Journal of Medicinal Chemistry

Article

pubs.acs.org/jmc

Synthesis and Pharmacological Characterization of 2-(Acylamino)thiophene Derivatives as Metabolically Stable, Orally Effective, Positive Allosteric Modulators of the $GABA_B$ Receptor

Claudia Mugnaini,[†] Valentina Pedani,[†] Angelo Casu,[‡] Carla Lobina,[§] Alberto Casti,[‡] Paola Maccioni,[§] Alessandra Porcu,[‡] Daniela Giunta,[∥] Stefania Lamponi,[†] Maurizio Solinas,[∥] Stefania Dragoni,[⊥] Massimo Valoti,[#] Giancarlo Colombo,[§] Maria Paola Castelli,[‡] Gian Luigi Gessa,[§] and Federico Corelli*[,†]

[†]Dipartimento di Biotecnologie, Chimica e Farmacia, Università degli Studi di Siena, Via A. Moro, Siena, Italy

[‡]Dipartimento di Scienze Biomediche, Divisione di Neuroscienze e Farmacologia Clinica, Università degli Studi di Cagliari, Cagliari, Italy

[§]Istituto di Neuroscienze, Consiglio Nazionale delle Ricerche, Sezione di Cagliari, Cagliari, Italy

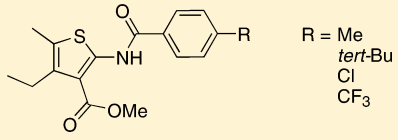
[∥]Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Sezione di Sassari, Sassari, Italy

[⊥]Dipartimento di Chimica, Università degli Studi di Perugia, Via Elce di Sotto, Perugia, Italy

[#]Dipartimento di Scienze della Vita, Università degli Studi di Siena, Via A. Moro, Siena, Italy

Supporting Information

ABSTRACT: Two recently reported hit compounds, COR627 and COR628, underpinned the development of a series of 2-(acylamino)thiophene derivatives. Some of these compounds displayed significant activity in vitro as positive allosteric modulators of the $GABA_B$ receptor by potentiating GTPγS stimulation induced by GABA at 2.5 and 25 μM while failing to exhibit intrinsic agonist activity. Compounds were also found to be effective in vivo, potentiating baclofen-induced sedation/hypnosis in DBA mice when administered either intraperitoneally or intragastrically. Although displaying a lower potency in vitro than the reference compound GS39783, the new compounds **6**, **10**, and **11** exhibited a higher efficacy in vivo: combination of these compounds with a per se nonsedative dose of baclofen resulted in shorter onset and longer duration of the loss of righting reflex in mice. Test compounds showed cytotoxic effects at concentrations comparable to or higher than those of GS39783 or BHF177.



INTRODUCTION

The concept of allosteric modulation of certain classes of proteins, such as enzymes, was introduced in the mid-1960s.[1] However, considerable interest only manifested in recent years following extraordinary developments in molecular pharmacology and screening technology[2,3] as well as in the clinical success of benzodiazepines, the first clinically useful drugs acting as allosteric modulators.[4]

Allosteric modulators act on a given receptor by binding at a topographically distinct site from the orthosteric one (active site), either potentiating (positive allosteric modulators, PAMs) or inhibiting (negative allosteric modulators, NAMs) binding of the orthosteric ligand, primarily by altering the three-dimensional receptor conformation. In principle, allosteric ligands afford several advantages over orthosteric drugs as potential therapeutic agents. First, lacking agonistic activity, their effect is only exerted in the presence of the endogenous agonist, thus promoting an increasingly integrated action with the temporal and spatial organization of physiological receptor activation, with a lower side effect potential than orthosteric agonists. Second, PAMs are expected to be devoid of or to display scarce propensity for receptor desensitization, whereas persistent agonist treatment often leads to receptor down-regulation. Third, PAMs and NAMs frequently display high selectivity for specific receptor subtypes, due to the lack of pressure during evolution to maintain the apparently unessential allosteric binding sites unchanged.[5]

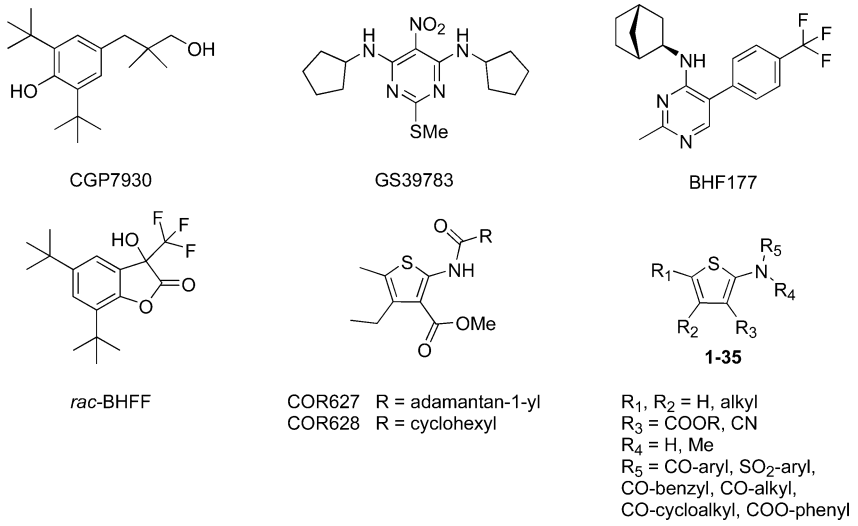
In recent years, remarkable progress has been made in the discovery, chemical optimization, and pharmacological understanding of allosteric modulators of G-protein-coupled receptors (GPCRs), as demonstrated by the large number of reports published in literature on this subject.[5−12] The marketing of cinacalcet[13] (a PAM of the calcium-sensing receptor) and maraviroc[14] (a NAM of the chemokine receptor CCR5) provided clear demonstration of the clinical relevance of GPCRs allosteric modulators. In addition to cinacalcet, several highly selective PAMs have been identified for family C GPCRs including calcium-sensing receptors, mGlu receptors,[5] and $GABA_B$ receptors.[15−18]

The $GABA_B$ receptor is implicated in a wide range of neurological disorders, namely, spasticity, pain, drug addiction, anxiety and depression, absence epilepsy, and cognition.[19−23] Nevertheless, the only currently marketed drug targeting the $GABA_B$ receptor is baclofen, a $GABA_B$ agonist that is widely used as a muscle relaxant in the treatment of spasticity in hemi-

Received: January 29, 2013
Published: April 1, 2013

   dx.doi.org/10.1021/jm400144w | J. Med. Chem. 2013, 56, 3620−3635

Chart 1. Structure of Selected $GABA_B$ PAMs and New Compounds 1-35



and tetraplegic subjects and in multiple sclerosis. Moreover, baclofen effectively reduces craving for and intake of drugs of abuse such as alcohol, nicotine, and cocaine.[24] However, the poor blood–brain barrier penetration, short duration of action, rapid tolerance, and narrow therapeutic window of baclofen[20] greatly limit its clinical use.

Research in the area of $GABA_B$ receptor PAMs may lead to the development of new potentially useful drugs for the treatment of a series of diseases and syndromes, including anxiety, alcohol, nicotine, and cocaine dependence, osteoarthritic pain, chronic nociceptive pain, and gastroesophageal reflux disease.[25] The mode of action displayed by these novel therapeutic agents suggests that they may be devoid of drawbacks that characterize full agonists, such as receptor desensitization, tolerance, muscle-relaxant effects, hypothermia, and central and gastrointestinal side effects.[5,25b] Surprisingly, to date, only a small number of pharmaceutical companies have made substantial investments aimed at identifying $GABA_B$ PAMs.[26] Several compounds have been shown to have positive $GABA_B$ modulatory activity in vitro, with 3,5-bis(1,1-dimethylethyl)-4-hydroxy-β,β-dimethyl-benzenepropanol (CGP7930),[15,27] *N*,*N*′-dicyclopentyl-2-(methylthio)-5-nitro-4,6-pyrimidinediamine (GS39783),[16] (*R*,*S*)-5,7-di-*tert*-butyl-3-hydroxy-3-trifluoromethyl-3*H*-benzofuran-2-one (*rac*-BHFF),[18] and *N*-[(1*R*,2*R*,4*S*)-bicyclo[2.2.1]hept-2-yl]-2-methyl-5-[4-(trifluoromethyl)phenyl]-4-pyrimidinamine (BHF177)[17] (Chart 1) being well studied and characterized as PAMs.

CGP7930 and GS39783, the first two $GABA_B$ PAMs discovered at Novartis, enhance both the potency and maximal response of GABA. Tested in behavioral models in vivo, these compounds reproduced the effects of baclofen but were devoid of side effects produced by the drug. Specifically, treatment with CGP7930 and GS39783 decreased multiple behaviors related to drugs of abuse, including (a) alcohol drinking[28] and operant oral alcohol self-administration in selectively bred, alcohol-preferring rats,[29] (b) intravenous nicotine self-administration[30] and nicotine-induced conditioned place preference[31] in rats, as well as nicotine-stimulated hyperlocomotion in mice,[32] (c) intravenous cocaine self-administration[33] and reinstatement of cocaine seeking[34] in rats, and (d) metamphetamine-induced conditioned place preference in rats.[35] Additionally, treatment with CGP7930 and GS39783 exerted anxiolytic effects in rats and mice.[36]

Despite its interesting pharmacological profile, GS39783 was found to be genotoxic, likely because of the presence of the aromatic nitro group.[17] In an attempt to obtain molecules devoid of the genotoxic potential of GS39783, a number of analogues were synthesized at Novartis. Among them, BHF177 was found to be the most potent, displaying an optimum profile in genotoxicity and mutagenicity tests.[17] *rac*-BHFF, a molecule structurally related to CGP7930 discovered at Hoffmann-La Roche, is capable of enhancing both the potency and maximal effect of GABA while acting as an allosteric agonist.[37] Recent studies have demonstrated that, similar to treatment with CGP7930 and GS39783, treatment with BHF177 and rac-BHFF decreased (a) alcohol drinking[38] and operant oral alcohol self-administration in alcohol-preferring rats[39,40] and (b) intravenous nicotine self-administration[30,41] and reinstatement of nicotine seeking[41] in rats.

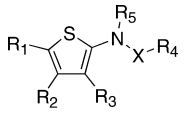
Research efforts undertaken have resulted in an expansion of the number of chemical scaffolds to be taken into account in the search for new $GABA_B$ PAMs and provided an insight into structure–activity relationships (SARs). The latter is particularly relevant in the context of GPCR allosteric modulators, frequently characterized by "flat, nontractable" SAR, thus complicating the choice of structural modifications of lead compounds.[5]

Very recently, our group identified COR627 and its simplified analogue COR628 (Chart 1) as new $GABA_B$ PAMs by means of a virtual screening protocol.[42] Both compounds were found to potentiate GABA- and baclofen-stimulated [^{35}S]guanosine 5′-*O*-(3-thio)triphosphate ([^{35}S]GTPγS) binding to native $GABA_B$ receptors while producing no effect when given alone. In vivo experiments indicated that pretreatment with per se ineffective doses of the two compounds potentiated the sedative/hypnotic effect of baclofen.[42a]

These results prompted us to further investigate the PAM activity toward $GABA_B$ receptor of a small library of 2-(acylamino)thiophene derivatives. Accordingly, the synthesis and in vitro and in vivo pharmacological evaluation of 2-(acylamino)thiophene derivatives **1**–**35** (Chart 1, Table 1), which represent a new class of compounds in the $GABA_B$ PAMs scenario, are reported. In particular, these new

dx.doi.org/10.1021/jm400144w | J. Med. Chem. 2013, 56, 3620–3635

Table 1. Structure of the New Compounds 1−35



Compd	R_1	R_2	R_3	X	R_4	R_5	Method	Yield (%)
1	Me	Et	CO_2Me	CO	methyl	H	A[a]	94
2	Me	Et	CO_2Me	CO	cyclopropyl	H	A	70
3	Me	Et	CO_2Me	CO	*tert*-butyl	H	A	92
4	Me	Et	CO_2Me	CO	cyclopentyl	H	A	90
5	Me	Et	CO_2Me	CO	phenyl	H	A	60
6	Me	Et	CO_2Me	CO	4-methylphenyl	H	B	85
7	Me	Et	CO_2Me	CO	4-*tert*-butylphenyl	H	B	96
8	Me	Et	CO_2Me	CO	4-methoxyphenyl	H	B	86
9	Me	Et	CO_2Me	CO	4-nitrophenyl	H	C	44
10	Me	Et	CO_2Me	CO	4-(trifluoromethyl)phenyl	H	B	82
11	Me	Et	CO_2Me	CO	4-chlorophenyl	H	B	70
12	Me	Et	CO_2Me	CO	3-chlorophenyl	H	B	85
13	Me	Et	CO_2Me	CO	2-chlorophenyl	H	B	80
14	Me	Et	CO_2Me	CO	4-bromophenyl	H	B	74
15	Me	Et	CO_2Me	CO	4-iodophenyl	H	B	92
16	Me	Et	CO_2Me	CO-O	phenyl	H	A[b]	37
17	Me	Et	CO_2Me	$CO-CH_2$	phenyl	H	B	75
18	H	H	CO_2Me	CO	phenyl	H	A	66
19	H	H	CO_2Me	CO	4-chlorophenyl	H	B	85
20			CO_2Me	CO	cyclohexyl	H	C	94
21			CO_2Me	CO	phenyl	H	A	99
22			CO_2Me	CO	3,4-dimethoxyphenyl	H	C	43
23			CN	CO	cyclohexyl	H	C	75
24			CN	CO	3,4-dimethoxyphenyl	H	C	25
25	Me	Et	CO_2Me	SO_2	phenyl	H	E	80
26	Me	Et	CO_2Me	SO_2	4-methylphenyl	H	D	69
27	Me	Et	CO_2Me	SO_2	cyclohexyl	H	D	46
28	Me	Et	CO_2Me	CO	4-chlorophenyl	Me	F	70
29	Me	Et	CO_2Me	CO	4-biphenyl	H	G	33
30	Me	Et	CO_2Me	CO	4-(4'-methyl)-biphenyl	H	G	14
31	Me	Et	CO_2Me	CO	4-(3-piridyl)phenyl	H	G	21
32	Me	Et	CO_2-*n*Pr	CO	4-methylphenyl	H	H	10
33	Me	Et	CO_2-*t*Bu	CO	4-methylphenyl	H	H	21
34	Me	Et	CO_2-*n*Pr	CO	4-chlorophenyl	H	I	86
35	Me	Et	CO_2-*t*Bu	CO	4-chlorophenyl	H	I	26

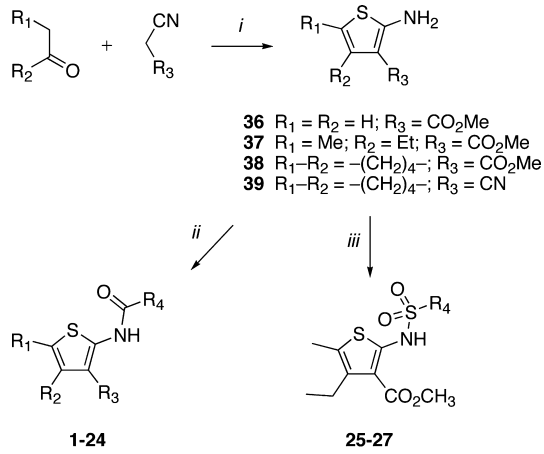
[a]Acetic anhydride was used as the acylating agent. [b]Reaction solvent: THF/DCM 1:1 at reflux temperature.

compounds were obtained by systematic modification of the substituents appended to the tiophene ring of COR627, with particular emphasis on modulation of the amide group. Further studies based on alternative approaches, such as scaffold hopping through bioisosteric replacement of thiophene nucleus by other heterocycles, are still in progress and will be reported on in due course.

