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New Fluorinated 1,2,4-Benzothiadiazine 1,1-Dioxides: Discovery of an Orally Active Cognitive Enhancer Acting through Potentiation of the 2-Amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic Acid Receptors

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In the search of a potent cognitive enhancer, a series of 3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-dioxides have been synthesized and evaluated as positive allosteric modulators of the AMPA receptors. In the present work, we focused our efforts on the insertion of mono- or polyfluoro-substituted alkyl chains at the 4-position of the thiadiazine ring in an attempt to enhance the pharmacokinetic behavior of previously described compounds. Among all the described compounds, 7-chloro-4-(2-fluoroethyl)-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-dioxide, **12b**, was shown to exert a strong activity on AMPA receptors in vitro and a marked cognitive-enhancing effect in vivo after oral administration to Wistar rats. Considering its in vivo activity, the metabolic degradation of **12b** was studied and compared to that of its nonfluorinated analogue **9b**. Taken together, results of this study clearly validated the positive impact of the fluorine atom on the alkyl chain at the 4-position of benzothiadiazine dioxides on activity and metabolic stability.

Introduction

As life expectancy increases in industrialized countries, inducing drastic changes in the population pyramid, cognitive disorders (namely, Alzheimer's disease) represent a major health problem. Only four symptomatic drugs are currently used to treat AD[a] patients. Three of them are acetylcholinesterase inhibitors (donepezil, rivastigmine, and galantamine), while memantine acts through uncompetitive antagonism of glutamate receptors of the NMDA subtype. Since these treatments have only limited efficacy, there is a great need for innovative treatment, targeting other aspects of the disease.

The AMPA receptors represent an interesting target to develop cognitive enhancers, since this subtype of ionotropic glutamate receptors is involved in the expression and the maintenance of long-term potentiation (LTP), a phenomenon closely linked to learning and memory processes.[1] Contrary to AMPA agonists that may cause severe adverse effects such as neurotoxicity, the AMPA potentiators seem to be a more interesting and less toxic pharmacological class because of their properties. Indeed, they are allosteric positive modulators able to potentiate the AMPA signals in the presence of glutamate, having no effect on the receptor in the absence of the endogenous neurotransmitter. In addition to their strong interest as cognitive enhancers, AMPA potentiators could also be valuable candidates for the management of schizophrenia or depression.[2]

Since the discovery that **1** (cyclothiazide),[3] **2** (diazoxide),[4] and its saturated derivative **3** (IDRA-21)[5] were able to allosterically activate AMPA receptors, many efforts have been devoted to develop novel 1,2,4-benzothiadiazine 1,1-dioxides and analogues such as **4** (S18986),[6] **5**,[7] and **6**[8] (see Figure 1).

Besides 4-alkyl-substituted 1,2,4-pyridothiadiazine 1,1-dioxides (including **7**[9] and **8**[10]), our team also reported a preliminary work focused on benzenic analogues (with **9** as a general structure) where the 7-position of the heterocycle was chosen to insert various groups.[11] This explorative approach led to the synthesis of new 1,2,4-benzothiadiazine 1,1-dioxides, from which emerged in vitro **9a** and **9b** (Figure 1). These two compounds were characterized by an ethyl chain on the nitrogen atom at the 4-position and bearing a fluorine or a chlorine atom at the 7-position of the fused ring system, respectively.

If the second derivative was quickly shown not to fulfill the pharmacokinetic criteria to reach the CNS after oral administration, the first one was validated as a new lead compound according to its marked positive effect in the object recognition task with Wistar rats after oral administration (0.3 mg/kg). Unfortunately, further pharmacological evaluations later pointed out its toxicity in rats after intraperitoneal injection at a dose of 30 mg/kg.

Considering the toxicity of **9a**, we focus our interest here on **9b**. The weakness of this compound was postulated to reside in

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[a] Abbreviations: AD, Alzheimer's disease; AMPA, 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid; CA1, Cornu Ammonis 1; CA3, Cornu Ammonis 3; CNS, central nervous system; DMSO, dimethyl sulfoxide; EPSfP, excitatory postsynaptic field potentials; LTP, long-term potentiation; NMDA, *N*-methyl-D-aspartate; SAR, structure−activity relationship.

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the metabolic reactions occurring on the alkyl chain at the 4-position. Hence, knowing the impact of fluorine atoms on the pharmacokinetic properties,[12] the introduction of this atom on the alkyl chain at the 4-position was hypothesized to block specific metabolism processes, such as hydroxylation and N-dealkylation. This idea led to the preparation of a series of compounds characterized by a fluoroalkyl chain at the 4-position and patented in 2005.[13] Moreover, taking into account the possible bioisosterism between the hydroxyl group and the fluorine atom, preparation of a compound bearing a 2-hydroxyethyl chain instead of a 2-fluoroethyl chain was also envisaged.

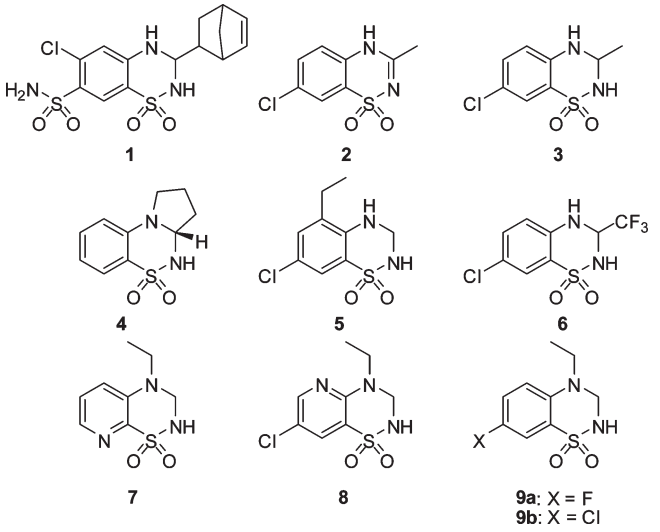


Figure 1. Chemical structures of some 1,2,4-benzothiadiazine 1,1-dioxides and pyridinic analogues acting as positive allosteric modulators of the AMPA receptors.

Chemistry

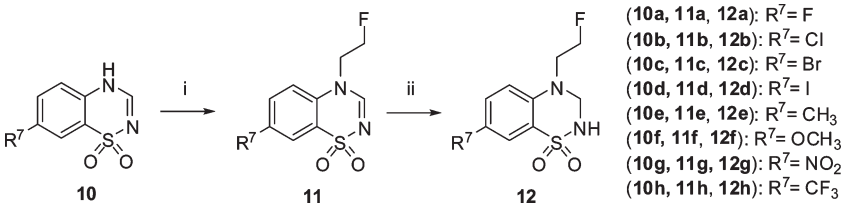
The synthetic pathways used to prepare the diversely 7-substituted 3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-dioxides bearing a mono- or polyfluoroalkyl chain at the 4-position reported here are illustrated in Schemes 1, 2, and 3.

Starting from **10** (obtained as previously described from the corresponding aminobenzenesulfonamides[11]), the fluoroethylation was performed by using the same conditions as those used for the classical methylation or ethylation[9] (Scheme 1). Initially, fluoroethyl bromide was chosen as the alkylating agent for the preparation of compounds **11**. However, since the reaction rate with fluoroethyl bromide was very slow (completion of the reaction required several days), this reagent was replaced by fluoroethyl iodide. Compounds **11** were then converted into their saturated analogues **12** by means of sodium borohydride in 2-propanol (Scheme 1).

Concerning the preparation of the difluorinated analogues **17**, all attemps to alkylate the nitrogen atom at the 4-position using difluorethyl bromide failed. However, selective acylation of the aniline **13** with difluoracetyl chloride in the presence of pyridine as a base was carried out at low temperature to avoid the ring closure of the acylated aminobenzensulfonamide into the corresponding unsaturated 1,2,4-benzothiadiazine 1,1-dioxide, as this was known to occur upon heating[14] (Scheme 2). The intermediate **14** was converted to **15** through a careful reduction of the carboxamide function using lithium aluminum hydride in diethyl ether at low temperature. Then ring closure into the corresponding benzothiadiazine dioxide was performed with triethyl orthoformate, and the subsequent reduction with sodium borohydride led to the final compound **17** (Scheme 2).

