food (Rodent Lab Diet 5001, Purina Mills Inc., St Louis, MO) and water in the home cage at all times, except when testing procedures took place. Experimental procedures were conducted during the light phase of a 12:12 light:dark cycle (lights off at 19:00). All experimental procedures were approved by the Purdue Animal Care and Use Committee and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

Drugs. All drugs were administered ip using a 10 mL/kg injection volume. (±)-Baclofen was dissolved in distilled water and administered at a dose of 7.5 mg/kg. *rac*-BHFF and compound (±)-**10** were dissolved in 10% DMSO/45% (w/v) 2-hydroxypropyl-β-cyclodextrin in distilled water and administered at a doses of 12.5 and 36.16 mg/kg, respectively. The dose for (±)-baclofen[45] and *rac*-BHFF was obtained from prior studies,[20] and a relative dose for the novel agent (±)-**10** was selected using the pharmacological data (see Table 1).

Apparatus. The acoustic startle response was assessed using sound-attenuated Coulbourn Instruments (Allentown, PA, USA) Animal Acoustic Startle System chambers, as previously described.[43] Startle stimuli consisted of 100 dB, 40 ms white noise bursts (frequency range: 20 Hz−20 kHz). The startle response was measured as the amount of force in grams exerted against a weight-sensitive platform during the 200 ms after the onset of each acoustic stimulus. The force measurement does not include the subject's body weight. A ventilating fan provided continuous 70−71 dB background noise.

Acoustic Startle Procedures. Mice received one conditioning and one test session separated by 24 h. During each conditioning session, mice received 40 trials of a 30 s, 7 W light stimulus paired with a 0.5 s, 0.8 mA foot shock (2 min intertrial interval). The foot shock occurred during the last 0.5 s of the light stimulus presentation. The test session consisted of a 5 min habituation period followed by 36 total trials (2 min ITI) presented on a random schedule (range: 12−108 s) to reduce habituation to any single trial type. Twelve of the trials were blank (no stimuli; 40 ms), 12 were noise alone (100 dB, 40 ms), and 12 were light (7 W, 30 s) + noise (100 dB, 40 ms). On light + noise trials, the noise stimulus was presented immediately after the light stimulus ended.

Statistical Analyses. All 12 startle responses on each trial type were averaged for each mouse. Data were analyzed using repeated measures analysis of variance (ANOVA) followed by posthoc one-way ANOVAs. Probability values <0.05 were considered to be significant.

■ ASSOCIATED CONTENT

Supporting Information

X-ray data of compound **10** and crystallographic data (CIF). This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Phone: (765) 496-3962. Fax: (765) 494-1414. E-mail: dcolby@purdue.edu. Address: Purdue University, 575 Stadium Mall Drive, West Lafayette, IN 47907.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

CRE-Luc, CRE-luciferase; GERD, gastroesophageal reflux; HAP, high-alcohol preferring; HEPES, 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid; ip, intraperitoneal; Osm, osmolality; *rac*-BHFF, (*R*,*S*)-5,7-di-*tert*-butyl-3-hydroxy-3-trifluoromethyl-3*H*-benzofuran-2-one

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