RESULTS

Chemistry. Synthesis of New 2-(Acylamino)thiophene Derivatives. Among the aminothiophene derivatives **36**−**39** (Scheme 1) used as starting substrates, compound **36** was commercially available, while the amino ester and aminonitrile derivatives **37**−**39** were synthesized in-house according to the Gewald synthesis.[43,44] This multicomponent reaction, which has been widely exploited in combinatorial/medicinal chemistry to yield a variety of compounds exhibiting different pharmacological activities,[44c] allowed for the straightforward preparation of the target compounds. Thus, condensation of 3-pentanone or cyclohexanone with methyl cyanoacetate in the presence of sulfur and morpholine yielded methyl 2-amino-thiophene-3-carboxylates **37** and **38**, respectively. Similarly, aminonitrile **39** was prepared by reacting cyclohexanone with malononitrile. Subsequent reaction of **36**−**39** with different acyl and sulfonyl chlorides (acetic anhydride for compound **1**) under standard conditions provided carboxamides **1**−**24** and sulfonamides **25**−**27**, respectively. However, the outcome of this apparently ordinary N-acylation reaction was strongly dependent on the experimental conditions used. Specifically, when relatively strong bases, such as TEA or DIPEA, were employed in combination with acyl chlorides and particularly sulfonyl chlorides, considerable amounts of the N,N-diacylated products were isolated. Most probably, these conditions favored an efficient deprotonation of the initially produced secondary (sulfon)amides, rendered strongly acidic by intramolecular conjugation with the electron-withdrawing ester moiety. Particularly in the case of sulfonamides, their conjugated bases efficiently compete with aminothiophenes for the acylating agent, giving rise to the corresponding sulfonimides. Accordingly, secondary sulfonamides could be generally

Scheme 1[a]



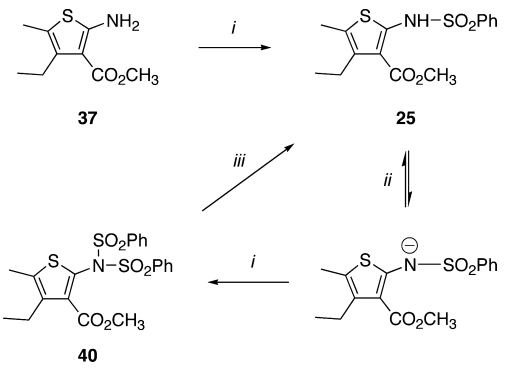
For R_1–R_4, see Table 1.

[a]Reagents and conditions: (i) S_8, morpholine, EtOH, reflux; (ii) R_4COCl, TEA, DCM, rt (method A) or R_4COCl, dioxane, reflux (method B) or R_4CO_2H, DIC, DMAP, DCM, 0 °C to rt (method C); (iii) R_4SO_2Cl, pyridine, 0 °C to rt (method D).

dx.doi.org/10.1021/jm400144w | J. Med. Chem. 2013, 56, 3620−3635

prepared in good yield by using a weaker base, such as pyridine. Nevertheless, the best way to synthesize sulfonamide **25** (R_4 = phenyl), achieving a 55% overall yield, was to apply a two-step procedure (Scheme 2) involving selective hydrolysis (16 equiv

Scheme 2[a]

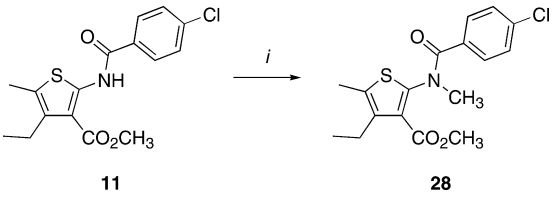


[a]Reagents and conditions: (i) benzenesulfonyl chloride, TEA, DCM, rt; (ii) TEA; (iii) NaOH, THF, H_2O, 50 °C (method E).

of NaOH, THF/H_2O, 45−50 °C, 8 h) of the initially formed sulfonimide **40**.[45] No hydrolysis of the ester function occurred under these conditions. Carboxamides **1**−**24** were synthesized by reacting **36**−**39** with acyl chlorides either in the presence of TEA, with only traces of diacylated products (method A), or simply in refluxing dioxane without added base (method B). In some cases, reaction with carboxylic acids in the presence of diisopropylcarbodiimide (DIC) (method C) was also used.

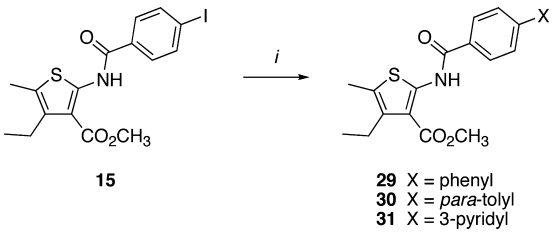
To explore the steric and electronic properties of the amide moiety, compound **11** was alkylated with methyl iodide to the corresponding tertiary amide **28** (Scheme 3), while the iodo derivative **15** was transformed into the biphenyl derivatives **29**−**31** by Suzuki coupling with the appropriate arylboronic acids (Scheme 4).

Scheme 3[a]



[a]Reagents and conditions: (i) methyl iodide, potassium *tert*-butoxide, DMF, rt (method F).

Scheme 4[a]



[a]Reagents and conditions: (i) appropriate arylboronic acid, $Pd(OAc)_2$, PPh_3, 1 M Na_2CO_3, EtOH/DME, MW (110 °C, 5 min) (method G).

Selected modifications of the methyl ester group into more bulky/lipophilic moieties were also performed (Scheme 5). Thus, basic hydrolysis of **6** and **11** yielded the corresponding carboxylic acids **41** and **42** under rather vigorous reaction conditions (15 equiv of NaOH in MeOH/water at 80 °C overnight or successive additions of LiOH in THF/H_2O during 24 h). Subsequent reaction of **41** and **42** with an excess of 1-propanol or *tert*-butanol in the presence of coupling reagents, such as carbodiimides or HBTU/HOBt, afforded the expected esters **32**−**35** in only low yield (10−21%). Esters **34** and **35** were ultimately found to be best prepared in 85% and 26% yield, respectively, by treatment of **42** with 1-propanol or *tert*-butanol under Mitsunobu conditions.

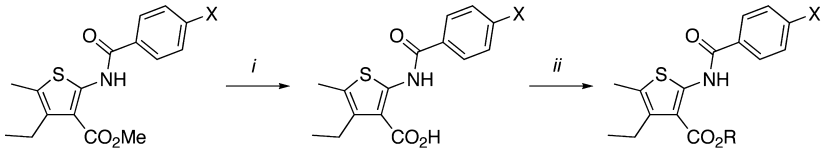
In Vitro Pharmacology. Effects of 2-(Acylamino)-thiophene Derivatives on Native $GABA_B$ Receptor Activity in [^{35}S]GTPγS Binding Assay. The functional characterization of the novel compounds was performed using GTPγS binding assay, a well validated functional assay for GPCRs, using membranes from rat brain cortex. To assay for $GABA_B$ PAM activity, the compounds were coapplied with the endogenous ligand GABA. As shown in Table 2, in the presence of 10 μM GABA, the test compounds at 25 μM potentiated [^{35}S]GTPγS stimulation induced by GABA alone. Considering the data (and respective SEM values) in Table 2, a clear-cut activity ranking for all the tested compounds can be hardly found. However, compounds **6**, **7**, **10**, and **11** stand out to some extent within the series: at 25 μM they stimulated [^{35}S]GTPγS binding to rat membranes to approximately 27−18% of GABA alone, with a potency similar to that of COR627 and COR628, though lower than that of GS39783, used as reference compounds. Compounds **7**, **10**, and **11** at 2.5 μM potentiated [^{35}S]GTPγS binding to approximately 19−9% of GABA alone, displaying a potency comparable to or higher than that of the prototypical compound COR627. When tested in the absence of GABA, all the compounds produced no stimulation of basal [^{35}S]GTPγS binding up to 100 μM (data not shown), demonstrating no intrinsic agonist activity. All the other compounds reported in Table 1 did not stimulate [^{35}S]GTPγS binding either alone or when coapplied with GABA (data not shown).

To further investigate the $GABA_B$ PAM profiles of **10** and **11**, two of the most active compounds in vitro and in vivo (vide infra), GABA concentration curves were performed in the absence and presence of a fixed concentration (30 μM) of both compounds. As shown in Figure 1, GABA alone stimulated [^{35}S]GTPγS in a concentration-dependent manner with an EC_{50} of 3.28 ± 0.29 μM and a maximal stimulation of 45 ± 4.40% over the basal value. In the presence of compounds **10** and **11**, the EC_{50} for GABA decreased by approximately 11-fold (EC_{50} = 0.28 ± 0.03 μM) and 3-fold (EC_{50} = 1.11 ± 0.08 μM) for compounds **10** and **11**, respectively. Only a slight concomitant increase in maximal GABA stimulation (E_{max} of 60 ± 5.50% and 51 ± 3.80% over the basal values for compounds **10** and **11**, respectively) was observed for both compounds. Contrary to other well-known $GABA_B$ PAMs, such as CGP7930, GS39783, and *rac*-BHFF, but similar to COR627 and COR628,[42a] **10** and **11** mainly affected the potency of GABA rather than its efficacy.

Cytotoxicity Assay. Cytotoxicity assay was performed to establish the effects of selected compounds on cell viability in vitro in comparison with reference compounds COR627, COR628, GS39783, and BHF177. IC_{50} values on NIH3T3 cell line are reported in Table 3. These data confirmed a low

dx.doi.org/10.1021/jm400144w | J. Med. Chem. 2013, 56, 3620−3635

Scheme 5[a]



6 X = CH_3
11 X = Cl

41 X = CH_3
42 X = Cl

32 X = CH_3; R = *n*-propyl
33 X = CH_3; R = *tert*-butyl
34 X = Cl; R = *n*-propyl
35 X = Cl; R = *tert*-butyl

[a]Reagents and conditions: (i) LiOH, THF, H_2O, rt; (ii) ROH, DIC, DCM, rt (for **32** and **33**, method H) or ROH, DEAD, PPh_3, THF, rt (for **34** and **35**, method I).

Table 2. Effects of 2-Acylaminothiophene Derivatives on the Stimulation of [^{35}S]GTPγS Binding via $GABA_B$ Receptor in Rat Cortical Membranes

	increase (%) relative to 10 μM GABA	
compd	2.5 μM[a]	25 μM[a]
4	3.0 ± 3.2	12.1 ± 3.7
5	3.1 ± 4.6	12.0 ± 5.9
6	3.7 ± 2.3	18.2 ± 2.8
7	19.0 ± 2.3	23.2 ± 3.9
10	17.8 ± 2.8	27.3 ± 5.3
11	9.2 ± 1.8	18.3 ± 3.6
12	4.0 ± 5.3	11.0 ± 8.3
16	3.9 ± 2.6	8.2 ± 4.5
20	−5.6 ± 2.9	12.0 ± 0.7
22	−4.1 ± 2.7	9.0 ± 3.7
25	2.9 ± 5.3	5.9 ± 1.6
COR627	9.2 ± 1.8	22.0 ± 4.9
COR628	2.0 ± 1.4	15.1 ± 3.3
GS39783	59.0 ± 1.3 (at 10 μM)	

[a]Data are the mean ± SEM of five experiments, each performed in triplicate. As the maximal effect of 10 μM GABA alone differed between experiments, data were normalized to the effect of 10 μM GABA (control, set as 0%). GABA at 10 μM stimulated [^{35}S]GTPγS binding to approximately 24.0 ± 1.1% of the basal activity. COR627, COR628, and GS39783 were used as reference compounds.

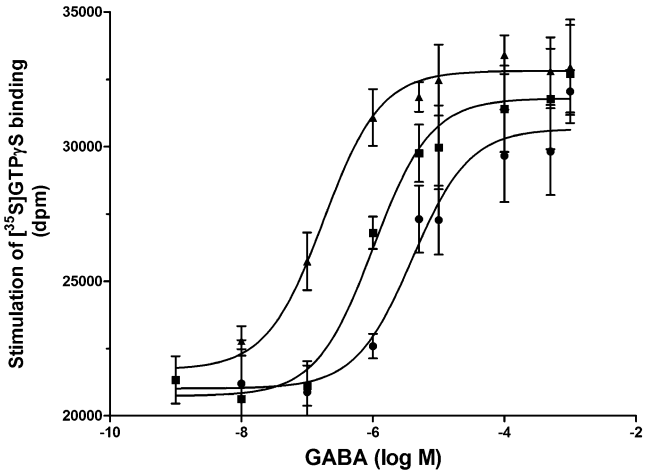


Figure 1. Concentration−response curves for GABA in the [^{35}S]GTPγS binding assay in the absence (●) and presence (▲) of **10** or (■) **11** (30 μM). GABA responses were measured in rat cortical membranes. Data shown are from a typical experiment performed in triplicate, expressed as the mean ± SEM dpm values.

Table 3. Cytotoxic Effect (IC_{50}, μM) of Selected 2-Acylaminothiophene Derivatives on NIH3T3 Cells in Comparison with Reference Compounds COR627, COR628, GS39783, and BHF177

compd	IC_{50} ± SD (μM)[a]
4	34 ± 1
5	66 ± 3
6	95 ± 4
7	140 ± 4
10	54 ± 2
11	59 ± 2
20	62 ± 3
25	146 ± 5
COR627	32 ± 1
COR628	33 ± 1
GS39783	60 ± 2
BHF177	35 ± 2

[a]Each value is the mean of six determinations.

cytotoxic potential for all the tested compounds toward NIH3T3. Some of the most active $GABA_B$ enhancers, such as **6**, **7**, **10**, and **11**, displayed cytotoxicity values comparable to or lower than GS39783 and BHF177, respectively. In particular, **7** and **25** were approximately 4-fold less cytotoxic than lead compounds COR627 and COR628.