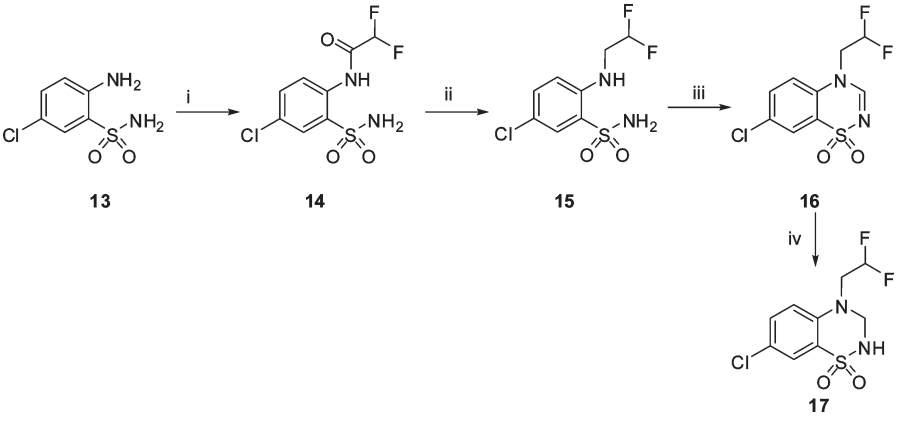
Several attempts at direct alkylation of **10b** with 2,2,2-trifluoroethyl chain resulted in poor yields. While the approach

Scheme 1[a]



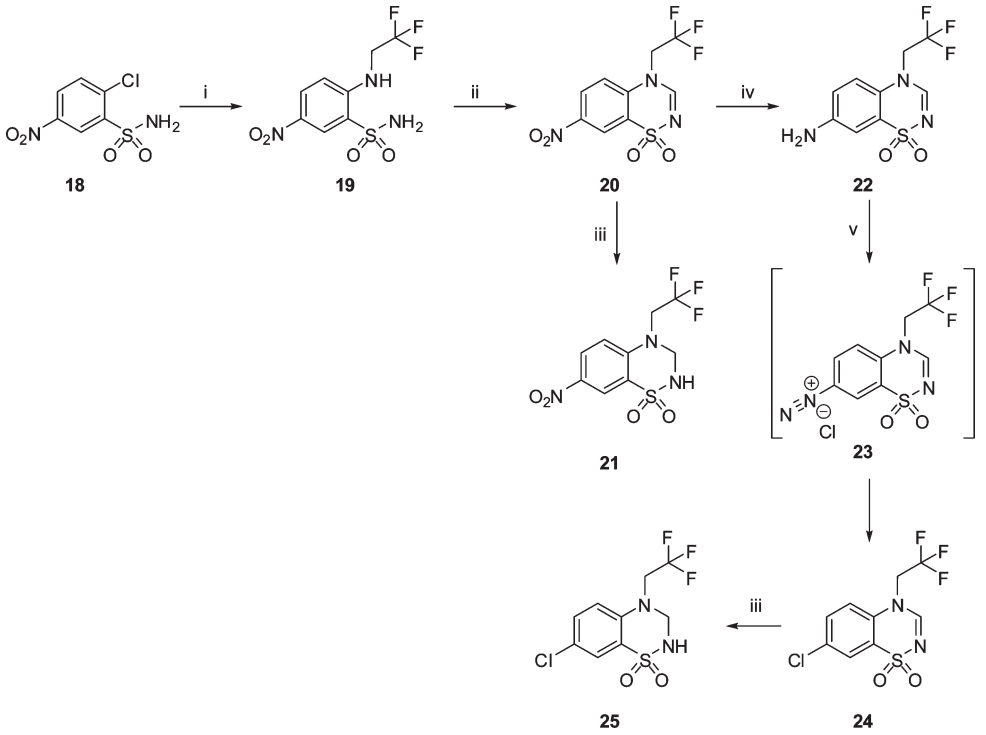
[a] (i) FCH_2CH_2I, K_2CO_3, CH_3CN, 70 °C, 30 h.; (ii) $NaBH_4$, 2-propanol, 45 min.

Scheme 2[a]



[a] (i) CF_2HCOCl, pyridine, 5 °C, 10 min; (ii) $LiAlH_4$, ether, 30 min; (iii) $CH(OEt)_3$, 180 °C, 45 min.; (iv) $NaBH_4$, 2-propanol, 45 min.

Scheme 3[a]



[a] (i) $CF_3CH_2NH_2$, 160 °C, 50 h; (ii) $CH(OEt)_3$, 160 °C, 2 h; (iii) $NaBH_4$, 2-propanol, 45 min; (iv) Fe/NH_4Cl, 80 °C, 45 min; (v) diazotization.

consisting of the acylation of the appropriate aminobenzenesulfonamide and the subsequent reduction could be applied in this case, the preference was to start from **18**. Taking advantage of the lability of the chlorine atom positioned para to the nitro group and ortho to the sulfonamide function, substitution of chlorine with 2,2,2-trifluoroethylamine, at high temperature, in a closed vessel followed by the ring closure using the classical procedure was achieved and afforded **20** (Scheme 3).

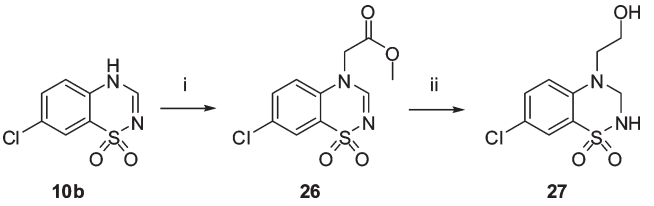
Since the synthetic pathway to **25** included the preparation of the 7-nitro intermediate **20**, a reduced congener of the latter was synthesized (Scheme 3) and tested to corroborate the observation made in the 7-chloro series. While it was possible to selectively reduce the double bond in the 2,3-positions with sodium borohydride (leading to **21**), a reduction of the nitro group was performed using powdered iron in the presence of ammonium chloride. This reaction did not affect the double bond in the 2,3-positions and gave access to **22**. The presence of an aromatic primary amino function in **22** allowed a diazotization on this group and the subsequent introduction of a chlorine atom at the 7-position. Then the classic double bond saturation with sodium borohydride led to the final compound **25** (Scheme 3).

As shown in Scheme 4, alkylation of **10b** with methyl bromoacetate was achieved with potassium carbonate as a base in refluxing acetonitrile. As expected, the alkylation reaction occurred at the 4-position, affording **26** in good yield. The subsequent reduction using sodium borohydride not only permitted the reduction of the double bond in the 2,3-positions but also converted the methyl acetate residue into the alcohol **27** (Scheme 4).

Results and Discussion

The newly prepared 1,2,4-benzothiadiazine 1,1-dioxides were tested as AMPA potentiators using voltage clamp recordings of AMPA-induced current on *Xenopus laevis*

Scheme 4[a]



[a] (i) $BrCH_2COOCH_3$, K_2CO_3, CH_3CN, 70°C, 5 h; (ii) $NaBH_4$, 2-propanol, 45 min.

oocytes injected with rat cortex poly(A^+) mRNA as previously described.[9] In addition to the voltage clamp method used for the first set of compounds, the Servier Research Institute developed a new technique permitting a high-throughput approach. More rapid than the "classical" voltage clamp method, this new technique was thus used to make a first selection among the newly synthesized compounds prior to their possible evaluation on *Xenopus* oocytes.

The new "flipR" method was performed on a 96-well plate containing primary neuronal cultures isolated from rat embryonic cortex, and AMPA-evoked membrane depolarization was measured through a fluorescent probe sensitive to membrane potential value. This new technique was assessed comparing the activity of 44 compounds obtained using the voltage clamp method and the new method. Some data of this comparison are reported in Table 1 in order to illustrate the correlation between the EC_{2X} values obtained in both models.

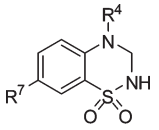
As observed from data in Table 1, the EC_{2X} obtained through the flipR method was found to be higher than the EC_{2X} given by the voltage clamp method. Although not identical to the voltage clamp's EC_{2X}, the flipR's EC_{2X} may be considered as a predictive value, allowing a preliminary screening.

Table 1. Comparison of the Results Obtained with a Selection of Hit Compounds and References Using the New FlipR Method and the Voltage Clamp Method

compd	name	EC_{2X} flipR (μM)[a]	EC_{2X} VC (μM)[b]
1	cyclothiazide	5.2 ± 1.1	1.7 ± 0.4
9a		16.8 ± 3.9	3.2 ± 0.1
7		47.7 ± 6.5	8.8 ± 1.3
8		127 ± 40.2	28.3 ± 3.0
4	S 18986	228[c]	25 ± 3
3	IDRA 21	260[c]	134.3 ± 7.3

[a] Drug concentration giving a 2-fold increase of the fluorescence induced by AMPA (300 μM) with the flipR method ($n \geq 3$, mean ± SEM). [b] Drug concentration giving a 2-fold increase of the amplitude of the current induced by (*S*)-AMPA (10 μM) with the voltage clamp method ($n \geq 3$, mean ± SEM). [c] $n = 2$ (no SEM).

Table 2. Effects of 3,4-Dihydro-2*H*-1,2,4-benzothiadiazine 1,1-Dioxides on the Fluorescence Induced by 300 μM AMPA on Primary Cultures of Neurons from Rat Embryonic Cortex and on the Amplitude of the Current Induced by 10 μM (*S*)-AMPA in *Xenopus laevis* Oocytes Injected with Rat Cortex mRNA



9, 12

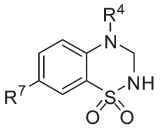
compd	R^7	R^4	EC_{2X} flipR (μM)[a,e]	EC_{2X} VC (μM)[b]
9a[c]	F	CH_2CH_3	16.8 ± 3.9	3.2 ± 0.1
12a	F	CH_2CH_2F	11.3[d]	3.6 ± 0.4
9b[c]	Cl	CH_2CH_3	nd	5.6 ± 0.9
12b	Cl	CH_2CH_2F	18.8 ± 1.1	6.7 ± 0.9
9c[c]	Br	CH_2CH_3	nd	29 ± 6
12c	Br	CH_2CH_2F	77.4[d]	17[d]
9d[c]	I	CH_2CH_3	nd.	95 ± 31
12d	I	CH_2CH_2F	> 100[d]	120[d]
9e[c]	CH_3	CH_2CH_3	nd	25 ± 8
12e	CH_3	CH_2CH_2F	76.7[d]	47.5[d]
9f[c]	OCH_3	CH_2CH_3	nd	68 ± 7
12f	OCH_3	CH_2CH_2F	> 100[d]	71[d]
9g[c]	NO_2	CH_2CH_3	nd	16 ± 1
12g	NO_2	CH_2CH_2F	72.8[d]	27[d]
9h[c]	CF_3	CH_2CH_3	nd	> 300
12h	CF_3	CH_2CH_2F	> 100[d]	> 300[d]

[a] Drug concentration giving a 2-fold increase of the fluorescence induced by AMPA (300 μM) with the flipR method ($n \geq 3$, mean ± SEM). [b] Drug concentration giving a 2-fold increase of the amplitude of the current induced by (*S*)-AMPA (10 μM) with the voltage clamp method ($n \geq 3$, mean ± SEM). [c] Published results.[11] [d] $n = 2$ (no SEM). [e] nd: not determined.

Comparing **9a**−**d**,**f**,**h** with their corresponding fluorinated analogues **12a**−**d**,**f**,**h**, it was clear that the fluorine atom did not negatively or positively influence the in vitro activity (Table 2). This assertion seemed less obvious, comparing **9e** and **9g** with **12e** and **12g**, although the in vitro activity was not greatly altered (Table 2). We could explain this well conserved in vitro activity by the small size of the fluorine atom, the introduction of one fluorine atom leading to a limited steric effect. Since the introduction of one fluorine atom did not exert any effects on the in vitro activity, it was therefore likely that the fluorine atom at this specific position did not play any role (i.e., no hydrogen-bond acceptance) in the binding of the compound to the AMPARs.

As can be seen in Table 3, the in vitro activity (voltage clamp method) was slightly decreased with the insertion of two

Table 3. Effects of 3,4-Dihydro-2*H*-1,2,4-benzothiadiazine 1,1-Dioxides on the Fluorescence Induced by 300 μM AMPA on Primary Cultures of Neurons from Rat Embryonic Cortex and on the Amplitude of the Current Induced by 10 μM (*S*)-AMPA in *Xenopus laevis* Oocytes Injected with Rat Cortex mRNA



9, 12, 17, 21, 25, 27

compd	R^7	R^4	EC_{2X} flipR (μM)[a,d]	EC_{2X} VC (μM)[b]
9b	Cl	CH_2CH_3	nd	5.6 ± 0.9
12b	Cl	CH_2CH_2F	18.8 ± 1.1	6.7 ± 0.9
17	Cl	CH_2CHF_2	9.7 ± 0.7	13.5[c]
25	Cl	CH_2CF_3	23.3[c]	19[c]
27	Cl	CH_2CH_2OH	66.3[c]	54[c]
12g	NO_2	CH_2CH_2F	72.8[c]	27[c]
21	NO_2	CH_2CF_3	110 ± 65	55[c]

[a] Drug concentration giving a 2-fold increase of the fluorescence induced by AMPA (300 μM) with the flipR method ($n \geq 3$, mean ± SEM). [b] Drug concentration giving a 2-fold increase of the amplitude of the current induced by (*S*)-AMPA (10 μM) with the voltage clamp method ($n \geq 3$, mean ± SEM). [c] $n = 2$ (no SEM). [d] nd: not determined.

fluorine atoms. This trend was confirmed considering the in vitro activity of compound **25** bearing the (2,2,2)-trifluoroethyl chain in the 4-position. The flipR method did not clearly confirm this observation.

With the second example of (2,2,2)-trifluoroethylated compound **21**, we noted that the presence of three fluorine atoms at this position seemed unfavorable for the in vitro activity (compare **12g** vs **21**, Table 3). This gradual loss of activity could be explained by the gradual steric hindrance obtained when introducing additional fluorine atoms. Considering Taft's Es value, or van der Waals radii (vdwr), the size of a trifluoromethyl group (Taft's Es of CF_3, −2.40; vdwr of CF_3, 2.2) could be compared to that of an isopropyl chain (Taft's Es of *i*-Pr, −2.17; vdwr of iPr: 2.2) rather than to that of a methyl group (Taft's Es of CH_3:-1.24; vdwr of CH_3, 1.8); however, when the molar refractivity is compared, the trifluoromethyl moiety may be described as smaller than a methyl group. Another explanation that may be given is the powerful electron-withdrawing effect exerted by more than one fluorine atom. However, the introduction of fluorine atoms at the end of the ethyl chain was found to have little electronic impact on the electronic distribution within the thiadiazine ring, comparing the quite similar pK_a values experimentally determined for **9b**, **12b**, and **25**: 9.33 ± 0.02, 9.35 ± 0.02, and 9.01 ± 0.07, respectively, expressed as the mean ± SEM (the pK_a value corresponding to the acid function due to the proton borne by the nitrogen atom at the 2-position). This pharmacomodulation was found to have much less effect than the substitution at the 7-position, regarding pK_a values obtained with the nitro-substituted compound **12g**, which was found to be 8.45 ± 0.07.