Metabolic Stability Assays. The intrinsic clearance (Cl_{intr}) of compounds **6**, **7**, **10**, and **11** was assessed in the presence of mouse liver microsomes. The plot of the natural logarithm of the percentage of nonmetabolized compounds versus time was linear, indicating that substrate depletion by CYPs followed a monoexponential relationship. The calculated *k* (Table 4) ranged from 0.0074 to 0.01155 min^{-1} for compound **6** and compound **10**, respectively. The resulting Cl_{intr} ranged from 7.7 to 12.03 (μL min^{-1} mg^{-1}), and the amount of unmetabolized compound after 1 h of incubation was ≥60%. These data

Table 4. Elimination Rate Constant and Intrinsic Clearance of Compounds 6, 7, 10, and 11

compd	*k* (min^{-1})	CL_{intr} (μL·min^{-1}·mg protein^{-1})	compd metabolized (%)[a]
6	0.007424	7.73	28
7	0.008628	8.98	32
10	0.01155	12.03	40
11	0.007627	7.9	28

[a]Amount of compound metabolized after 60 min of incubation in the presence of mouse liver microsomal preparations and NADPH-generating system, expressed as % of the initial amount added.

dx.doi.org/10.1021/jm400144w | J. Med. Chem. 2013, 56, 3620−3635

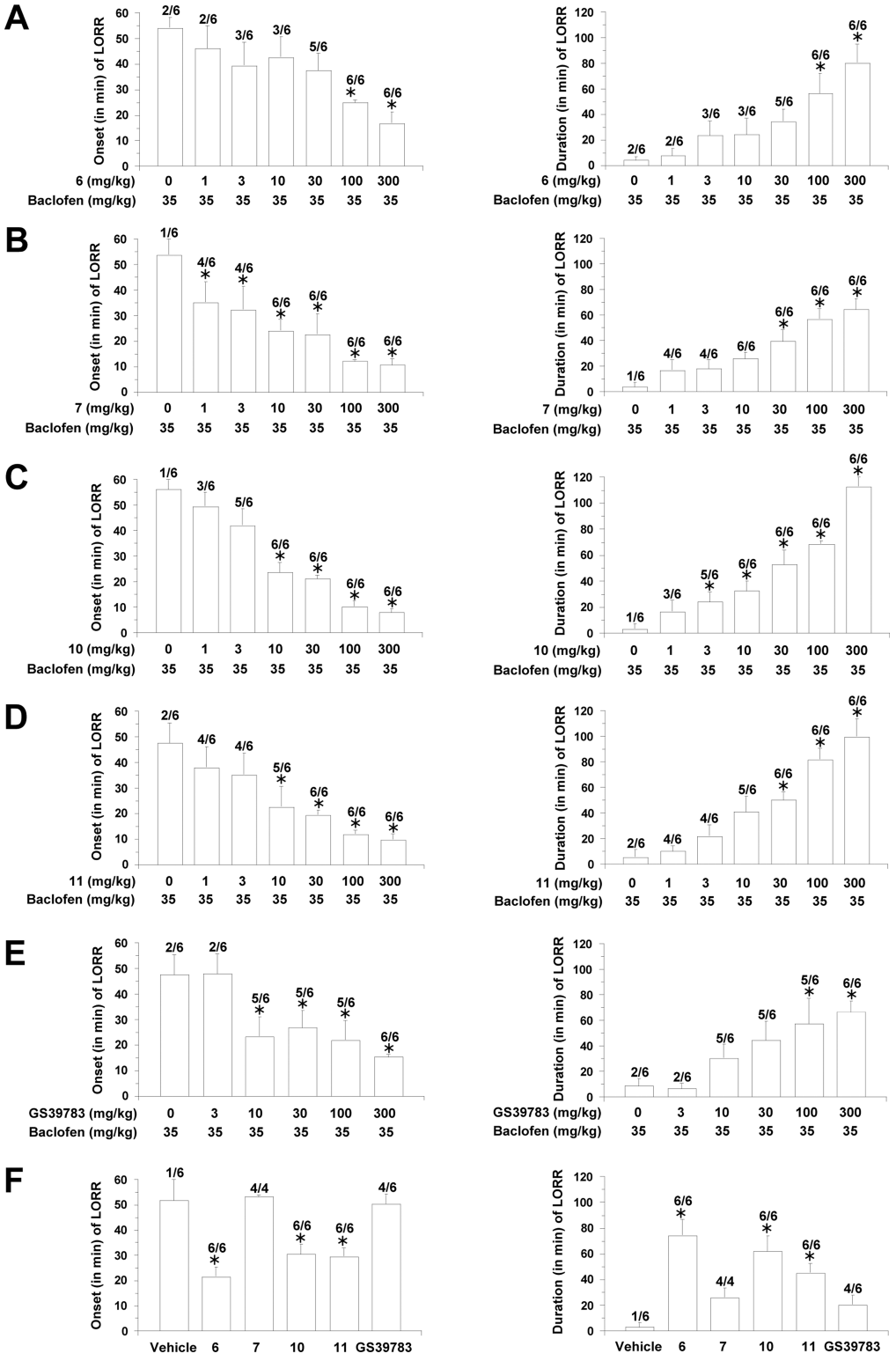


Figure 2. Effect of **6**, **7**, **10**, **11**, and GS39783 on different parameters [number of mice losing the righting reflex; onset and duration of loss of righting reflex (LORR)] of the sedative/hypnotic effect of baclofen in DBA mice. Panels A–E depict the effect of the intraperitoneal administration of different doses of test compounds and GS39783 on onset (left panels) and duration (right panels) of LORR. Panel F depicts the effect of the intragastric administration of the fixed dose of 300 mg/kg test compounds and GS39783 on onset (left panels) and duration (right panels) of LORR. Test compounds and GS39783 were administered 30 min before administration of 35 mg/kg baclofen. Each bar is the mean ± SEM of $n = 4–6$ mice. Mice that did not lose the righting reflex were assigned the values of 6 -min onset and 0 min duration, respectively: (*) $P < 0.05$ in comparison to vehicle-treated mouse group (Newman–Keuls test). Numbers on top of each bar represent the number of mice that lost the righting reflex over the total number of mice tested.

dx.doi.org/10.1021/jm400144w | J. Med. Chem. 2013, 56, 3620–3635

indicated that all compounds studied featured high metabolic stability, similar to that observed for well established clinical drugs such as felbamate, lorazepam, and propranolol.[46] Furthermore, irrespective of the compound tested, HPLC–MS analysis performed on samples obtained by incubating the compounds in mouse plasma preparations failed to show the presence of additional peaks, with the calculated amount of the parent being equal to initial concentrations. These data indicated that plasma esterases do not promote hydrolytic reactions on the studied compounds within 30 min of incubation time.

In Vivo Pharmacology. Baclofen-Induced Sedation/Hypnosis. Compounds **6**, **7**, **10**, and **11**, four significantly active $GABA_B$ enhancers in vitro, were selected for in vivo pharmacological evaluation. Intraperitoneal administration of all four new compounds, as well as GS39783 (included in the experimental design as reference compound), resulted in a dose-dependent potentiation of the sedative/hypnotic effect produced by treatment with a subthreshold dose of 35 mg/kg baclofen. Specifically:

*Experiment with Compound **6**.* Analysis of quantal data indicated that the number of mice that lost their righting reflex was 2/6, 2/6, 3/6, 3/6, 5/6, 6/6, and 6/6 in mouse groups pretreated with 0, 1, 3, 10, 30, 100, and 300 mg/kg **6**, respectively (X^2 = 13.69, df = 6, P < 0.05). Onset of loss of righting reflex (LORR) was dose-dependently reduced by pretreatment with **6** [$F_{(6,35)}$ = 3.60, P < 0.01]; post hoc analysis indicated that onset of LORR in 100 and 300 mg/kg **6**-treated mouse groups was significantly lower than in vehicle-treated mouse group (Figure 2, panel A, left). Duration of LORR was dose-dependently increased by pretreatment with **6** [$F_{(6,35)}$ = 5.91, P < 0.0005]; post hoc analysis indicated that duration of LORR in 100 and 300 mg/kg **6**-treated mouse groups was significantly longer than in vehicle-treated mouse group (Figure 2, panel A, right).

*Experiment with Compound **7**.* Analysis of quantal data indicated that the number of mice that lost their righting reflex was 1/6, 4/6, 4/6, 6/6, 6/6, 6/6, and 6/6 in mouse groups pretreated with 0, 1, 3, 10, 30, 100, and 300 mg/kg **7**, respectively (X^2 = 25.62, df = 6, P < 0.0005). Onset of LORR was dose-dependently reduced by pretreatment with **7** [$F_{(6,35)}$ = 5.81, P < 0.0005]; post hoc analysis indicated that onset of LORR in mouse groups treated with doses of **7** equal to or higher than 1 mg/kg was significantly lower than in vehicle-treated mouse group (Figure 2, panel B, left). Duration of LORR was dose-dependently increased by pretreatment with **7** [$F_{(6,35)}$ = 9.64, P < 0.0001]; post hoc analysis indicated that duration of LORR in mouse groups treated with doses of **7** equal to or higher than 30 mg/kg was significantly longer than in vehicle-treated mouse group (Figure 2, panel B, right).

*Experiment with Compound **10**.* Analysis of quantal data indicated that the number of mice that lost their righting reflex was 1/6, 3/6, 5/6, 6/6, 6/6, 6/6, and 6/6 in mouse groups pretreated with 0, 1, 3, 10, 30, 100, and 300 mg/kg **10**, respectively (X^2 = 23.19, df = 6, P < 0.001). Onset of LORR was dose-dependently reduced by pretreatment with **10** [$F_{(6,35)}$ = 25.58, P < 0.0001]; post hoc analysis indicated that onset of LORR in mouse groups treated with doses of **10** equal to or higher than 10 mg/kg was significantly lower than in vehicle-treated mouse group (Figure 2, panel C, left). Duration of LORR was dose-dependently increased by pretreatment with **10** [$F_{(6,35)}$ = 23.99, P < 0.0001]; post hoc analysis indicated that duration of LORR in mouse groups treated with doses of **10** equal to or higher than 3 mg/kg was significantly longer than in vehicle-treated mouse group (Figure 2, panel C, right).

*Experiment with Compound **11**.* Analysis of quantal data indicated that the number of mice that lost their righting reflex was 2/6, 4/6, 4/6, 5/6, 6/6, 6/6, and 6/6 in mouse groups pretreated with 0, 1, 3, 10, 30, 100, and 300 mg/kg **11**, respectively (X^2 = 13.29, df = 6, P < 0.05). Onset of LORR was dose-dependently reduced by pretreatment with **11** [$F_{(6,35)}$ = 5.28, P < 0.001]; post hoc analysis indicated that onset of LORR in mouse groups treated with doses of **11** equal to or higher than 10 mg/kg was significantly lower than in vehicle-treated mouse group (Figure 2, panel D, left). Duration of LORR was dose-dependently increased by pretreatment with **11** [$F_{(6,35)}$ = 16.59, P < 0.0001]; post hoc analysis indicated that duration of LORR in mouse groups treated with doses of **11** equal to or higher than 10 mg/kg was significantly longer than in vehicle-treated mouse group (Figure 2, panel D, right).

Experiment with GS39783. Analysis of quantal data indicated that the number of mice that lost their righting reflex was 2/6, 2/6, 5/6, 5/6, 5/6, and 6/6 in mouse groups pretreated with 0, 3, 10, 30, 100, and 300 mg/kg GS39783, respectively (X^2 = 11.65, df = 5, P < 0.05). Onset of LORR was dose-dependently reduced by pretreatment with GS39783 [$F_{(5,30)}$ = 4.03, P < 0.01]; post hoc analysis indicated that onset of LORR in mouse groups treated with doses of GS39783 equal to or higher than 10 mg/kg was significantly lower than in vehicle-treated mouse group (Figure 2, panel E, left). Duration of LORR was dose-dependently increased by pretreatment with GS39783 [$F_{(5,30)}$ = 4.37, P < 0.005]; post hoc analysis indicated that duration of LORR in 100 and 300 mg/kg GS39783-treated mouse groups was significantly longer than in vehicle-treated mouse group (Figure 2, panel E, right).

Experiment with Intragastric Administration. Intragastric administration of 300 mg/kg **6**, **7**, **10**, and **11**, together with 35 mg/kg baclofen, resulted in the occurrence of LORR in all treated mice (6/6 in **6**-, **10**-, and **11**-treated mouse groups; 4/4 in **7**-treated mouse group), while pretreatment with vehicle produced LORR only in 1/6 mouse (Figure 2, panels F). Pretreatment with 300 mg/kg reference compound GS39783 produced LORR in 4/6 mice (Figure 2, panels F). Onset of LORR was differentially altered by pretreatment with vehicle, **6**, **7**, **10**, **11**, and GS39783 [$F_{(5,28)}$ = 8.29, P < 0.0001]; post hoc analysis indicated that onset of LORR in mouse groups treated with **6**, **10**, and **11** was significantly lower than in vehicle-treated mouse group (Figure 2, panel F, left). Duration of LORR was differentially altered by pretreatment with vehicle, **6**, **7**, **10**, **11**, and GS39783 [$F_{(5,28)}$ = 9.33, P < 0.0001]; post hoc analysis indicated that duration of LORR in mouse groups treated with **6**, **10**, and **11** was significantly longer than in vehicle-treated mouse group (Figure 2, panel F, right).

Pentobarbital-Induced Sedation/Hypnosis. These experiments were conducted to assess the specificity of the potentiating effect of compounds **6**, **10**, and **11** on baclofen-induced sedation/hypnosis. To this end, the effect of their pretreatment on the sedative/hypnotic effect of the barbiturate pentobarbital was assessed. In all experiments, pentobarbital was administered at the subthreshold dose of 20 mg/kg. Specifically:

*Experiment with Compound **6**.* Analysis of quantal data indicated a tendency toward a dose-dependent increase in the number of mice that lost their righting reflex [0/6, 1/6, 2/6, and 4/6 in mouse groups pretreated with 0, 30, 100, and 300 mg/kg **6**, respectively (X^2 = 7.06, df = 3, P > 0.05)]. ANOVA

revealed a significant effect of pretreatment with **6** on onset of LORR [$F_{(3,20)}$ = 3.20, P < 0.05]; post hoc analysis indicated that onset of LORR in the 300 mg/kg **6**-treated mouse group was significantly lower than in vehicle-treated mouse group (Table 5). Conversely, duration of LORR was not significantly altered by pretreatment with **6** [$F_{(3,20)}$ = 1.17, P > 0.05] (Table 5).

Table 5. Effect of Compounds 6, 10, 11, and GS39783 on Different Parameters [Number of Mice Losing the Righting Reflex and Onset and Duration of Loss of Righting Reflex (LORR)] of the Sedative/Hypnotic Effect of Pentobarbital in DBA Mice

compd[a]	dose (mg/kg)	no. of rats losing the righting reflex	onset of LORR (min)[b]	duration of LORR (min)[b]
6	0	0/6	60.0 ± 0.0	0.0 ± 0.0
	30	1/6	53.3 ± 6.7	4.7 ± 4.7
	100	2/6	43.8 ± 11.2	11.2 ± 7.9
	300	4/6	26.7 ± 10.6*	14.0 ± 6.7
10	0	0/6	60.0 ± 0.0	0.0 ± 0.0
	30	1/6	54.0 ± 6.0	2.3 ± 2.3
	100	2/6	45.2 ± 9.4	7.0 ± 5.4
	300	5/6	31.3 ± 6.5*	23.3 ± 6.9*
11	0	0/6	60.0 ± 0.0	0.0 ± 0.0
	30	0/6	60.0 ± 0.0	0.0 ± 0.0
	100	1/6	51.8 ± 8.9	4.8 ± 5.3
	300	2/6	45.8 ± 9.1	7.3 ± 5.0
GS39783	0	0/6	60.0 ± 0.0	0.0 ± 0.0
	30	0/6	60.0 ± 0.0	0.0 ± 0.0
	100	0/6	60.0 ± 0.0	0.0 ± 0.0
	300	1/6	57.3 ± 2.7	2.3 ± 2.3

[a]Test compounds were administered 30 min before administration of 20 mg/kg pentobarbital. [b]Values in the "onset" and "duration" columns are the mean ± SEM of n = 6 mice. Mice that did not lose the righting reflex were assigned the values of 60 min onset and 0 min duration. *P < 0.05 in comparison to vehicle-treated mouse group (Newman–Keuls test).