The fluorine atom is commonly cited in the literature as a potential bioisoster of the hydroxy function. **27** could thus be considered at first as a potential bioisosteric congener of **12b**. However, as expected, considering the first in vitro results, **27** did not display the same activity as **9b** and **12b** (Table 3). The weak activity of **27** clearly showed that, in our series, the fluorine atom could not be considered as a bioisosteric group for the hydroxy function. Moreover, by comparison of **27** to

9b (Table 3), it clearly appeared that the introduction of a polar function, which is able to act as a hydrogen bond acceptor or donor, was not favorable for the in vitro activity.

On the basis of their EC_{2X} values observed with the voltage clamp method, five new benzothiadiazine dioxides emerged: **12a**−**c**, **17**, and **25**. Nevertheless, **12c** and **17** were shown to act as weak agonists of AMPARs when applied without AMPA. These effects led to the exclusion of these two derivatives from the selection of compounds for further experiments.

Prior to their in vivo evaluation, the safety of the three compounds (**12a**, **12b**, and **25**) was assessed in Irwin's test. This test is a common procedure in the pharmaceutical industry to assess and quantify the effects of a new compound on the behavioral and physiological state of the mouse.[15] Such data allow appreciation of the potential toxicity (in particular, the acute neurotoxicity) of a new substance.

In this test, while **12a** was shown to induce toxic effects, **12b** and **25** up to 30 mg/kg ip were found to be atoxic, although in mice an unexpected hypothermia was observed 1.5 h after the oral administration of **12b** at a dose of 30 mg/kg (−3.2 °C).

The object recognition test in Wistar rats performed with **12b** served to highlight its dose-dependent activity after oral administration, while **25** was found to be inactive in this test.

As shown in Figure 2, oral administration of **12b** 60 min before the three sessions of the test increased the object recognition at doses as low as 0.03 mg/kg. This effect was shown to be dose-dependent and significant ($p < 0.05$, one way Anova followed by Dunnett's test) from 0.1 to 1 mg/kg. This test also demonstrated that compound **12b** was well absorbed after oral administration and was also able to cross the blood−brain barrier and to reach the central nervous system.

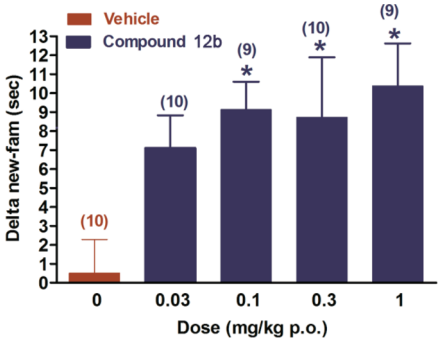


Figure 2. Effect of oral treatment with **12b** on the object recognition test in Wistar rat ($n = 10$ for doses 0, 0.03, 0.3 mg/kg; $n = 9$ for doses 0.1 and 1 mg/kg): (∗) $p \leq 0.05$ vs control, one-way ANOVA followed by Dunnett's test.

In an attempt to assess the potential interest of **12b**, an ex vivo electrophysiological test was performed to study its effect in the hippocampus, the brain region that plays an essential role in learning and memory processes.[16] Importantly, this ex vivo experiment is possible owing to the maintenance of the major elements of the trisynaptic circuitry of the hippocampus (dentate gyrus, CA3−CA1).[17,18]

The effects of **12b** on the excitatory postsynaptic field potentials (EPSfPs) evoked in the CA1 region of the hippocampus after electrical stimulation of the Schaffer collateral were studied in rat slices. Since this test was performed in the presence of 1.2 mM Mg^{2+} in the perfusion medium, EPSfPs were primarily mediated by activation of AMPARs. As can be seen in Figure 3, **12b** applied at increasing concentrations to the slices was shown to elicit a 23 ± 5% increase of amplitude of the EPSfP at 50 μM. This result suggested that **12b** interacted with postsynaptic AMPA receptors located on hippocampal CA1 neurons, as it was demonstrated for **9a**.[11]

Although the interest in **12b** was confirmed, the observation of epileptiform activity (in $^2/_5$ cases) at 100 μM gave a second warning concerning the potential toxicity of **12b**.

Knowing the cognition-enhancing effects displayed by **12b** in vivo after oral administration and also considering its ex vivo effects on the AMPA-mediated postsynaptic response, it was tempting to examine the effect of **12b** on the long-term potentiation of the postsynaptic response in an in vivo experiment. This was achieved on anaesthetized rats, measuring LTP evoked in the dentate gyrus: 1 h after the ip administration of **12b**, the LTP was induced by a titanic stimulation (brief high frequent stimulation), and the subsequent EPSfPs were then recorded during 3 h. While **12b** was not shown to exert a significant activity at a dose of 3 mg/kg, **12b** was found to increase the duration of the LTP at a dose of 10 mg/kg (Figure 4).

This activity, which correlated well with the effects observed in the previous experiment, is interesting because it was obtained in vivo and may be viewed as an explanation, at least in part, for the cognition-enhancing effects displayed by **12b** in the object recognition test. Moreover, it is noted here that the dose needed to obtain a significant activity in the present experiment (10 mg/kg, ip) was much higher than the dose showing a first significant activity in the object recognition task (0.1 mg/kg, po). This could be partially explained by anesthesia. Another reason for such a variation is the fundamental difference between the two in vivo evaluations.

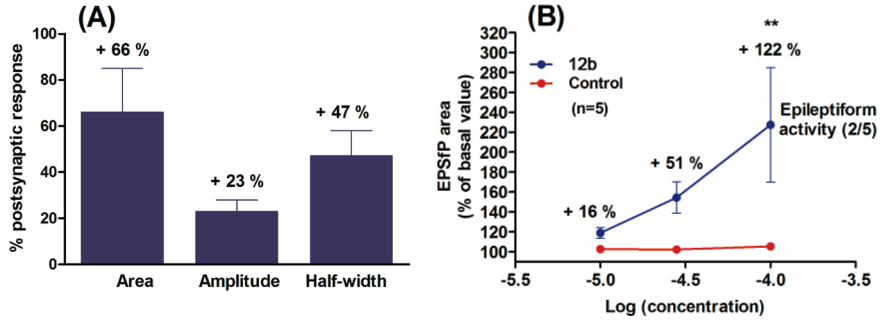


Figure 3. (A) Increase of the parameters (area, amplitude, and half width) of the AMPA-mediated postsynaptic response recorded in CA1 area of the hippocampus on slices in vitro after application of **12b** at 50 μM ($n = 3$). (B) Concentration−effect of **12b**: **12b** increased the EPSfP area in a concentration-dependent manner. The amplitude of the AMPA-mediated EPSfP in the presence of each concentration of **12b** or vehicle was normalized as a percentage of that evoked before application (basal value taken as 100%): (∗∗) $p \leq 0.01$ vs control, two-way ANOVA followed by Dunnett's test.

While the present test consists of the local recording of the response of neurons located around the recording electrode in a cerebral region (CA1 area), the object recognition task is a global measurement of the effect of the compound on the brain (a behavioral test).

As **12b** was conceived to avoid the rapid metabolic degradation undergone by **9b**, it seemed interesting to verify if the introduction of the fluorine atom indeed afforded a metabolic stabilization. In the context of the preliminary in vivo experiments (memory test in rats) and since the use of rat liver microsomes induced by phenobarbital represents an attractive tool to generate many phase I metabolites, the metabolic degradation was achieved on microsomes from rats. Hence, these experiments were expected to reflect the metabolic degradation undergone by the compound during the object recognition test achieved in rats. The interest of this study was even reinforced considering the potential implication of **12b**'s metabolites in the hypothermic effect occurring after its oral administration. An additional interesting feature of this study came from our capacity to synthesize the identified metabolites and to test them in order to check their activity and/or toxicity.