Experiment with Compound 10. Analysis of quantal data indicated a significant, dose-dependent increase in the number of mice that lost their righting reflex [0/6, 1/6, 2/6, and 5/6 in mouse groups pretreated with 0, 30, 100, and 300 mg/kg **10**, respectively (X^2 = 10.50, df = 3, P < 0.05)]. ANOVA revealed a significant effect of pretreatment with **10** on onset of LORR [$F_{(3,20)}$ = 3.71, P < 0.05]; post hoc analysis indicated that onset of LORR in the 300 mg/kg **10**-treated mouse group was significantly lower than in vehicle-treated mouse group (Table 5). ANOVA revealed a significant effect of pretreatment with **10** on duration of LORR [$F_{(3,20)}$ = 5.34, P < 0.01]; post hoc analysis indicated that duration of LORR in the 300 mg/kg **10**-treated mouse group was significantly longer than in vehicle-treated mouse group (Table 5).

Experiment with Compound 11. Analysis of quantal data [0/6, 0/6, 1/6, and 2/6 in mouse groups pretreated with 0, 30, 100, and 300 mg/kg **11**, respectively (X^2 = 4.19, df = 3, P > 0.05)], onset of LORR [$F_{(3,20)}$ = 1.28, P > 0.05], and duration of LORR [$F_{(3,20)}$ = 1.01, P > 0.05] revealed no effect of pretreatment with **11** (Table 5).

Experiment with GS39783. Analysis of quantal data [0/6, 0/6, 0/6, and 1/6 in mouse groups pretreated with 0, 30, 100, and 300 mg/kg GS39783, respectively (X^2 = 3.13, df = 3, P > 0.05)], onset of LORR [$F_{(3,20)}$ = 1.00, P > 0.05], and duration of LORR [$F_{(3,20)}$ = 1.00, P > 0.05] revealed no effect of pretreatment with GS39783 (Table 5).

DISCUSSION

In Vitro Activity and SAR. Out of 35 new compounds reported in Table 1, 11 (namely, **4**, **5**, **6**, **7**, **10**, **11**, **12**, **16**, **20**, **22**, **25**) given at 25 μM increased GABA activity in vitro by >5% compared to controls, thus being considered "active" as $GABA_B$ PAMs (Table 2, third column). Among these, compounds **7**, **10**, and **11** were also found to be active at 2.5 μM. Structural modifications of the prototypical compounds COR627 and COR628, undertaken with the aim of improving their in vitro and in vivo pharmacological profile, involved (a) substituents at positions 4 and 5 of the thiophene ring, (b) ester moiety at position 3, and (c) amide group at position 2.

(a) 4-Ethyl and 5-methyl groups were found to be the best substituents at positions 4 and 5 of the thiophene ring. Removal of the latter resulted in completely inactive derivatives (compare **6** and **11** with **18** and **19**, respectively), while their replacement with a tetramethylene chain provided the corresponding tetrahydrobenzo[*b*]thiophene derivatives **20**–**24** showing reduced or no activity. In particular, although **20** exhibited moderate activity, comparison with COR628 demonstrates that the conversion of 4-ethyl-5-methylthiophene moiety into a tetrahydrobenzo[*b*]thiophene ring was not capable of enhancing in vitro activity.

(b) Replacement of the methyl ester group at position 3 of compounds **6** and **11** with bulkier/more lipophilic alkyl esters led to derivatives **32**–**35** that were devoid of activity. Replacement of the ester moiety with a nitrile (**23** and **24**) or carboxylic group (**41** and **42**) proved to be equally unfavorable.

(c) Amide functionality at position 2 of the thiophene scaffold was the main target of SAR investigations, proving to be a crucial structural feature for in vitro activity. Thus, replacement of the acylamide moiety with carbamate or sulfonamide groups provided scarcely active (**16**, **25**) or inactive (**26**, **27**) compounds. Likewise, conversion of the secondary amide of the active derivative **11** into a tertiary one by N-methylation (compound **28**) completely abolished activity. These results may infer involvement of the NH of the amide group as a donor in H-bonding interaction with the target receptor. As a result, structural modifications that either prevent (N-methylation) or hinder, possibly by lowering the NH pK_a value (carbamate and sulfonamide groups), this ligand–receptor interaction can be expected to decrease the biological activity of the compounds.

The residue linked to the amide carbonyl group produced a marked modulatory effect on activity of the compounds. When the cyclohexyl group of COR628 was replaced with progressively smaller alkyl groups (**4** to **1**), as well as a with a benzyl group (**17**), a decrease of activity was observed, with the sole cyclopentyl derivative **4** retaining moderate activity. Conversely, substitution of a phenyl group for the cyclohexyl group did not result in an unfavorable outcome, with compound **5** displaying a similar activity profile as **4** at both concentrations tested. This "bioequivalence" between cycloalkyl and phenyl residues appeared to be quite promising with regard to additional SAR studies, particularly in view of the wide availability of diversely substituted arylcarboxylic acid derivatives for potential use in the structural optimization program. According to the conventional Topliss operational scheme for hit-to-lead optimization,[47] the 4-methylphenyl (**6**) and 4-

dx.doi.org/10.1021/jm400144w | J. Med. Chem. 2013, 56, 3620–3635

chlorophenyl (**11**) analogues were selected for in vitro assay at 25 μM. Both compounds were found to be equipotent and slightly more active than the corresponding phenyl derivative **5**. These findings led us to hypothesize that increased activity obtained with methyl and chloro substituents might be due to their steric, rather than electronic, effects. Accordingly, methoxy analogues were less potent (**22**) or inactive (**8**, **24**) compared to **6**, while 3-chloro (**12**) and 2-chloro (**13**) analogues were respectively 60% less active than **11** and completely inactive. All other compounds bearing electron withdrawing substituents, such as halogens (**14** and **15**) or a nitro group (**9**), located at the para position of the phenyl ring were devoid of activity in an in vitro assay. On the other hand, no correlation was found between CLogP values (data not shown) and activity of the tested compounds, thus supporting a minor role, if any, for lipophilicity in determining in vitro activity. Indeed, although equipotent compounds **4** and **5** share the same CLogP of 4.28, other compounds featuring a similar lipophilicy (e.g., **11**, **13**, **14**) did not elicit similar in vitro potency.

Among the four active compounds **6**, **7**, **10**, and **11**, the 4-*tert*-butylphenyl derivative **7** and the 4-trifluorophenyl derivative **10** emerged as the most potent $GABA_B$ PAMs, displaying significant activity even at the lowest tested concentration of 2.5 μM. Notably, both **7** and **10** are characterized by substituents (*tert*-butyl and trifluoromethyl) that possess opposite electronic properties but similar steric effects. In particular, both present three fluorine atoms or isosteric methyl groups linked to a quaternary carbon atom at the para position of the phenyl ring. This feature, which ensures the best in vitro profile, would also appear to be in line with the difference in activity observed between COR627 and COR628. In other words, the presence of an aliphatic and sterically demanding tertiary group, likely occupying a large and basically hydrophobic pocket of the $GABA_B$ receptor, is required at position 4 of the phenyl ring linked to the amide carbonyl group, in order for these new $GABA_B$ PAMs to elicit the best modulatory activity. While this assumption seems to be confirmed by the inactivity of 4-arylphenyl derivatives **29**–**31**, on the other hand it cannot account for activity displayed by the 4-chlorophenyl derivative **11** or for its difference in potency compared to the corresponding 4-bromophenyl and 4-iodophenyl compounds **14** and **15**. Briefly, although steric effects appear to be implicated in determining in vitro potency, the involvement of electronic effects cannot be definitely ruled out.

Early efforts aimed at developing SARs for GPCR allosteric modulators revealed that the phenomenon known as "flat SAR" appears considerably more widespread than with orthosteric ligands,[5,48] introducing significant challenges in the hit-to-lead stage of drug discovery process.[49] Accordingly, even slight modifications of the hit structure may result in inactive compounds or point toward very shallow and narrow SAR patterns. Flat SAR is frequently observed with PAMs,[50] and the 2-(acylamino)thiophene compounds described herein do not appear to be an exception. As a result, further studies should be undertaken to develop a consistent and comprehensive SAR model, which may act as a guide in the rational design of novel analogues and the hit-to-lead optimization process.

In Vivo Activity. Potentiation of the sedative/hypnotic effect of baclofen, or any other $GABA_B$ receptor agonist, constitutes a reliable test to assess the in vivo potential of putative $GABA_B$ PAMs. This procedure has indeed been extensively and successfully used to characterize the in vivo $GABA_B$-PAM profile of CGP7930[18,42b,51] and *rac*-BHFF.[42b,51] The results of the in vivo experiments performed in the present study demonstrate that acute pretreatment with ineffective doses of **6**, **7**, **10**, and **11** synergistically increased the sedative/hypnotic effect produced by the acute administration of a subthreshold dose of baclofen (Figure 2, panels A–D). Indeed, all three parameters of baclofen-induced sedation/hypnosis [specifically, (1) proportion of mice losing the righting reflex, (2) onset of LORR, and (3) duration of LORR] were markedly and dose-dependently altered by pretreatment with the four compounds. Together, these data support the conclusion that **6**, **7**, **10**, and **11** may also behave as $GABA_B$ PAMs in an in vivo assay.

Comparison of the effect of **6**, **7**, **10**, and **11** with that of the prototypic $GABA_B$ PAM, GS39783 (Figure 2, panel E) suggests that the four novel compounds had similar, if not superior, potency and efficacy in potentiating the sedative/hypnotic effect of baclofen.

Notably, all four compounds were still active after intragastric administration, as administration of 300 mg/kg of each compound resulted in LORR in all tested mice (Figure 2, panel F). Additionally, pretreatment with **6**, **10**, and **11**, but not **7** and GS39783, reduced the onset and increased the duration of LORR. Among the tested compounds, **6** elicited fast onset, basically comparable to that of **10** and **11**, and the longest duration of LORR, followed by compounds **10** and **11** (Figure 2, panel F). Nevertheless, considering that the intragastric experiment was performed with a single and high dose of the compounds, these results should be taken with due caution. Thus, a full in vivo pharmacokinetic profiling of these compounds should be performed in order to better define their actual value.

Finally, the results of the pentobarbital experiment (Table 5) suggest that the potentiating effect of **11**, as well as those of GS39783, was specific for baclofen, as pretreatment with these two compounds failed to alter the sedative/hypnotic effect of pentobarbital. Conversely, pretreatment with **6** and **10** potentiated pentobarbital-induced sedation/hypnosis, posing some questions on their specificity for the baclofen effect.

Taking into account the three above-mentioned parameters of baclofen-induced sedation/hypnosis, as well as the results of the experiments testing the intragastric administration and drug specificity, a tentative rank of order of potency and efficacy of the four novel compounds may include **11** as the most interesting compound, as it (a) potentiated baclofen-induced sedation/hypnosis in all mice at doses as low as 30 mg/kg, (b) produced significant changes in baclofen-induced onset and duration of LORR at doses as low as 10 mg/kg, (c) produced one of largest potentiation of baclofen-induced duration of LORR at the 300 mg/kg dose (ip), (d) was active after intragastric administration, and (e) failed to affect pentobarbital-induced sedation/hypnosis, providing evidence in favor of its specificity for the baclofen effect.

CONCLUSION

A new family of $GABA_B$ PAMs has been identified through chemical manipulation of the prototypes COR627 and COR628. In this series, several aspects of SAR have been clarified and the pharmacological activity both in vitro and in vivo has been assessed. Although less potent than GS39783, used as a reference compound, a number of 2-(acylamino)-thiophene derivatives proved to be capable of significantly potentiating $GABA_B$ activity by increasing potency rather than efficacy of the endogenous ligand GABA. The lack of intrinsic

activity and interaction with the orthosteric binding site should result in the lack of any unwanted side effects typical of $GABA_B$ receptor agonists and ago-allosteric agents.

Findings obtained in in vivo experiments demonstrated that pretreatment with ineffective doses of compounds **6**, **7**, **10**, and **11** synergistically increased the sedative/hypnotic effect of a subthreshold dose of baclofen in mice. In particular, all three parameters of baclofen-induced sedation/hypnosis were indeed markedly altered in a dose-related fashion by pretreatment with the test compounds. Notably, the potentiating effect of compound **11** was specific for baclofen because no dose altered the sedative/hypnotic effect of pentobarbital.

In conclusion, the present study has disclosed structurally novel and noncytotoxic $GABA_B$ PAMs, which may constitute additional tools for use in investigating $GABA_B$ receptor function and physiopathology. Some of these compounds proved to be active in vivo, even after intragastric administration, and displayed significant metabolic stability toward mouse liver microsomes and plasma esterases. Nevertheless, the in vivo pharmacokinetic profile of these compounds should be further evaluated in order to better understand the value of the series. Although the requirement of an optimized drug is to display greater potency, several of the new compounds described here may be viewed as potentially innovative leads in medicinal chemistry research focused on the design of effective, orally available, positive allosteric modulators of the $GABA_B$ receptor.

EXPERIMENTAL SECTION

Chemistry. General Methods. Reagents were purchased from commercial suppliers and used without further purification. Anhydrous reactions were run under a positive pressure of dry N_2. Merck silica gel 60 was used for flash chromatography (23−400 mesh) with the following eluent systems: DCM (A), DCM/petroleum ether 1:1 (B), DCM/petroleum ether 9:1 (C), DCM/petroleum ether 2:1 (D), petroleum ether/AcOEt 6:1 (E), petroleum ether/AcOEt 4:1 (F), petroleum ether/AcOEt 1:1 (G). IR spectra were recorded on a Perkin-Elmer BX FT-IR system ($CHCl_3$ solution or Nujol dispersion) or on KBr disks using a Thermo Nicolet Avatar 330 FT-IR instrument. ^1H NMR and ^{13}C NMR were recorded at 200 and 50 MHz, respectively, on a Bruker AC200F spectrometer and at 400 and 100 MHz on a Bruker Advance DPX400 or on a Varian Mercury instrument. Chemical shifts are reported in ppm and multiplicity is specified as follow: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, bs = broad signal. Mass spectrometry (MS) data were obtained using Agilent 1100 LC/MSD VL system (G1946C) with a 0.4 mL/min flow rate using a binary solvent system of 95:5 methanol/water. UV detection was monitored at 254 nm. Mass spectra were acquired either in positive or in negative mode scanning over the mass range of 105−1500, and data are reported as m/z. MALDI-TOF analysis were performed using a Waters Micromass Micro Mx spectrometer. Melting points were determined on either a Gallenkamp apparatus or a Büchi B-540 instrument and are uncorrected. Microwave irradiations were conducted using a CEM Discover synthesis unit (CEM Corp., Matthews, NC). Elemental analyses were performed on a Perkin-Elmer PE 2004 elemental analyzer, and the data for C, H, and N are within 0.4% of the theoretical values. HPLC analyses were performed using a Thermo HPLC apparatus equipped with an RP80A 250 mm × 4.6 mm column, an SCM1000 vacuum membrane degasser, a P4000 gradient pump, an AS3000 autosampler, and a UV6000LP detector. HPLC eluent systems were the following: A, water (0.03% TFA)−acetonitrile (0.03% TFA) 30:70; B, water (0.03% TFA)−acetonitrile (0.03% TFA) 15:85; C, water (0.01% HCOOH)−acetonitrile (0.03% TFA) 30:70. The purity of each compound was ≥95% in either analysis.

Aminothiophene precursors **37**−**39** were prepared according to known procedures.[44b,c]

General Procedure for the Synthesis of Methyl 2-(Acylamino)thiophene-3-carboxylates 1−5, 16, 18, and 21. Method A. The appropriate acyl chloride (1.5 mmol) was slowly added under a nitrogen atmosphere to a cooled (0 °C) solution of aminothiophene derivative **36**−**38** (1 mmol) and TEA (210 μL, 1.5 mmol) in dry DCM (10 mL). After being stirred at room temperature for 18 h, the solution was washed with 1 N HCl, saturated solution of $NaHCO_3$ and brine, then dried over anhydrous Na_2SO_4 and evaporated to dryness. The crude product was purified by recrystallization from MeOH or flash column chromatography using the reported eluent system.

Methyl 2-(Acetylamino)-4-ethyl-5-methylthiophene-3-carboxylate (1). Eluent A. Orange oil. ^1H NMR (600 MHz, $CDCl_3$): δ 11.26 (s, 1 H,), 3.89 (s, 3 H), 2.73 (q, J = 7.34 Hz, 2 H), 2.26 (s, 3 H), 2.25 (s, 3 H), 1.06 (t, J = 7.34 Hz, 3 H). MALDI-TOF: 241.624 $[M]^+$.

Methyl 2-[(Cyclopropylcarbonyl)amino]-4-ethyl-5-methylthiophene-3-carboxylate (2). Eluent B. Mp 69 °C. ^1H NMR (200 MHz, $CDCl_3$): δ 11.44 (s, 1H), 3.85 (s, 3H), 2.68 (q, J = 7.4 Hz, 2H), 2.20 (s, 3H), 1.66−1.56 (m, 1H), 1.13−1.01 (m, 5H), 0.92−0.83 (m, 2H). MS (ESI): 290 $[M + Na]^+$.

Methyl 2-[(*tert*-Butylcarbonyl)amino]-4-ethyl-5-methylthiophene-3-carboxylate (3). Eluent B. Mp 83 °C. ^1H NMR (200 MHz, $CDCl_3$): δ 11.62 (s, 1H), 2.68 (q, J = 7.6 Hz, 2H), 3.85 (s, 3H), 2.21 (s, 3H), 1.28 (s, 9H), 1.01 (t, J = 7.6 Hz, 3H). MS (ESI): 284 $[M + H]^+$, 306 $[M + Na]^+$.

Methyl 2-[(Cyclopentylcarbonyl)amino]-4-ethyl-5-methylthiophene-3-carboxylate (4). Eluent B. Yellow oil. ^1H NMR (200 MHz, $CDCl_3$): δ 11.30 (s, 1H), 3.83 (s, 3H), 2.82−2.52 (m, 3H), 2.20 (s, 3H), 1.97−1.54 (m, 8H), 1.00 (t, J = 7.0 Hz, 3H). MS (ESI): 296 $[M + H]^+$, 318 $[M + Na]^+$.

Methyl 2-(Benzoylamino)-4-ethyl-5-methylthiophene-3-carboxylate (5). Mp 110 °C. ^1H NMR (200 MHz, $CDCl_3$): δ 12.33 (s, 1H), 8.01−7.97 (m, 2H), 7.60−7.45, (m, 3H), 3.92 (s, 3H), 2.29 (s, 3H), 2.74 (q, J = 7.5 Hz, 2H), 1.07 (t, J = 7.5 Hz, 3H). MS (ESI): 304 $[M + H]^+$, 326 $[M + Na]^+$.

Methyl 4-Ethyl-5-methyl-2-[(phenoxycarbonyl)amino]-thiophene-3-carboxylate (16). Eluent E. Mp 78−82 °C. ^1H NMR (200 MHz, $CDCl_3$): δ 10.82 (s, 1H), 7.42−7.34 (m, 2H), 7.26−7.17 (m, 3H), 3.82 (s, 3H), 2.71 (q, J = 7.2 Hz, 2H), 2.25 (s, 3H), 1.06 (t, J = 7.2 Hz, 3H). MS (ESI): 320 $[M + H]^+$, 342 $[M + Na]^+$.

Methyl 2-(Benzoylamino)thiophene-3-carboxylate (18). Eluent D. Mp 115 °C. ^1H NMR (200 MHz, $CDCl_3$): δ 11.97 (s, 1H), 8.02−7.98 (m, 2H), 7.53−7.50 (m, 3H), 7.21 (d, J = 5.4 Hz, 1H), 6.75 (d, J = 5.4 Hz, 1H), 3.90 (s, 3H). MS (ESI): 284 $[M + Na]^+$.

Methyl 2-(Benzoylamino)-4,5,6,7-tetrahydrobenzo[*b*]-thiophene-3-carboxylate (21). Eluent D. Mp 117−119 °C. ^1H NMR (400 MHz, $CDCl_3$): δ 12.31 (s, 1 H), 8.03 (m, 2 H), 7.58 (m, 1 H), 7.54 (m, 2 H), 3.92 (s, 3 H), 2.80 (m, 2 H), 2.70 (m, 2 H), 1.82 (m, 4 H). MALDI-TOF: 315.457 $[M]^+$, 338.276 $[M + Na]^+$, 354.159 $[M + K]^+$.

General Procedure for the Synthesis of Methyl 2-(Acylamino)thiophene-3-carboxylates 6−8, 10−15, 17, and 19. Method B. A solution of the appropriate acyl chloride (1.3 mmol) in dry dioxane (3 mL) was added dropwise to a solution of **36** or **37** (1.0 mmol) in 10 mL of the same solvent, maintained at 70 °C. After the addition was complete, the mixture was refluxed until the formation of hydrogen chloride stopped (1−18 h). The solution was concentrated in vacuo, and the residue was dissolved in DCM, washed with $NaHCO_3$ solution, dried over anhydrous Na_2SO_4, and evaporated to dryness. The crude product was purified by recrystallization from MeOH or flash column chromatography using the reported eluent system.

Methyl 4-Ethyl-5-methyl-2-[((4-methylphenyl)carbonyl)-amino]thiophene-3-carboxylate (6). Mp 116 °C. ^1H NMR (400 MHz, $CDCl_3$): δ 12.27 (s, 1H), 7.87 (d, J = 8.0 Hz, 2H), 7.27 (d, J = 8.0 Hz, 2H), 3.90 (s, 3H), 2.74 (q, J = 7.4 Hz, 2H), 2.39 (s, 3H), 2.27 (s, 3H), 1.06 (t, J = 7.4 Hz, 3H). MS (ESI): 340 $[M + Na]^+$.

dx.doi.org/10.1021/jm400144w | J. Med. Chem. 2013, 56, 3620−3635

Methyl 2-[((4-*tert*-Butylphenyl)carbonyl)amino]-4-ethyl-5-methylthiophene-3-carboxylate (7). Mp 102−104 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.93 (d, J = 8.4 Hz, 2H), 7.51 (d, J = 8.4 Hz, 2H), 3.91 (s, 3H), 2.74 (q, J = 7.4 Hz, 2H), 2.28 (s, 3H), 1.33 (s, 9H), 1.07 (t, J = 7.4 Hz, 3H). MS (ESI): 382 $[M + Na]^+$.

Methyl 4-Ethyl-2-[((4-methoxyphenyl)carbonyl)amino]-5-methyl-thiophene-3-carboxylate (8). Eluent A. Mp 150−153 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 12.22 (s, 1H), 7.93 (d, J = 8.7 Hz, 2H), 6.95 (d, J = 8.7 Hz, 2H), 3.89 (s, 3H), 3.82 (s, 3H), 2.71 (q, J = 7.4 Hz, 2H), 2.26 (s, 3H), 1.05 (t, J = 7.4 Hz, 3H). MS (ESI): 338 $[M + H]^+$, 360 $[M + Na]^+$.

Methyl 4-Ethyl-5-methyl-2-[((4-trifluoromethylphenyl)-carbonyl)amino]thiophene-3-carboxylate (10). Eluent D. Mp 122−123 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.48 (s, 1 H), 8.12 (d, J = 8.2 Hz, 2 H), 7.79 (d, J = 8.2 Hz, 2 H), 3.95 (s, 3 H), 2.77 (q, J = 7.4 Hz, 2 H), 2.32 (s, 3 H), 1.10 (t, J = 7.4 Hz, 3 H). MALDI-TOF: 370.913 $[M]^+$, 371.914 $[M + H]^+$.

Methyl 2-[((4-Chlorophenyl)carbonyl)amino]-4-ethyl-5-methylthiophene-3-carboxylate (11). Mp 113 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.38 (s, 1H), 7.97 (d, J = 8.5 Hz, 2H), 7.51 (d, J = 8.5 Hz, 2H), 3.96 (s, 3H), 2.79 (q, J = 7.4 Hz, 2H), 2.34 (s, 3H), 1.11 (t, J = 7.4 Hz, 3H). MS (ESI): 360 $[M + Na]^+$.

Methyl 2-[((3-Chlorophenyl)carbonyl)amino]-4-ethyl-5-methylthiophene-3-carboxylate (12). Eluent B. Mp 99−102 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 12.31 (s, 1H), 7.97 (s, 1H), 7.83−7.79 (m, 1H), 7.49−7.41 (m, 2H), 3.91 (s, 3H), 2.71 (q, J = 7.2 Hz, 2H), 2.27 (s, 3H), 1.05 (t, J = 7.2 Hz, 3H). MS (ESI): 338 $[M + H]^+$, 360 $[M + Na]^+$.

Methyl 2-[((2-Chlorophenyl)carbonyl)amino]-4-ethyl-5-methylthiophene-3-carboxylate (13). Eluent B. Mp 95−97 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 11.90 (s, 1H), 7.77−7.72 (m, 1H), 7.47−7.31 (m, 3H), 3.85 (s, 3H), 2.73 (q, J = 7.4 Hz, 2H), 2.28 (s, 3H), 1.05 (t, J = 7.4 Hz, 3H). MS (ESI): 338 $[M + H]^+$, 360 $[M + Na]^+$.

Methyl 2-[((4-Bromophenyl)carbonyl)amino]-4-ethyl-5-methylthiophene-3-carboxylate (14). Eluent B. Mp 128−130 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.30 (s, 1H), 7.82 (d, J = 8.1 Hz, 2H), 7.61 (d, J = 8.1 Hz, 2H), 3.91 (s, 3H), 2.72 (q, J = 7.5 Hz, 2H), 2.27 (s, 3H), 1.06 (t, J = 7.5 Hz, 3H). MS (ESI): 383 $[M + H]^+$.

Methyl 4-Ethyl-2-[((4-iodophenyl)carbonyl)amino]-5-methylthiophene-3-carboxylate (15). Eluent B. Mp 164−165 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.85 (d, J = 8.4 Hz, 2H), 7.69 (d, J = 8.4 Hz, 2H), 3.91 (s, 3H), 2.74 (q, J = 7.4 Hz, 2H), 2.29 (s, 3H), 1.06 (t, J = 7.4 Hz, 3H). MS (ESI): 452 $[M + Na]^+$.

Methyl 2-[(Phenylacetyl)amino]-4-ethyl-5-methylthiophene-3-carboxylate (17). Mp 80 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 10.96 (s, 1H), 7.33 (m, 5H), 3.76 (s, 2H), 3.67 (s, 3H), 2.66 (q, J = 7.5 Hz, 2H), 2.21 (s, 3H), 0.99 (t, J = 7.5 Hz, 3H). MS (ESI): 340 $[M + Na]^+$.

Methyl 2-[((4-Chlorophenyl)carbonyl)amino]thiophene-3-carboxylate (19). Mp 189−191 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.00 (s, 1H), 7.97 (d, J = 8.5 Hz, 2H), 7.51 (d, J = 8.5 Hz, 2H), 7.25 (d, J = 5.8 Hz, 1H), 6.80 (d, J = 5.8 Hz, 1H), 3.94 (s, 3H). MS (ESI): 318 $[M + Na]^+$, 296 $[M + H]^+$.

General Procedure for the Synthesis of Methyl 2-(Acylamino)thiophene-3-carboxylates 9, 20, 22 and 2-(Acylamino)thiophene-3-carbonitriles 23, 24. Method C. *N*,*N*′-Diisopropylcarbodiimide (DIC, 190 mg, 1.5 mmol) was added under inert atmosphere to a 0 °C solution of the appropriate carboxylic acid (1.5 mmol) in dry DCM (15 mL). After 0.5 h at 0 °C a DCM solution (15 mL) of the appropriate aminothiophene **37**−**39** (1.0 mmol) and 4-dimethylaminopyridine (DMAP, 122 mg, 1.0 mmol) were added and the reaction mixture was stirred at room temperature overnight. The organic solution was extracted with 1 N HCl, dried over Na_2SO_4, filtered and the solvent removed under vacuum. The crude product was purified by flash column chromatography using the reported eluent system.

Methyl 4-Ethyl-5-methyl-2-[((4-nitrophenyl)carbonyl)-amino]thiophene-3-carboxylate (9). Eluent C. Mp 152−154 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.58 (s, 1 H), 8.38 (d, J = 8,6 Hz, 2 H), 8.18 (d, J = 8,6 Hz, 2 H), 3.96 (s, 3 H), 2.78 (q, J = 7.4 Hz, 2 H), 2.33 (s, 3 H), 1.10 (t, J = 7.4 Hz, 3 H). MALDI-TOF: 349.865 $[M + H]^+$.

Methyl 2-[(Cyclohexylcarbonyl)amino]-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate (20). Eluent C. Colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.34 (s, 1 H), 3.85 (s, 3 H), 2.73 (m, 2 H), 2.62 (m, 2 H), 2.37 (tt, J_1 = 11.6, J_2 = 3.5 Hz, 1 H), 2.01 (m, 2 H), 1.85−1.68 (m, 6 H), 1.54 (dd, J_1 = 12.1, J_2 = 2.9 Hz, 1 H), 1.48 (dd, J_1 = 12.1, J_2 = 2.9 Hz, 1 H), 1.38−1.22 (m, 4 H). MALDI-TOF: 321.816 $[M + H]^+$.

Methyl 2-[((3,4-Dimethoxyphenyl)carbonyl)amino]-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate (22). Eluent G. Mp 164−165 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.25 (s, 1 H), 7.63 (d, J = 2.1 Hz, 1 H), 7.57 (dd, J = 8.2, 2.1 Hz, 1 H), 6.95 (d, J = 8.2 Hz, 1 H), 3.97 (s, 3 H), 3.95 (s, 3 H), 3.90 (s, 3 H), 2.78 (m, 2 H), 2.68 (m, 2 H), 1.81 (m, 4 H). MALDI-TOF: 375.863 $[M + H]^+$.

2-[(Cyclohexylcarbonyl)amino]-4,5,6,7-tetrahydrobenzo[*b*]-thiophene-3-carbonitrile (23). Eluent C. Mp 186−188 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.33 (s, 1 H), 2.65−2.58 (m, 4 H), 2.37 (tt, J = 11.7, 3.5 Hz, 1 H), 1.99−1.95 (m, 2 H), 1.88−1.79 (m, 6 H), 1.74−1.71 (m, 1 H), 1.59−1.50 (m, 2 H), 1.39−1.24 (m, 3 H). MALDI-TOF: 288.951 $[M]^+$.