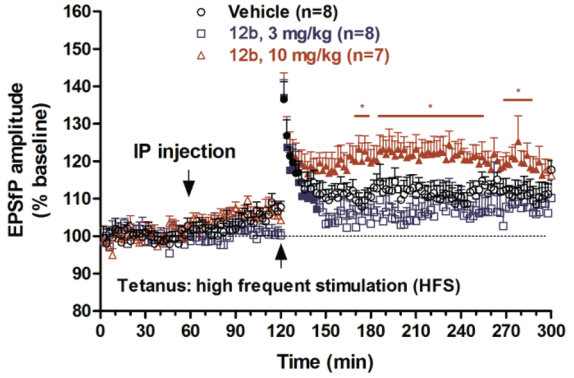


Figure 4. Effects of **12b** on LTP induced in the dentate gyrus of the hippocampus on anesthetised Wistar rat. The amplitude of the EPSfP was averaged over four stimuli and was normalized as a percentage of the averaged amplitude of the response during the 1 h baseline period prior to ip injection (control value taken as 100%). **12b** significantly increased the maintenance of the LTP compared to vehicle rats: (∗) $p \leq 0.05$ **12b** vs vehicle, two-way ANOVA followed by Dunnett's test. Closed symbols: $p < 0.05$ vs the average amplitude of EPSfPs obtained during a 6 min period preceding the tetanus: two-way Anova + Dunnett test.

The metabolic stability study was therefore performed with **12b** and its non fluorinated analogue, **9b**. Two complementary techniques were used to identify the metabolites formed by the microsomes: while the first one was LC−MS/MS (liquid chromatography coupled to tandem mass spectrometry) and used a small amount of sample, the second one was LC−SPE−NMR (a liquid chromatography coupled to a nuclear magnetic resonance apparatus through a solid phase extraction step) and required much more sample (due to the relatively poor sensitivity of the NMR technique). In spite of this disadvantage, the use of the LC−SPE−NMR technique seemed interesting because it was expected to unequivocally identify the structure of the metabolites when combined with MS data. In any case, this technique was more interesting than older techniques involving LC−NMR, in particular concerning the signal-to-noise ratio, which was greatly enhanced by successive collection on the same cartridge. Another interesting characteristic of this technique was the use of classical HPLC solvents, which were not allowed in the standard "stop-flow" method.

The first experiments were achieved with **9b** and **12b**, where the metabolic reactions were halted at the start by the addition of organic solvents in the medium ("T_0 experiments"). This provided samples with unchanged **12b** or **9b** to test the LC separation conditions. As shown in Figure 5, **12b** and **9b** were found to have nearly the same retention time.

After incubation of **12b** for 30 min at 37 °C and the subsequent RP-HPLC separation of the incubation medium, the metabolic degradation of **12b** exhibited two major metabolites, called **M1** and **M2**, and represented in the LC chromatogram together with the parent compound (see Figure 6A). As could be expected, the metabolites generated from **12b** were found to be less lipophilic than the parent compound **12b**, considering their retention times.

Under the same experimental conditions, **9b** was completely transformed into two metabolites, named **M3** and **M4**, as shown in Figure 6B. Here again, the two metabolites were found to be less lipophilic than the parent compound **9b**.

While **12b** was found to be partially transformed into two metabolites, the same experimental conditions led to the nearly total remove of **9b** from the medium. Interestingly, this first observation suggested that **12b** was characterized by an enhanced metabolic stability compared to **9b**. This remark was well in accordance with the hypothesis that led to the introduction of the fluorine atom on the ethyl chain at the 4-position.

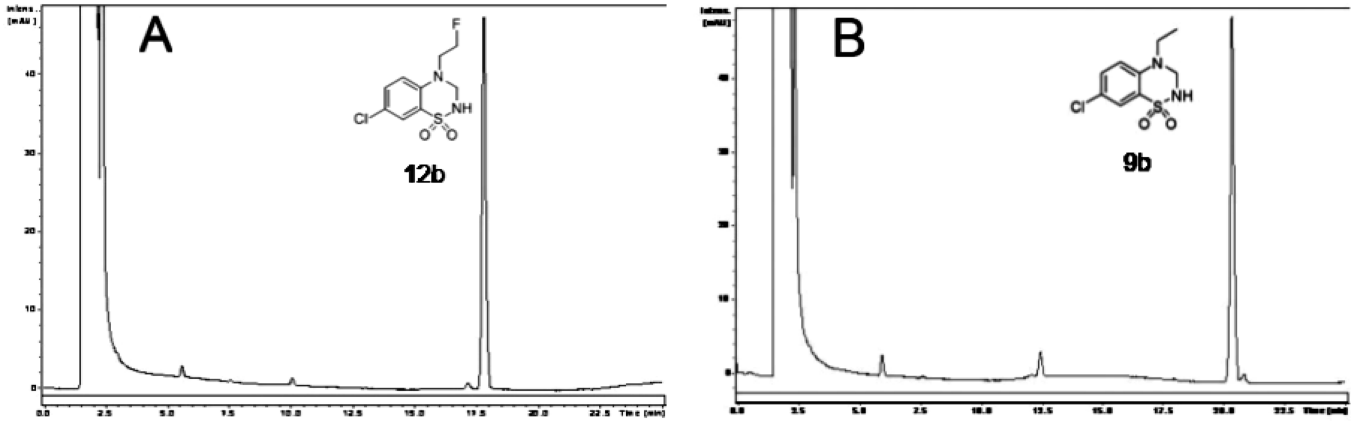


Figure 5. Chromatograms obtained after stopping the metabolism reaction at the start ("T_0"): (A) HPLC profile obtained with **9b**; (B) HPLC profile obtained with **12b**.

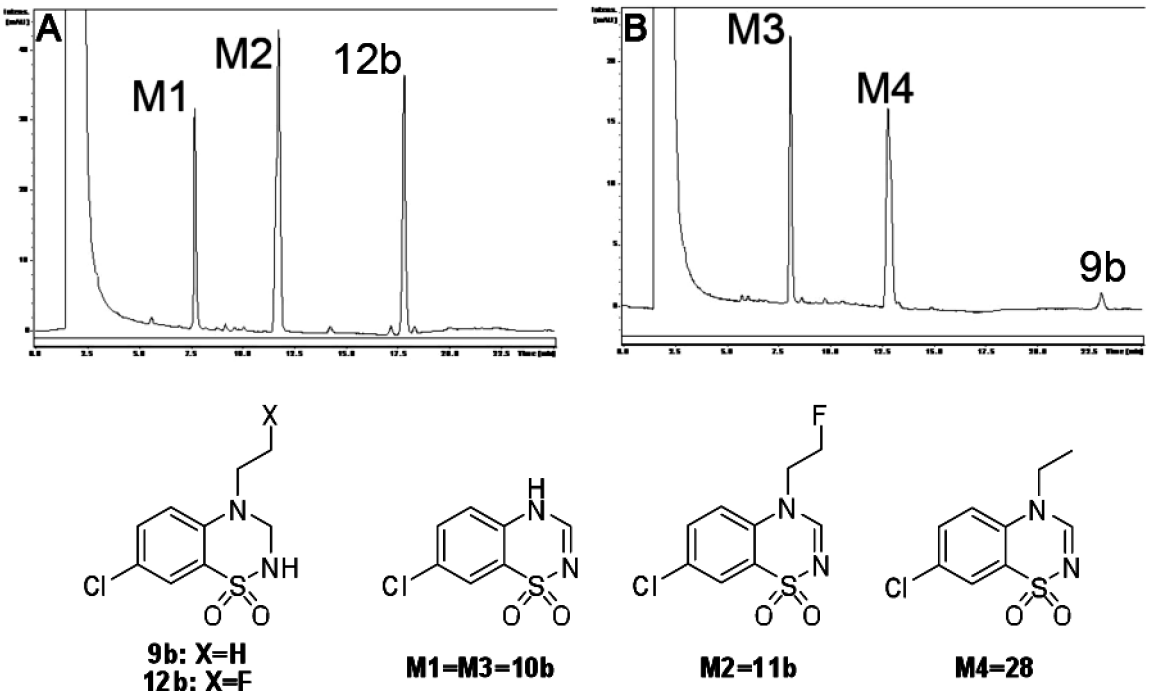


Figure 6. (A) HPLC profile obtained after the metabolic degradation of **12b** performed on rat liver microsomes induced by phenobarbital with the structures of the two major metabolites. (B) HPLC profile obtained after the metabolic degradation of **9b** performed on rat liver microsomes induced by phenobarbital with the structures of the two major metabolites.