2-[((3,4-Dimethoxyphenyl)carbonyl)amino]-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carbonitrile (24). Eluent G. Mp 230−232 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.80 (s, 1 H), 7.53 (d, J = 2.1 Hz, 1 H), 7.44 (dd, J = 8.3, 2.1 Hz, 1 H), 6.94 (d, J = 8.3 Hz, 1 H), 3.97 (s, 3 H), 3.96 (s, 3 H), 2.68−2.61 (m, 4 H), 1.87−1.83 (m, 2 H). MALDI-TOF: 342.936 $[M]^+$.

General Procedure for the Synthesis of Methyl 2-(Sulfonylamino)thiophene-3-carboxylates 26 and 27. Method D. The appropriate sulfonyl chloride (1.0 mmol equiv) was slowly added to a 0 °C solution of **37** (199 mg, 1 mmol) in pyridine (10 mL). The reaction mixture was stirred at room temperature under inert atmosphere for 6 h. After addition of EtOAc the solution was washed with 1 N HCl and brine. The organic phase was dried over $MgSO_4$ and evaporated under high vacuum. The residue was purified by flash chromatography using the reported eluent system.

Methyl 4-Ethyl-5-methyl-2-[((4-methylphenyl)sulfonyl)-amino]thiophene-3-carboxylate (26). Eluent F. Mp 92−94 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.32 (s, 1 H), 7.78 (d, J = 8.2 Hz, 2 H), 7.26 (d, J = 8.2 Hz, 2 H), 3.79 (s, 3 H), 2.62 (q, J = 7.4 Hz, 2 H), 2.40 (s, 3 H), 2.23 (s, 3 H) 0.99 (t, J = 7.4 Hz, 3 H). MALDI-TOF: 353.791 $[M]^+$.

Methyl 2-[(Cyclohexylsulfonyl)amino]-4-ethyl-5-methylthiophene-3-carboxylate (27). Eluent C. Mp 81−83 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.18 (s, 1 H), 3.88 (s, 3 H), 3.08 (tt, J = 12.1, 3.5 Hz, 1 H), 2.69 (q, J = 7.4 Hz, 2 H), 2.24 (s, 3 H), 2.13 (bs, 2 H), 1.87 (bs, 2 H), 1.69−1.51 (bs, 4 H), 1.26−1.18 (bs, 2 H), 1.06 (t, J = 7.4 Hz, 3 H). MALDI-TOF: 344.982 $[M]^+$, 345.981 $[M + H]^+$.

Synthesis of Methyl 4-Ethyl-5-methyl-2-[(phenylsulfonyl)-amino]thiophene-3-carboxylate (25). Method E. To a solution of **37** (199 mg, 1 mmol) and TEA (210 μL, 1.5 mmol) in DCM (20 mL) cooled at 0 °C, a solution of benzensulfonyl chloride (264 mg, 1.5 mmol) in 10 mL of DCM was added dropwise. The reaction mixture was stirred at room temperature under N_2 atmosphere for 36 h. The organic layer was washed with 1 N HCl and saturated solution of $NaHCO_3$, dried over anhydrous Na_2SO_4, and evaporated to dryness. The crude product was purified by flash chromatography using eluent D to obtained the intermediate **40** (68%).

This compound (290 mg, 0.6 mmol) was dissolved in THF/H_2O (4:1, 3 mL). Sodium hydroxide (400 mg, 10 mmol) was added, and the solution was warmed at 50 °C under stirring for 7 h. The solution was concentrated in vacuo, and the residue was dissolved in water, cooled to 0 °C, and acidified to pH 3−4 with 12 N HCl. The solid was filtered and purified by flash chromatography using eluent D to give **25** in 85% (58% overall) yield. Mp 74−76 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 10.32 (s, 1H), 7.88−7.84 (m, 2H), 7.54−7.40 (m, 3H), 3.75 (s, 3H), 2.58 (q, J = 7.5 Hz, 2H), 2.19 (s, 3H), 0.95 (t, J = 7.5 Hz, 3H). MS (ESI): 340 $[M + H]^+$, 362 $[M + Na]^+$.

Synthesis of Methyl *N*-Methyl-2-[((4-chlorophenyl)-carbonyl)amino]-4-ethyl-5-methylthiophene-3-carboxylate (28). Method F. Solid 95% potassium *tert*-butoxide (140 mg, 1.2

mmol) was added to a solution of **11** (337 mg, 1 mmol) in dry DMF (5 mL), and the mixture was stirred at room temperature under N_2 atmosphere for 1 h. Then methyl iodide (398 mg, 2.8 mmol) was added to the mixture and stirring was continued for 2 h. The mixture was poured into water and extracted with DCM. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and evaporated to dryness. The crude product was purified by flash chromatography using eluent B to furnish **28** (246 mg, 70%) as a white solid. Mp 109−111 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.34 (d, J = 8.0 Hz, 2H), 7.20 (d, J = 8.0 Hz, 2H), 3.82(s, 3H), 3.39 (s, 3H), 2.60 (q, J = 7.4 Hz, 2H), 2.28 (s, 3H), 0.98 (t, J = 7.4 Hz, 3H). MS (ESI): 352 $[M + H]^+$, 390 $[M + Na]^+$.

General Procedure for the Synthesis of Biphenyl Derivatives 29−31 by Suzuki Coupling. Method G. A 5 mL process vial was charged with the iodo derivative **15** (107 mg, 0.25 mmol), the appropriate boronic acid (1 mmol), $Pd(OAc)_2$ (5.6 mg, 0.025 mmol), PPh_3 (19.6 mg, 0.075 mmol), 2 M Na_2CO_3 (0.5 mL, 1 mmol), EtOH (0.25 mL), and DME (1 mL). The vessel was sealed under air and exposed to microwave heating for 5 min at 110 °C. The reaction mixture was thereafter cooled to room temperature, diluted with AcOEt, and filtered through a short plug of Celite. The solution was washed with brine, dried over anhydrous sodium sulfate, and evaporated. The residue was purified by flash chromatography using eluent B.

Methyl 4-Ethyl-5-methyl-2-[4-(phenyl)benzoylamino]-thiophene-3-carboxylate (29). Mp 148−151 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.39 (s, 1H), 8.09 (d, J = 8.2 Hz, 2H), 7.74 (d, J = 8.2 Hz, 2H), 7.64 (d, J = 7.5 Hz, 2H), 7.50−7.46 (m, 2H), 7.42−7.39 (m, 1H), 3.96 (s, 3H), 2.78 (q, J = 7.3 Hz, 2H), 2.32 (s, 3H), 1.11 (t, J = 7.3 Hz, 3H). MS (ESI): 380 $[M + H]^+$, 402 $[M + Na]^+$.

Methyl 4-Ethyl-5-methyl-2-[((4′-methyl)-4-phenyl)-benzoylamino]thiophene-3-carboxylate (30). Mp 144−145 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.05 (d, J = 8.3 Hz, 2H), 7.71 (d, J = 8.3 Hz, 2H), 7.52 (d, J = 8.0 Hz, 2H), 7.27 (d, J = 8.0 Hz, 2H), 3.94 (s, 3H), 2.76 (q, J = 7.4 Hz, 2H), 2.40 (s, 3H), 2.30 (s, 3H), 1.08 (t, J = 7.4 Hz, 3H). MS (ESI): 416 $[M + Na]^+$.

Methyl 4-Ethyl-5-methyl-2-[4-(pyridin-3-yl)benzoylamino)-thiophene-3-carboxylate (31). Mp 181−182 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.48 (s, 1H), 8.96 (s, 1H), 8.72−8.70 (m, 1H), 8.13−8.11 (s, 1H), 8.18 (d, J = 8.3 Hz, 2H), 7.78 (d, J = 8.3 Hz, 2H), 7.30−7.28 (m, 1H), 3.99 (s, 3H), 2.81 (q, J = 7.4 Hz, 2H), 2.35 (s, 3H), 1.13 (t, J = 7.4 Hz, 3H). MS (ESI): 403 $[M + Na]^+$.

General Procedure for the Synthesis of Alkyl 2-(Acylamino)-thiophene-3-carboxylates 32 and 33. Method H. To a cold (0 °C) solution of acid **41** (304 mg, 1 mmol) in dry DCM (50 mL), under inert atmosphere, were added DIC (150 mg, 1.2 mmol), DMAP (12 mg, 0.1 mmol), and the appropriate alcohol (2 mmol). The reaction mixture was stirred at 35 °C for 3 h. The solution was washed with H_2O. The organic layers were dried over $MgSO_4$ and filtered, and the solvent was removed under vacuum. The pure esters were obtained by means of flash chromatography using eluent A.

***n*-Propyl 4-Ethyl-5-methyl-2-[((4-methylphenyl)carbonyl)-amino]thiophene-3-carboxylate (32).** Mp 111−113 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.36 (s, 1 H), 7.92 (d, J = 8.0 Hz, 2 H), 7.30 (d, J = 8.0 Hz, 2 H), 4.32 (t, J = 6.6 Hz, 2 H), 2.80 (q, J = 7.4 Hz, 2 H), 2.44 (s, 3 H), 2.32 (s, 3 H), 1.64 (sextuplet, J = 7.4, 6.6 Hz, 2 H), 1.12 (t, J = 7.4 Hz, 3 H), 1.07 (t, J = 7.4 Hz, 3 H). MALDI-TOF: 345.073 $[M]^+$, 384.014 $[M + K]^+$.

***tert*-Butyl 4-Ethyl-5-methyl-2-[((4-methylphenyl)carbonyl)-amino]thiophene-3-carboxylate (33).** Mp 165−167 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.44 (s, 1 H), 7.92 (d, J = 8.0 Hz, 2 H), 7.33 (d, J = 8.0 Hz, 2 H), 2.77 (q, J = 7.4 Hz, 2 H), 2.44 (s, 3 H), 2.31 (s, 3 H), 1.64 (s, 9 H), 1.11 (t, J = 7.4 Hz, 3 H). MALDI-TOF: 359.976 $[M]^+$.

General Procedure for the Synthesis of Alkyl 2-(Acylamino)-thiophene-3-carboxylates 34 and 35. Method I. To a solution of **42** (324 mg, 1 mmol) in dry THF (10 mL) were added the appropriate alcohol (1.5 mmol) and PPh_3 (392 mg, 1.5 mmol). Then DEAD (260 mg, 1.5 mmol) was added dropwise at 0 °C. The mixture was stirred at room temperature for 18 h, then poured into water and taken up into DCM. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and evaporated to dryness. The crude residue was purified by flash chromatography using eluent A.

***n*-Propyl 2-[((4-Chlorophenyl)carbonyl)amino]-4-ethyl-5-methylthiophene-3-carboxylate (34).** Mp 110 °C. ^{1}H NMR (400 MHz, $CDCl_3$): 12.38 (s, 1H), 7.91 (d, J = 8.0, 2H), 7.44 (d, J = 8.0, 2H), 4.28 (t, J = 6.5, 2H), 2.76 (q, J = 7.1, 2H), 2.28 (s, 3H), 1.85−1.76 (m, 2H), 1.11−1.02 (m, 6H). MS (ESI): 366 $[M + H]^+$.

***tert*-Butyl 2-[((4-Chlorophenyl)carbonyl)amino]-4-ethyl-5-methylthiophene-3-carboxylate (35).** Mp 142−144 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.52 (s, 1H), 7.96 (d, J = 8.1 Hz, 2H), 7.51 (d, J = 8.1 Hz, 2H), 2.77 (q, J = 7.1 Hz, 2H), 1.64 (s, 9H), 1.11 (t, J = 7.1 Hz, 3H). MS (ESI): 380 $[M + H]^+$.

General Procedure for the Synthesis of Acids 41 and 42 by Ester Hydrolysis. A mixture of the appropriate ester **6** or **11** (1 mmol) and LiOH monohydrate (1.5 mmol) in THF (23 mL) and water (7 mL) was stirred at room temperature for 24 h. During this time, three more aliquots of LiOH monohydrate (1.5 mmol each) were added. The mixture was evaporated to dryness, and the residue was treated with 6 N HCl. The precipitate that formed was filtered, washed with water, and then dried under high vacuum to give the corresponding carboxylic acids.

4-Ethyl-5-methyl-2-[((4-methylphenyl)carbonyl)amino]-thiophene-3-carboxylic Acid (41). 41 was obtained in 60% yield after washing with petroleum ether. Mp 216−218 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.09 (s, 1 H), 7.90 (d, J = 8.0 Hz, 2 H), 7.32 (d, J = 8.0 Hz, 2 H), 2.86 (q, J = 7.4 Hz, 2 H), 2.45 (s, 3 H), 2.35 (s, 3 H), 1.18 (t, J = 7.4, 3 H). MALDI-TOF: 303.883 $[M]^+$; 342.847 $[M + K]^+$.

2-[((4-Chlorophenyl)carbonyl)amino]-4-ethyl-5-methylthiophene-3-carboxylic Acid (42). 42 was obtained in 85% yield after purification by flash chromatography using 0.5% HCO_2H in DCM as eluent. Mp 218−220 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.09 (s, 1H), 7.90 (d, J = 8.5 Hz, 2H), 7.47 (d, J = 8.5 Hz, 2H), 2.83 (q, J = 7.4 Hz, 2H), 2.32 (s, 3H), 1.14 (t, J = 7.4 Hz, 3H). MS (ESI): 322 $[M - H]^-$.

In Vitro Binding Studies. Tissue Preparation. Rats were killed by decapitation, their brains rapidly removed, and cerebral cortices dissected on ice. Cortical tissue was homogenized using a homogenizer system (Glass-Col, Terre Haute, IN, U.S.) in 20 volumes (v/w) of ice-cold 0.32 M sucrose containing 1 mM EDTA. The homogenate was centrifuged at 1000*g* for 10 min and the supernatant collected and recentrifuged at 20000*g* for 20 min. The pellet was resuspended in 20 volumes (v/w) of ice-cold water, homogenized using a Polytron homogenizer, and centrifuged at 8000*g* for 20 min. The supernatant together with the buffy layer on the pellet was then centrifuged at 45000*g* for 20 min. The resulting pellet was resuspended in ice-cold distilled water and once more centrifuged at 45000*g* for 30 min. The final pellet was frozen and stored at −80 °C for at least 18 h before use for [^{35}S]GTPγS binding assay for $GABA_B$. The Bradford protein assay[52] was used for protein determination using bovine serum albumin as a standard according to the supplier protocol (Bio-Rad, Milan, Italy).

[^{35}S]GTPγS Binding Assay in Rat Cortical Membranes. Rat cortex membranes were thawed at 4 °C, resuspended in 1−2 mL of ice-cold water, and homogenized using a homogenizer system (Glass-Col, Terre Haute, IN, U.S.). Membranes were incubated on ice for 1 h and then centrifuged at 4 °C for 15 min at 20000*g*. The pellet was resuspended in GTPγS buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 1.8 mM $CaCl_2$) to a final concentration of 10−15 μg of protein. Membrane homogenates and drugs were preincubated in Packard-Picoplates 96 (300 μL volume) in the presence of 30 μM GDP for 30 min at 30 °C. The main incubation was subsequently started by the addition of [^{35}S]GTPγS to a final concentration of 0.2 nM. After a 40 min incubation at 30 °C, the samples were filtered using a Packard Unifilter-GF/B, washed twice with 300 μL of buffer, and dried for 1 h at 30 °C. The radioactivity on the filters was counted in a liquid microplate scintillation counter (TopCount NXT, Packard, Meridian, ID, U.S.) using 50 μL of scintillation fluid (Microscint TM 20, Packard, Meridian, ID, U.S.). Basal binding was assessed in the absence of agonist and in the presence of GDP, and nonspecific binding was measured in the

dx.doi.org/10.1021/jm400144w | J. Med. Chem. 2013, 56, 3620−3635

presence of 10 μM unlabeled GTPγS. The stimulation by agonist was defined as a percentage increase above basal levels (i.e., {[dpm (agonist) − dpm (no agonist)]/[dpm (no agonist)]} × 100). Data are reported as the mean ± SEM of three to six experiments, performed in triplicate. Nonlinear regression analysis of concentration−response data was performed using Prism 2.0 software (GraphPad Prism Program, San Diego, CA, U.S.) to calculate E_{max} and EC_{50} values.