Concerning the metabolites of **12b**, the **M2**'s MS data highlighted the decrease by 2 units of its molecular mass compared to **12b** (moreover, **M2**'s mass spectrum presented the characteristic isotopic distribution "3:1" of monochlorinated compounds, assessing the presence of one chlorine atom in **M2**). These first clues, linked with the NMR data, suggested that **M2** corresponded to the intermediate **11b**. On the other hand, **M1** was found to have a mass profile ($[M + H]^+$ m/z) of 216.9842/218.9855 (3:1), compatible with the structure of **10b**. This hypothesis was confirmed by the NMR data obtained with the corresponding eluted peak.

On the other hand, as suggested by the retention time of **M3**, which was the same as that of **M1** and taking into account their matching MS profile, **M3** was clearly identified as **10b**.

On the basis of the MS data and the preliminary identification of **M2** as **11b**, **M4** was found to correspond to **28**.

Focusing our interest on the two major metabolites of **12b**, **10b** and **11b** were evaluated as AMPA potentiators using voltage clamp recordings of AMPA-induced current on *Xenopus laevis* oocytes injected with rat cortex poly(A^+) mRNA. As expected, considering the in vitro results obtained with **28**,[11] the major **12b** metabolites were found to be inactive. The potential toxicity of these two compounds was investigated through Irwin's test. After intraperitoneal injection in mice (**10b**, 10−20 mg/kg; **11b**, 10−30 mg/kg) no effect was observed for either derivative.

In addition to the formation of these major metabolites, it was possible to identify the minor formation of the corresponding N-substituted aminobenzenesulfonamides, possibly resulting from the chemical degradation of **28** and **11b**, since such derivatives are known to readily undergo a ring-opening in water in alkaline medium.

The identification of the metabolites was finally confirmed by comparing their retention times with those observed after injection of the corresponding synthetically obtained intermediates in the HPLC system.

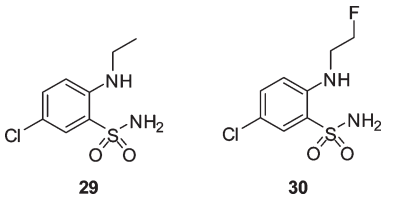


Figure 7. Minor metabolites of **9b** and **12b** obtained after metabolic degradation performed with hepatic microsomes from rats.

As achieved with **10b** and **11b**, **30** (Figure 7) was studied for its in vitro activity and in vivo toxicity. Interestingly, while completely inactive in vitro in the *Xenopus laevis* oocytes test ($EC_{2X} > 300\,\mu M$), **30** was found to induce hypothermic effect in Irwin's test (at 20 mg/kg ip: −3.6 °C 6 h after the injection). Recovery was observed after 24 h. These data suggest that the hypothermic effect observed after **12b**'s administration could be partially due to the in vivo formation of **30**.

Conclusion

Following the hypothesis that the introduction of a fluorine atom at a strategic position could induce modifications of the pharmacokinetic properties, a new pharmacomodulation was achieved, leading to the generation of novel in vitro active AMPA potentiators belonging to 1,2,4-benzothiadiazine 1,1-dioxides. While the introduction of one fluorine atom was shown to have no negative effect on the in vitro activity, a gradual loss of activity was observed concomitantly with the insertion of additional fluorine atoms. More importantly, the introduction of one fluorine atom was shown to induce the awaited beneficial changes on the in vivo profile: contrary to its nonfluorinated congener **9b**, **12b** displayed a significant and dose dependent activity after oral administration. In addition to this cognitive effect in vivo, **12b** was shown to unexpectedly induce hypothermic effects. Unfortunately,

first toxicological experiments of **12b** highlighted some security problems in animals. These warnings precluded the development of the new compound. In the near future, it should be interesting, however, to further examine the metabolism undergone by **12b** with human microsomes. After comparison with the metabolic degradation observed in rodents, these additional data should refine our knowledge about this new lead compound.

Experimental Section

Synthesis. Melting points were determined on a Stuart smp3 capillary apparatus and are uncorrected. IR spectra were recorded as KBr pellets on a Perkin-Elmer 1750 FT spectrophotometer. The ^{1}H NMR spectra were taken on a Bruker Advance 500 (500 MHz) instrument in DMSO-d_6 with TMS as an internal standard. Chemical shifts are reported in δ values (ppm) relative to internal TMS. The abbreviations s = singlet, d = doublet, t = triplet, q = quadruplet, quint = quintuplet, m = multiplet, and b = broad are used throughout. Elemental analysis results (C, H, N, S) were realized on a Carlo-Erba EA 1108 elemental analyzer or on a FlashEA 1112 series (Thermo-Interscience) and were within ±0.4% of the theoretical values (see Supporting Information). All compounds showed >95% purity, according to the elemental analyses. All reactions were routinely checked by TLC on silica gel Merck 60F254.

7-Fluoro-4-(2-fluoroethyl)-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (11a). A mixture of 7-fluoro-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**10a**)[11] (600 mg, 3.00 mmol), potassium carbonate (1.2 g), and 2-fluoroethyl iodide (0.6 mL) in acetonitrile (15 mL) was heated at 70 °C for 30 h in a closed vessel. The solvent was removed by distillation under reduced pressure, and the residue was suspended in water (5 mL). The resulting insoluble material was collected by filtration, washed with water, dried, and then suspended in a minimum of ethyl acetate and collected on a filter. The powder thereby obtained was recrystallized from warm acetone to yield the title compound (0.55 g, 75%): mp 211−218 °C; IR (KBr) 1614, 1568, 1488, 1467, 1410, 1303, 1163, 1152, 1106, 790 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 4.51 (dt, 2H, NCH_2CH$_2$F), 4.73 (dt, 2H, NCH$_2$CH_2F), 7.72 (m, 2H, 5-*H*/6-*H*), 7.82 (d, 1H, 8-*H*), 8.09 (s, 1H, 3-*H*). Anal. ($C_9H_8F_2N_2O_2S$) C, H, N, S.

7-Chloro-4-(2-fluoroethyl)-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (11b). The title compound was obtained as described for **11a** starting from 7-chloro-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**10b**)[11] (1 g, 4.62 mmol) (0.98 g, 81%): mp 208−212 °C; IR (KBr) 1613, 1478, 1464, 1307, 1161, 1038, 788 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 4.51 (dt, 2H, NCH_2CH$_2$F), 4.73 (dt, 2H, NCH$_2$CH_2F), 7.71 (d, 1H, 5-*H*), 7.85 (d, 1H, 6-*H*), 7.97 (s, 1H, 8-*H*), 8.12 (s, 1H, 3-*H*). Anal. ($C_9H_8ClFN_2O_2S$) C, H, N, S.

7-Bromo-4-(2-fluoroethyl)-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (11c). The title compound was obtained as described for **11a** starting from 7-bromo-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**10c**)[11] (0.3 g, 1.15 mmol) (0.25 g, 70%): mp 239−242 °C; IR (KBr) 1613, 1470, 1308, 1160, 1102, 787 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 4.50 (dt, 2H, NCH_2CH$_2$F), 4.73 (dt, 2H, NCH$_2$CH_2F), 7.64 (d, 1H, 5-*H*), 7.97 (d, 1H, 6-*H*), 8.06 (s, 1H, 8-*H*), 8.14 (s, 1H, 3-*H*). Anal. ($C_9H_8BrFN_2O_2S$) C, H, N, S.