Cytotoxicity Assay. Materials. Dulbecco's modified Eagle medium (DMEM) and Eagle's minimum essential medium (EMEM), trypsin solution, and all the solvents used for cell culture were purchased from Lonza (Switzerland). Mouse immortalized fibroblasts NIH3T3 were purchased from American Type Culture Collection (U.S.).

Cell Cultures and Cytotoxicity Assay. NIH3T3 cells were utilized for cytotoxicity experiments. NIH3T3 cells were maintained in DMEM at 37 °C in a humidified atmosphere containing 5% CO_2. Culture medium was supplemented with 10% fetal calf serum (FCS), 1% L-glutamine−penicillin−streptomycin solution, and 1% MEM nonessential amino acid solution. On reaching confluence, cells were washed with PBS 0.1 M, taken up with trypsin−EDTA solution, and then centrifuged at 1000 rpm for 5 min. The pellet was resuspended in medium solution (dilution 1:15), and cells were seeded.

After 24 h of incubation, the test compounds solubilized in DMSO were added to the cells. Concentrations ranging from 5 to 200 μM were tested. Each concentration was tested in six replicates. Cell viability after 24 h of incubation with the different compounds was evaluated by Neutral Red Uptake (Sigma-Aldrich, Switzerland) by the procedure previously reported.[53] Briefly, the following solutions were prepared in order to determine the percentage of viable cells: (1) Neutral Red (NR) stock solution, 0.33 g NR dye powder in 100 mL of sterile H_2O; (2) NR medium, 1.0 mL of NR stock solution (99.0 routine culture medium prewarmed to 37 °C); (3) NR desorb solution, 1% glacial acetic acid solution + 50% ethanol + 49% H_2O.

At the end of the incubation the routine culture medium was removed from each well, and cells were carefully rinsed with 1 mL of prewarmed D-PBS. Multiwells were then gently blotted with paper towels. Then 1.0 mL of NR medium was added to each well and further incubated at 37 °C, 95% humidity, 5.0% CO_2 for 3 h. The cells were checked during the NR incubation for NR crystal formation. After incubation, NR medium was removed, and cells were carefully rinsed with 1 mL of prewarmed D-PBS. PBS was subsequently decanted and blotted from the wells, and exactly 1 mL of NR desorb solution was added to each sample. Multiwells were then put on a shaker for 20−45 min to extract NR from the cells and form a homogeneous solution. During this step, samples were covered in order to protect them from light. Five minutes after removal from the plate shaker absorbance was read at 540 nm by a UV/visible spectrophotometer (Lambda 25, Perkin-Elmer).

Metabolic Stability Assays. In Vitro Intrinsic Clearance (Mouse Liver Microsomes). Test compounds **6**, **7**, **10**, and **11** were incubated separately at 5 μM in 100 mM phosphate buffer (pH 7.4) and 1 mM EDTA in the presence of 0.48 mg/mL mouse liver microsomal proteins as previously reported.[54] The enzymatic reaction was initiated by addition of a NADPH regenerating system (final concentrations: 2 mM β-nicotinamide adenine dinucleotide phosphate reduced (NADPH), 10 mM glucose 6-phosphate (G6P), 0.4 U/mL glucose 6-phosphate dehydrogenase (G6PDH)). Reactions were terminated at regular time intervals (0−60 min) by adding a double volume of acetonitrile. All incubations were performed in duplicate.

Samples were analyzed by Agilent 6540 UHD Accurate-Mass Q-TOF LC/MS. The MS detection was conducted using an Agilent Accurate Mass Q-TOF high resolution mass spectrometer operated in positive electrospray mode. The experimental data obtained (mass spectra acquisition using LC/MSMS) were elaborated using Mass-MetaSite Mass-Metasite, a computer assisted method for the interpretation of LC−MSMS data that combines prediction of a compound site of metabolism (SoM) with the processing of MS spectra and rationalization based on fragment analysis.[55] The intrinsic clearance (Cl_{intr}) was calculated by the following equation

$$Cl_{intr} = k(\mathrm{min}^{-1})[V]/[P]$$

where k is the rate costant for the depletion of substrate, V is the volume of incubation in μL, and P is the amount of microsomal proteins in the incubation medium in mg according to Baranczewski et al.[56] Compounds were classified based on their Cl_{intr} value (expressed as μL·min^{-1}·mg protein^{-1}, where in mouse <7 indicates very low metabolic turnover, 8−50 indicates low clearance, 50−150 indicates moderate clearance, and >150 indicates high clearance.[46]

Stability to Plasma Esterases. The stability of test compounds toward esterases was evaluated in mice plasma according to the method previously described[57] with slight modifications. Compounds **6**, **7**, **10**, and **11** were added to plasma (5 μM final concentration) and incubated at 37 °C for 30 min. At the end of the incubation time, the reaction was stopped by addition of a double volume of acetonitrile and each sample was subjected to HPLC−MS analysis as reported above.

In Vivo Pharmacology. The experimental procedures employed in the present study were in accordance with the European Communities Council Directive (86/609/EEC) and the subsequent Italian law on the protection of animals used for experimental and other scientific reasons.

Animals. DBA mice (Charles River Laboratories, Calco, Italy) weighing 17−20 g were used. Mice were housed 6−7 per cage in standard plastic cages with wood chip bedding under an inverted 12:12 h artificial light−dark cycle (lights on at 9:30 p.m.) at a constant temperature of 22 ± 2 °C and relative humidity of approximately 60%. Tap water and standard laboratory rodent chow (Mucedola, Settimo Milanese, Italy) were provided ad libitum in the home cage.

Experimental Procedures. Each experiment [testing a single compound, administered either intraperitoneally (ip) or intragastrically (ig)] used independent sets of mice.

Baclofen-Induced Sedation/Hypnosis. In each experiment, mice were divided into six to seven groups of n = 6 each. Mice were treated acutely and ip with 0, 1, 3, 10, 30, 100, and 300 mg/kg **6**, **7**, **10**, and **11** or with 0, 3, 10, 30, 100, and 300 mg/kg GS39783 (included in the experimental design as reference compound). Thirty minutes later, mice were treated acutely and ip with a fixed dose of 35 mg/kg baclofen. All test compounds and GS39783 were suspended in saline with a few drops of Tween 80. Baclofen was dissolved in saline. All drugs were injected at a volume of 12.5 mL/kg. The dose ranges of test compounds and GS39783 were chosen on the basis of the results of a series of preliminary experiments demonstrating that they were totally devoid of any sedative/hypnotic effect in DBA mice (this laboratory, unpublished results). Baclofen dose was chosen on the basis of the results of previous experiments demonstrating that it was the highest ineffective dose (in terms of sedation/hypnosis) in DBA mice.[42a]In each experiment and according to the procedure used in previous studies,[51] after baclofen injection each mouse was placed on its back once every 60 s until it was unable to right itself within 60 s. The time between baclofen injection and the start of the 60 s interval during which the mouse was unable to right itself was measured as onset of LORR. Each mouse was then left undisturbed on its back until it spontaneously regained its righting reflex (determined as having at least three paws under its body). Complete recovery of righting reflex was defined as the animal being able to turn itself upright twice more within 60 s. If this criterion was not fulfilled, the mouse was left undisturbed until it spontaneously regained its righting reflex. The time between loss and recovery of righting reflex was monitored in each mouse and defined as duration of LORR. Observations were conducted by an operator unaware of the drug treatment.

An additional experiment, testing the effect of ig administration of the test compounds, was conducted. Mice were fasted for 2 h before drug administration. Mice were divided into six groups. Five mouse groups were treated acutely and ig with a fixed dose of 300 mg/kg **6** (n = 6), **7** (n = 4), **10** (n = 6), **11** (n = 6), and GS39783 (n = 6); the sixth mouse group (n = 6) was treated with vehicle. Thirty minutes later, mice were treated acutely and ip with a fixed dose of 35 mg/kg baclofen. All the test compounds were suspended or dissolved as

described above and injected at a volume of 12.5 mL/kg. Onset and duration of LORR were recorded as described above.

Pentobarbital-Induced Sedation/Hypnosis. In each experiment, mice were divided into four groups of $n = 6$ each and treated acutely and ip with 0, 30, 100, and 300 mg/kg **6**, **10**, **11**, and GS39783. Thirty minutes later, mice were treated acutely and ip with a fixed dose of 20 mg/kg pentobarbital. Test compounds and GS39783 were suspended or dissolved as described above. Pentobarbital was dissolved in saline. All drugs were injected at a volume of 12.5 mL/kg. Pentobarbital dose was chosen on the basis of the results of previous experiments demonstrating that it was minimally effective (in terms of sedation/hypnosis) in DBA mice.[42a] Onset and duration of LORR were recorded as described above.

Statistical Analysis. In each experiment, occurrence of LORR was evaluated by a χ^2 test. Data on onset and duration (both expressed in minutes) of LORR were evaluated by separate one-way ANOVAs, followed by the Newman–Keuls test for post hoc comparisons. Mice that did not lose their righting reflex were assigned the values of 60 min onset and 0 min duration.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional spectroscopic and analytical data of compounds **1**–**35**, **41**, and **42**. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +39 0577 234308. Fax: +39 0577 234333. E-mail: corelli@unisi.it.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was partially supported by Grant CRP-17596 to G.C., Grant CRP2_6 to D.G., and Grant CRP-25257 to M.P.C. and M.S. from Regione Autonoma della Sardegna (L.R. 7 Agosto 2007, No. 7). The authors thank Roberto Pellegrino for invaluable assistance in the HPLC–MS analyses.

■ ABBREVIATIONS USED

ANOVA, analysis of variance; DBA, dilute brown non-agouti; DCM, dichloromethane; DEAD, diethyl azodicarboxylate; DIC, *N*,*N*′-diisopropylcarbodiimide; DIPEA, diisopropylethylamine; DME, 1,2-dimethoxyethane; DMAP, 4-dimethylaminopyridine; DMEM, Dulbecco's modified Eagle medium; DMF, *N*,*N*-dimethylformamide; D-PBS, Dulbecco's phosphate buffered saline; EMEM, Eagle's minimum essential medium; HBTU, *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; GPCR, G-protein-coupled receptor; LORR, loss of righting reflex; MW, microwave; PAM, positive allosteric modulator; PBS, phosphate buffered saline; SAR, structure–activity relationship; [^{35}S]GTPγS, [^{35}S]guanosine 5′-*O*-(3-thio)triphosphate; TEA, triethylamine; THF, tetrahydrofuran

■ REFERENCES

(1) Monod, J.; Wyman, J.; Changeux, J. P. On the nature of allosteric transition. *J. Mol. Biol.* **1965**, *12*, 88–118.

(2) Rees, S.; Morrow, D.; Kenakin, T. GPCR drug discovery through the exploitation of allosteric drug binding sites. *Recept. Channel* **2002**, *8*, 261–268.

(3) Christopoulos, A.; May, L. T.; Avlani, V. A.; Sexton, P. M. G-protein-coupled receptor allosterism: the promise and the problem(s). *Biochem. Soc. Trans.* **2004**, *32*, 873–877.

(4) Mohler, H. F.; Fritschy, J. M.; Rudolph, U. A new benzodiazepine pharmacology. *J. Pharmacol. Exp. Ther.* **2002**, *300*, 2–8.

(5) Urwyler, S. Allosteric modulation of family C G-protein-coupled receptors: from molecular insights to therapeutic perspectives. *Pharmacol. Rev.* **2011**, *63*, 59–126.

(6) Melancon, B. J.; Hopkins, C. R.; Wood, M. R.; Emmitte, K. A.; Niswender, C. M.; Christopoulos, A.; Conn, P. J.; Lindsley, C. W. Allosteric modulation of seven transmembrane spanning receptors: theory, practice, and opportunities for central nervous system drug discovery. *J. Med. Chem.* **2012**, *55*, 1445–1464.

(7) Burford, N. T.; Watson, J.; Bertekap, R.; Alt, A. Strategies for the identification of allosteric modulators of G-protein-coupled receptors. *Biochem. Pharmacol.* **2011**, *81*, 691–702.

(8) Keov, P.; Sexton, P. M.; Christopoulos, A. Allosteric modulation of G protein-coupled receptors: a pharmacological perspective. *Neuropharmacology* **2011**, *60*, 24–35.

(9) De Amici, M.; Dallanoce, C.; Holzgrabe, U.; Trankle, C.; Mohr, K. Allosteric ligands for G protein-coupled receptors: a novel strategy with attractive therapeutic opportunities. *Med. Res. Rev.* **2010**, *30*, 463–549.

(10) Franco, R.; Canela, E. I.; Casado, V.; Ferre, S. Platform for identification of GPCR targets, and orthosteric and allosteric modulators. *Expert Opin. Drug Discovery* **2010**, *5*, 391–403.

(11) Wang, L.; Martin, B.; Brenneman, R.; Luttrell, L. M.; Maudsley, S. Allosteric modulators of G protein-coupled receptors: future therapeutics for complex physiological disorders. *J. Pharmacol. Exp.Ther.* **2009**, *331*, 340–348.

(12) Conn, P. J.; Christopoulos, A.; Lindsley, C. W. Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. *Nat. Rev. Drug Discovery* **2009**, *8*, 41–54.

(13) Harrington, P. E.; Fotsch, C. Calcium sensing receptor activators: calcimimetics. *Curr. Med. Chem.* **2007**, *14*, 3027–3034.

(14) Dorr, P.; Westby, M.; Dobbs, S.; Griffin, P.; Irvine, B.; Macartney, M.; Mori, J.; Rickett, G.; Smith-Burchnell, C.; Napier, C.; Webster, R.; Armour, D.; Prince, D.; Stammen, B.; Wood, A.; Perros, M. Maraviroc (UK-427, 857), a potent, orally bioavailable, and selective small-molecule inhibitor of chemokine receptor CCR5 with broad-spectrum anti-human immunodeficiency virus type 1 activity. *Antimicrob. Agents Chemother.* **2005**, *49*, 4721–4732.

(15) Urwyler, S.; Mosbacher, J.; Lingenhoehl, K.; Heid, J.; Hofstetter, K.; Froestl, W.; Bettler, B.; Kaupmann, K. Positive allosteric modulation of native and recombinant γ-aminobutyric acid$_B$ receptors by 2,6-di-*tert*-butyl-4-(3-hydroxy-2,2-dimethylpropyl)-phenol (CGP7930) and its aldehyde analog CGP13501. *Mol. Pharmacol.* **2001**, *60*, 963–971.

(16) Urwyler, S.; Pozza, M. F.; Lingenhoehl, K.; Mosbacher, J.; Lampert, C.; Froestl, W.; Koller, M.; Kaupmann, K. *N*,*N*′-Dicyclopentyl-2-methylsulfanyl-5-nitro-pyrimidine-4,6-diamine (GS39783) and structurally related compounds: novel allosteric enhancers of γ-aminobutyric acid$_B$ receptor function. *J. Pharmacol. Exp. Ther.* **2003**, *307*, 322–330.

(17) Guery, S.; Floersheim, P.; Kaupmann, K.; Froestl, W. Syntheses and optimization of new GS39783 analogues as positive allosteric modulators of GABA B receptors. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 6206–6211.

(18) Malherbe, P.; Masciadri, R.; Norcross, R. D.; Knoflach, F.; Kratzeisen, C.; Zenner, M. T.; Kolb, Y.; Marcuz, A.; Huwyler, J.; Nakagawa, T.; Porter, R. H.; Thomas, A. W.; Wettstein, J. G.; Sleight, A. J.; Spooren, W.; Prinssen, E. P. Characterization of (*R*,*S*)-5,7-di-*tert*-butyl-3-hydroxy-3-trifluoromethyl-3*H*-benzofuran-2-one as a positive allosteric modulator of GABAB receptors. *Br. J. Pharmacol.* **2008**, *154*, 797–811.

(19) Bettler, B.; Kaupmann, K.; Mosbacher, J.; Gassmann, M. Molecular structure and physiological functions of GABA(B) receptors. *Physiol. Rev.* **2004**, *84*, 835–867.

(20) Vacher, C.-M.; Bettler, B. GABA$_B$ receptors as potential therapeutic targets. *Curr. Drug Targets: CNS Neurol. Disord.* **2003**, *2*, 248–259.

(21) Ong, J.; Kerr, D. I. B. Clinical potential of GABAB receptor modulators. *CNS Drug Rev.* **2005**, *11*, 317−334.
(22) Bowery, N. G. GABAB receptor: a site of therapeutic benefit. *Curr. Opin. Pharmacol.* **2006**, *6*, 37−43.
(23) Lujan, R.; Ciruela, F. GABAB receptors-associated proteins: potential drug targets in neurological disorders? *Curr. Drug Targets* **2012**, *13*, 129−144.
(24) Froestl, W. Chemistry and pharmacology of $GABA_B$ receptor ligands. *Adv. Pharmacol.* **2010**, *58*, 19−62.
(25) (a) Froestl, W. An historical perspective on GABAergic drugs. *Future Med. Chem.* **2011**, *3*, 163−175. (b) Pin, J. P.; Prézeau, L. Allosteric modulators of GABAB receptors: mechanism of action and therapeutic perspective. *Curr. Neuropharmacol.* **2007**, *5*, 195−201.
(26) Froestl, W. Novel $GABA_B$ receptor positive modulators: a patent survey. *Expert Opin. Ther. Pat.* **2010**, *20*, 1007−1017.
(27) Adams, C. L.; Lawrence, A. J. CGP7930: a positive allosteric modulator of the $GABA_B$ receptor. *CNS Drug Rev.* **2007**, *13*, 308−316.
(28) Orrù, A.; Lai, P.; Lobina, C.; Maccioni, P.; Piras, P.; Scanu, L.; Froestl, W.; Gessa, G. L.; Carai, M. A. M.; Colombo, G. Reducing effect of the positive allosteric modulators of the $GABA_B$ receptor, CGP7930 and GS39783, on alcohol intake in alcohol-preferring rats. *Eur. J. Pharmacol.* **2005**, *525*, 105−111.
(29) (a) Liang, J. H.; Chen, F.; Krstew, E.; Cowen, M. S.; Carroll, F. Y.; Crawford, D.; Beart, P. M.; Lawrence, A. J. The $GABA_B$ receptor allosteric modulator CGP7930, like baclofen, reduces operant self-administration of ethanol in alcohol-preferring rats. *Neuropharmacology* **2006**, *50*, 632−639. (b) Maccioni, P.; Pes, D.; Orrù, A.; Froestl, W.; Gessa, G. L.; Carai, M. A. M.; Colombo, G. Reducing effect of the positive allosteric modulator of the $GABA_B$ receptor, GS39783, on alcohol self-administration in alcohol-preferring rats. *Psychopharmacology* **2007**, *193*, 171−178. (c) Maccioni, P.; Fantini, N.; Froestl, W.; Carai, M. A. M.; Gessa, G. L.; Colombo, G. Specific reduction of alcohol's motivational properties by the positive allosteric modulator of the $GABA_B$ receptor, GS39783. Comparison with the effect of the $GABA_B$ receptor direct agonist, baclofen. *Alcohol.: Clin. Exp. Res.* **2008**, *32*, 1−7. (d) Maccioni, P.; Zaru, A.; Loi, B.; Lobina, C.; Carai, M. A. M.; Gessa, G. L.; Mugnaini, C.; Pasquini, S.; Corelli, F.; Hyytiä, P.; Lumeng, L.; Colombo, G. Comparison of the effect of the positive allosteric modulator of the $GABA_B$ receptor, GS39783, on alcohol self-administration in three different lines of alcohol-preferring rats. *Alcohol.: Clin. Exp. Res.* **2012**, *36*, 1748−1766.
(30) Paterson, N. E.; Vlachou, S.; Guery, S.; Kaupmann, K.; Froestl, W.; Markou, A. Positive modulation of $GABA_B$ receptors decreased nicotine self-administration and counteracted nicotine-induced enhancement of brain reward function in rats. *J. Pharmacol. Exp. Ther.* **2008**, *326*, 306−314.
(31) Mombereau, C.; Lhuillier, L.; Kaupmann, K.; Cryan, J. F. $GABA_B$ receptor-positive modulation-induced blockade of the rewarding properties of nicotine is associated with a reduction in nucleus accumbens ΔFosB accumulation. *J. Pharmacol. Exp. Ther.* **2007**, *321*, 172−177.
(32) Lobina, C.; Carai, M. A. M.; Froestl, W.; Mugnaini, C.; Pasquini, S.; Corelli, F.; Gessa, G. L.; Colombo, G. Activation of the $GABA_B$ receptor prevents nicotine-induced locomotor stimulation in mice. *Front. Psychiatry* **2011**, *2*, 76.
(33) (a) Smith, M. A.; Yancey, D. L.; Morgan, D.; Liu, Y.; Froestl, W.; Roberts, D. C. Effects of positive allosteric modulators of the $GABA_B$ receptor on cocaine self-administration in rats. *Psychopharmacology* **2004**, *173*, 105−111. (b) Filip, M.; Frankowska, M.; Przegaliński, E. Effects of $GABA_B$ receptor antagonist, agonists and allosteric positive modulator on the cocaine-induced self-administration and drug discrimination. *Eur. J. Pharmacol.* **2007**, *574*, 148−157.
(34) Filip, M.; Frankowska, M. Effects of $GABA_B$ receptor agents on cocaine priming, discrete contextual cue and food induced relapses. *Eur. J. Pharmacol.* **2007**, *571*, 166−173.
(35) Voigt, R. M.; Herrold, A. A.; Riddle, J. L.; Napier, T. C. Administration of $GABA_B$ receptor positive allosteric modulators inhibit the expression of previously established methamphetamine-induced conditioned place preference. *Behav. Brain Res.* **2011**, *216*, 419−423.
(36) (a) Cryan, J. F.; Kelly, P. H.; Chaperon, F.; Gentsch, C.; Mombereau, C.; Lingenhoehl, K.; Froestl, W.; Bettler, B.; Kaupmann, K.; Spooren, W. Behavioral characterization of the novel $GABA_B$ receptor positive modulator GS39783 (*N*,*N*-dicyclopentyl-2-methylsulfanyl-5-nitropyrimidine-4,6-diamine): anxiolytic-like activity without side effects associated with baclofen or benzodiazepines. *J. Pharmacol. Exp. Ther.* **2004**, *310*, 952−963. (b) Frankowska, M.; Filip, M.; Przegaliński, E. Effects of $GABA_B$ receptor ligands in animal tests of depression and anxiety. *Pharmacol. Rep.* **2007**, *59*, 645−655. (c) Jacobson, L. H.; Cryan, J. F. Evaluation of the anxiolytic-like profile of the $GABA_B$ receptor positive modulator CGP7930 in rodents. *Neuropharmacology* **2008**, *54*, 854−862.
(37) Schwartz, T. W.; Holst, B. Allosteric enhancers, allosteric agonists and ago-allosteric modulators: Where do they bind and how do they act? *Trends Pharmacol. Sci.* **2007**, *28*, 368−373.
(38) Loi, B.; Maccioni, P.; Lobina, C.; Carai, M. A. M.; Gessa, G. L.; Thomas, A. W.; Malherbe, P.; Colombo, G. Reduction of alcohol intake by the positive allosteric modulator of the $GABA_B$ receptor, *rac*-BHFF, in alcohol-preferring rats. *Alcohol* **2013**, *47*, 69−73.
(39) Maccioni, P.; Carai, M. A.; Kaupmann, K.; Guery, S.; Froestl, W.; Leite-Morris, K. A.; Gessa, G. L.; Colombo, G. Reduction of alcohol's reinforcing and motivational properties by the positive allosteric modulator of the GABAB receptor, BHF177, in alcohol-preferring rats. *Alcohol.: Clin. Exp. Res.* **2009**, *33*, 1749−1756.
(40) Maccioni, P.; Thomas, A. W.; Carai, M. A. M.; Gessa, G. L.; Malherbe, P.; Colombo, G. The positive allosteric modulator of the $GABA_B$ receptor, *rac*-BHFF, suppresses alcohol self-administration. *Drug Alcohol Depend.* **2010**, *109*, 96−103.
(41) Vlachou, S.; Guery, S.; Froestl, W.; Banerjee, D.; Benedict, J.; Finn, M. G.; Markou, A. Repeated administration of the GABAB receptor positive modulator BHF177 decreased nicotine self-administration, and acute administration decreased cue-induced reinstatement of nicotine seeking in rats. *Psychopharmacology* **2011**, *215*, 117−128.
(42) (a) Castelli, M. P.; Casu, A.; Casti, P.; Lobina, C.; Carai, M. A. M.; Colombo, G.; Solinas, M.; Giunta, D.; Mugnaini, C.; Pasquini, S.; Tafi, A.; Brogi, S.; Gessa, G. L.; Corelli, F. Characterization of methyl 2-(1-adamantanecarboxamido)-4-ethyl-5-methylthiophene-3-carboxylate (COR627) and methyl 2-(cyclohexanecarboxamido)-4-ethyl-5-methylthiophene-3-carboxylate (COR628), two novel positive allosteric modulators of the GABAB receptor. *J. Pharmacol. Exp. Ther.* **2012**, *340*, 529−538. (b) Koek, W.; France, C. P.; Cheng, K.; Rice, K. C. Effects of the $GABA_B$ receptor-positive modulators CGP7930 and rac-BHFF in baclofen- and γ-hydroxybutyrate discriminating pigeons. *J. Pharmacol. Exp. Ther.* **2012**, *341*, 369−376.
(43) (a) Gewald, K. 2-Aminothiophene aus α-oxo-mercaptanen und methylenaktiven nitrilen. *Chem. Ber.* **1965**, *98*, 3571−3576. (b) Gewald, K.; Schinke, E.; Böttcher, H. 2-Aminothiophene aus methylenaktiven nitrilen, carbonylverbindungen und schwefel. *Chem. Ber.* **1966**, *99*, 94−100.
(44) Reviews: (a) Sabnis, R. W. The Gewald synthesis. *Sulfur Rep.* **1994**, *16*, 1−17. (b) Sabnis, R. W.; Rangnekar, D. W.; Sonawane, N. D. 2-Aminothiophenes by the Gewald reaction. *J. Heterocycl. Chem.* **1999**, *36*, 333−345. (c) Huang, Y.; Dömling, A. The Gewald multicomponent reaction. *Mol. Diversity* **2011**, *15*, 3−33.
(45) Chan, M. F.; Kois, A.; Verner, E. J.; Raju, B. G.; Castillo, R. S.; Wu, C.; Okun, I.; Stavros, F. D.; Balaji, V. N. The discovery and structure−activity relationships of nonpeptide, low molecular weight antagonists selective for the endothelin ET_B receptor. *Bioorg. Med. Chem.* **1998**, *6*, 2301−2316.
(46) Richmond, W.; Wogan, M.; Isbell, J.; Gordon, W. P. Interstrain differences of in vitro metabolic stability and impact on early drug discovery. *J. Pharm. Sci.* **2010**, *99*, 4463−8.
(47) Silverman, R. B. *The Organic Chemistry of Drug Design and Drug Action*, 2nd ed.; Elsevier Academic Press: Burlington, MA, 2004; pp 71−73.

(48) Conn, P. J.; Kuduk, S. D.; Doller, D. Drug Design Strategies for GPCR Allosteric Modulators. In *Annual Reports in Medicinal Chemistry*; Desai, M. C., Ed.; Academic Press: San Diego, CA, 2012; Vol. *47*, pp 441−457.
(49) Williams, R.; Niswender, C. M.; Luo, Q.; Le, U.; Conn, P. J.; Lindsley, C. W. Positive allosteric modulators of the metabotropic glutamate receptor subtype 4 (mGluR4). Part II: Challenges in hit-to-lead. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 962−966.
(50) Hopkins, C. R.; Lindslay, C. W.; Niswender, C. M. mGluR4-positive allosteric modulation as potential treatment for Parkinson's disease. *Future Med. Chem.* **2009**, *1*, 501−513.
(51) Carai, M. A. M.; Colombo, G.; Froestl, W.; Gessa, G. L. In vivo effectiveness of CGP7930, a positive allosteric modulator of the $GABA_B$ receptor. *Eur. J. Pharmacol.* **2004**, *504*, 213−216.
(52) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the priciple of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.
(53) Rossi, C.; Foletti, A.; Magnani, A.; Lamponi, S. New perspectives in cell communication: bioelectromagnetic interactions. *Semin. Cancer Biol.* **2011**, *21*, 207−214.
(54) D'Elia, P.; De Matteis, F.; Dragoni, S.; Shah, A.; Sgaragli, G.; Valoti, M. DP7, a novel dihydropyridine multidrug resistance reverter, shows only weak inhibitory activity on human CYP3A enzyme(s). *Eur. J. Pharmacol.* **2009**, *614*, 7−13.
(55) Zamora, I.; Fontaine, F.; Serra, B.; Plasencia, G. High-throughput, fully automated, specific MetID. A revolution for drug discovery. *Drug Discovery Today: Technol.* **2013**, *10*, e199−e205.
(56) Baranczewski, P.; Stańczak, A.; Sundberg, K.; Svensson, R.; Wallin, A.; Jansson, J.; Garberg, P.; Postlind, H. Introduction to in vitro estimation of metabolic stability and drug interactions of new chemical entities in drug discovery and development. *Pharmacol. Rep.* **2006**, *58*, 453−472.
(57) Brizzi, A.; Cascio, M. G.; Frosini, M.; Ligresti, A.; Aiello, F.; Biotti, I.; Brizzi, V.; Pertwee, R. G.; Corelli, F.; Di Marzo, V. Resorcinol-*sn*-glycerol derivatives: novel 2-arachidonoylglycerol mimetics endowed with high affinity and selectivity for cannabinoid type 1 receptor. *J. Med. Chem.* **2011**, *54*, 8278−8288.