4-(2-Fluoroethyl)-7-iodo-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (11d). The title compound was obtained as described for **11a** starting from 7-iodo-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**10d**)[11] (0.3 g, 0.97 mmol) (0.21 g, 81%): mp 224−228 °C; IR (KBr) 1613, 1463, 1308, 1168, 1030, 788 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 4.48 (dt, 2H, NCH_2CH$_2$F), 4.72 (dt, 2H, NCH$_2$CH_2F), 7.46 (d, 1H, 5-*H*), 8.09 (m, 2H, 6-*H*/8-*H*), 8.12 (s, 1H, 3-*H*). Anal. ($C_9H_8FIN_2O_2S$) C, H, N, S.

4-(2-Fluoroethyl)-7-methyl-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (11e). The title compound was obtained as described for **11a** starting from 7-methyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**10e**)[11] (0.4 g, 2.04 mmol) (0.38 g, 77%): mp 189−191 °C; IR (KBr) 1618, 1602, 1495, 1407, 1305, 1160, 1111, 1037 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 2.41 (s, 3H, 7-CH_3), 4.48 (dt, 2H, NCH_2CH$_2$F), 4.72 (dt, 2H, NCH$_2$CH_2F), 7.53 (d, 1H, 5-*H*), 7.59 (d,, 1H, 6-*H*), 7.72 (s, 1H, 8-*H*), 8.04 (s, 1H, 3-*H*). Anal. ($C_{10}H_{11}FN_2O_2S$) C, H, N, S.

4-(2-Fluoroethyl)-7-methoxy-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (11f). The title compound was obtained as described for **11a** starting from 7-methoxy-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**10f**)[11] (0.25 g, 1.2 mmol) (0.17 g, 57%): mp 167−169 °C; IR (KBr) 1603, 1499, 1404, 1306, 1248, 1171, 1154, 1028 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 3.87 (s, 3H, 7-OCH_3), 4.51 (dt, 2H, NCH_2CH$_2$F), 4.73 (dt, 2H, NCH$_2$CH_2F), 7.34 (m, 2H, 6-*H*/8-*H*), 7.61 (d, 1H, 5-*H*), 8.01 (s, 1H, 3-*H*). Anal. ($C_{10}H_{11}FN_2O_3S$) C, H, N, S.

4-(2-Fluoroethyl)-7-nitro-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (11g). The title compound was obtained as described for **11a** starting from 7-nitro-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**10g**)[11] (0.4 g, 1.8 mmol) (0.32 g, 66%): mp 177−180 °C; IR (KBr) 1622, 1607, 1529, 1470, 1347, 1318, 1167, 747 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 4.59 (dt, 2H, NCH_2CH$_2$F), 4.78 (dt, 2H, NCH$_2$CH_2F), 7.92 (d, 1H, 5-*H*), 8.24 (s, 1H, 3-*H*), 8.53 (d, 1H, 6-*H*), 8.58 (s, 1H, 8-*H*). Anal. ($C_9H_8FN_3O_4S$) C, H, N, S.

4-(2-Fluoroethyl)-7-trifluoromethyl-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (11h). The title compound was obtained as described for **11a** starting from 7-trifluoromethyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**10h**)[19] (0.4 g, 1.6 mmol) (0.29 g, 61%): mp 155−158 °C; IR (KBr) 1630, 1603, 1380, 1331, 1169, 1132, 1094 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 4.57 (dt, 2H, NCH_2CH$_2$F), 4.75 (dt, 2H, NCH$_2$CH_2F), 7.88 (d, 1H, 5-*H*), 8.14 (d, 1H, 6-*H*), 8.20 (m, 2H, 3-*H*/8-*H*). Anal. ($C_{10}H_8F_4N_2O_2S$) C, H, N, S.

7-Fluoro-4-(2-fluoroethyl)-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-Dioxide (12a). A solution of 7-fluoro-4-(2-fluoroethyl)-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**11a**) (0.5 g, 2.0 mmol) in 2-propanol (15 mL) was supplemented under stirring with sodium borohydride (0.2 g, 5.3 mmol). After the mixture was stirred for 45 min at room temperature, the solvent was removed by distillation under reduced pressure and the residue was suspended in water (25 mL). The alkaline suspension was adjusted to pH 7 with 0.1 N HCl and extracted 3-fold with chloroform (3 × 60 mL). The combined organic layers were dried over MgSO$_4$ and filtered. The filtrate was concentrated to dryness under reduced pressure, and the residue of the title compound was recrystallized in methanol/water, 1:2. (0.38 g, 76%): mp 133−134 °C; IR (KBr) 3256, 1501, 1402, 1322, 1160, 988 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 3.74 (dt, 2H, NCH_2CH$_2$F), 4.61 (dt, 2H, NCH$_2$CH_2F), 4.74 (d, 2H, 3-H_2), 7.01 (d, 1H, 5-*H*), 7.32 (td, 1H, 6-*H*), 7,41 (d, 1H, 8-*H*), 8.12 (t, 1H, 2-*H*). Anal. ($C_9H_{10}F_2N_2O_2S$) C, H, N, S.

7-Chloro-4-(2-fluoroethyl)-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-Dioxide (12b). The title compound was obtained as described for **12a** starting from 7-chloro-4-(2-fluoroethyl)-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**11b**) (1 g, 3.81 mmol) (0.82 g, 81%): mp 121−122 °C; IR (KBr) 3287, 3257, 1601, 1497, 1332, 1318, 1302, 1156, 1045, 985 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 3.75 (dt, 2H, NCH_2CH$_2$F), 4.60 (dt, 2H, NCH$_2$-CH_2F), 4.77 (d, 2H, 3-H_2), 7.01 (d, 1H, 5-*H*), 7.43 (d, 1H, 6-*H*), 7,54 (s, 1H, 8-*H*), 8.17 (t, 1H, 2-*H*). Anal. ($C_9H_{10}ClFN_2O_2S$) C, H, N, S.

7-Bromo-4-(2-fluoroethyl)-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-Dioxide (12c). The title compound was obtained as described for **12a** starting from 7-bromo-4-(2-fluoroethyl)-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**11c**) (0.25 g, 0.81 mmol) (0.19 g, 75%): mp 98−102 °C; IR (KBr) 3237, 1598, 1496, 1332, 1318, 1302, 1153, 979 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 3.75 (dt, 2H, NCH_2CH$_2$F), 4.60 (dt, 2H, NCH$_2$CH_2F), 4.76 (s, 2H, 3-H_2), 6.96 (d, 1H, 5-*H*), 7.53 (d, 1H, 6-*H*),

7,63 (s, 1H, 8-*H*), 8.10 (t, 1H, N-*H*). Anal. ($C_9H_{10}BrFN_2O_2S$) C, H, N, S.

4-(2-Fluoroethyl)-7-iodo-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-Dioxide (12d). The title compound was obtained as described for **12a** starting from 7-iodo-4-(2-fluoroethyl)-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**11d**) (0.25 g, 0.71 mmol) (0.18 g, 71%): mp 156−158 °C; IR (KBr) 3244, 1591, 1494, 1332, 1315, 1300, 1151, 980 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 3.74 (dt, 2H, NCH_2CH$_2$F), 4.59 (dt, 2H, NCH$_2$CH_2F), 4.76 (s, 2H, 3-H_2), 6.83 (d, 1H, 5-*H*), 7.65 (d, 1H, 6-*H*), 7,73 (s, 1H, 8-*H*), 8.15 (t, 1H, N-*H*). Anal. ($C_9H_{10}FIN_2O_2S$) C, H, N, S.

4-(2-Fluoroethyl)-7-methyl-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-Dioxide (12e). The title compound was obtained as described for **12a** starting from 7-methyl-4-(2-fluoroethyl)-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**11e**) (0.3 g, 1.2 mmol) (0.24 g, 79%): mp 88−90 °C; IR (KBr) 3246, 1621, 1513, 1358, 1337, 1313, 1164, 1150, 983 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 2.21 (s, 3H, 7-CH_3), 3.70 (dt, 2H, NCH_2CH$_2$F), 4.58 (dt, 2H, NCH$_2$CH_2F), 4.71 (d, 2H, 3-H_2), 6.87 (d, 1H, 5-*H*), 7.21 (d, 1H, 6-*H*), 7.35 (s, 1H, 8-*H*), 7.96 (t, 1H, N-*H*). Anal. ($C_{10}H_{13}FN_2O_2S$) C, H, N, S.

4-(2-Fluoroethyl)-7-methoxy-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-Dioxide (12f). The title compound was obtained as described for **12a** starting from 4-(2-fluoroethyl)-7-methoxy-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**11f**) (0.2 g, 0.77 mmol) (0.13 g, 65%): mp 115−117 °C; IR (KBr) 3235, 3207, 1508, 1312, 1274, 1239, 1161, 992, 773 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 3.68 (dt, 2H, NCH_2CH$_2$F), 3.72 (s, 3H, 7-OCH_3), 4.58 (dt, 2H, NCH$_2$CH_2F), 4.69 (d, 2H, 3-H_2), 6.90 (d, 1H, 5-*H*), 7.03 (m, 1H, 6-H/8-*H*), 7.93 (t, 1H, N-*H*). Anal. ($C_{10}H_{13}FN_2O_3S$) C, H, N, S.

4-(2-Fluoroethyl)-7-nitro-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-Dioxide (12g). The title compound was obtained as described for **12a** starting from 4-(2-fluoroethyl)-7-nitro-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**11g**) (0.25 g, 0.91 mmol) (0.18 g, 72%): mp 165−166 °C; IR (KBr) 3188, 1605, 1521, 1314, 1185, 1170, 1131, 984 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 3.93 (dt, 2H, NCH_2CH$_2$F), 4.67 (dt, 2H, NCH$_2$CH_2F), 4.94 (d, 2H, 3-H_2), 7.18 (d, 1H, 5-*H*), 8.19 (d, 1H, 6-*H*), 8.31 (s, 1H, 8-*H*), 8.49 (bs, 1H, N*H*). Anal. ($C_9H_{10}FN_3O_4S$) C, H, N, S.

4-(2-Fluoroethyl)-7-trifluoromethyl-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-Dioxide (12h). The title compound was obtained as described for **12a** starting from 4-(2-fluoroethyl)-7-trifluoromethyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**11h**) (0.25 g, 0.84 mmol) (0.19 g, 75%): mp 142−144 °C; ^1H NMR (DMSO-d_6, 500 MHz) δ 3.84 (dt, 2H, NCH_2CH$_2$F), 4.64 (dt, 2H, NCH$_2$-CH_2F), 4.86 (d, 2H, 3-H_2), 7.16 (d, 1H, 5-*H*), 7.69 (d, 1H, 6-*H*); 7.76 (s, 1H, 8-*H*), 8.29 (t, 1H, N-*H*). Anal. ($C_{10}H_{10}F_4N_2O_2S$) C, H, N, S.

5-Chloro-2-(2,2-difluoroacetamido)benzenesulfonamide (14). To 2-amino-5-chlorobenzenesulfonamide (**13**)[11](1 g, 4.84 mmol) dissolved in dioxane (4 mL) were added in the cold state (+5 °C) pyridine (0.6 mL) and difluoroacetic acid chloride (0.6 mL). The flask was hermetically closed immediately, and contents were vigorously stirred at ambient temperature for 10 min. The solvents were then removed under reduced pressure. The solid residue was taken up in water (12 mL) and the insoluble material was collected by filtration, washed with water, and dried to yield the expected compound (1.25 g, 91%): mp 180−181 °C; IR (KBr) 3352, 3278, 3235, 1705, 1607, 1542, 1396, 1328, 1157, 1145, 1104, 1092 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 6.51 (t, 1H, 2-NHCOCHF$_2$), 7.76 (d, 1H, 4-*H*), 7.90 (bs, 3H, SO$_2$NH_2/3-*H*), 8.18 (d, 1H, 6-*H*), 10.29 (bs, 1H, 2-N*H*COCHF$_2$). Anal. ($C_8H_7ClF_2N_2O_3S$) C, H, N, S.

5-Chloro-2-(2,2-difluoroethylamino)benzenesulfonamide (15). 5-Chloro-4-(2,2-difluoroacetamido)benzenesulfonamide (**14**) (1 g, 3.51 mmol) was suspended in dry ether (15 mL) prior to the addition of $LiAlH_4$ (500 mg). After being stirred during 30 min, the mixture was cooled in an ice bath and water was slowly added. The mixture was then carefully ajusted to pH 4 by adding concentrated HCl. The mixture was extracted with ethyl acetate (3 × 15 mL). The combined organic layers were dried over $MgSO_4$ and filtered, and the filtrate was concentrated under reduced pressure. The dry residue was then recrystallized from a mixture of acetone/water (1:10) (0.47 g, 50%): mp 127−131 °C; IR (KBr) 3418, 3362, 3273, 1602, 1509, 1452, 1313, 1147, 1057 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 3.73 (t, 2H, 2-NHCH_2CHF$_2$), 6.18 (m, 2H, 4-NHCH$_2$CHF$_2$), 7.01 (d, 1H, 3-*H*), 7.42 (d, 1H, 4-*H*), 7.54 (bs, 2H, SO$_2$NH_2), 7.61 (s, 1H, 6-*H*). Anal. ($C_8H_9ClF_2N_2O_2S$) C, H, N, S.

7-Chloro-4-(2,2-difluoroethyl)-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (16). 5-Chloro-4-(2,2-difluoroethylamino)benzenesulfonamide (**15**) (300 mg, 1.10 mmol) was suspended in triethyl orthoformate (1.5 mL). The mixture was heated at 180 °C in an open vessel for 45 min. After the mixture was cooled, the insoluble material that appeared was collected by filtration, washed with diethyl ether, and dried (0.26 g, 84%): mp 183−186 °C; IR (KBr) 1617, 1590, 1482, 1459, 1428, 1412, 1307, 1171, 1158, 1138, 1071, 784 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 4.73 (t, 2H, NCH_2CHF$_2$), 6.49 (td, 1H, NCH$_2$CHF$_2$), 7.78 (d, 1H, 5-*H*), 7.88 (d, 1H, 6-*H*), 7.97 (s, 1H, 8-*H*), 8.14 (s, 1H, 3-*H*). Anal. ($C_9H_7ClF_2N_2O_2S$) C, H, N, S.

7-Chloro-4-(2,2-difluoroethyl)-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-Dioxide (17). The title compound was obtained as described for **12a** starting from 7-chloro-4-(2,2-difluoroethyl)-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**16**) (1 g, 3.56 mmol) (0.75 g, 75%): mp 128−130 °C; ^1H NMR (DMSO-d_6, 500 MHz) δ 3.91 (td, 2H, NCH_2CHF$_2$), 4.81 (d, 2H, 3-H_2), 6.26 (td, 1H, NCH$_2$CHF$_2$), 7.09 (d, 1H, 5-*H*), 7.46 (d, 1H, 6-*H*), 7,56 (s, 1H, 8-*H*), 8.25 (t, 1H, N*H*). Anal. ($C_9H_9ClF_2N_2O_2S$) C, H, N, S.

2-(2,2,2-Trifluoroethylamino)-5-nitrobenzenesulfonamide (19). 2-Chloro-5-nitrobenzenesulfonamide (**18**) (1 g, 4.23 mmol) previously dissolved in dioxane (15 mL) and 2,2,2-trifluoroethylamine (1.6 mL, 2.1 g, 21.15 mmol) were introduced in a closed vessel and heated during 50 h at 160 °C. The solvent was then removed under reduced pressure. Water was added to the residue, and the resulting precipitate was collected by filtration, washed with water, and dried (0.76 g, 60%): mp 169−172 °C; IR (KBr) 3354, 3263, 1603, 1589, 1503, 1329, 1185, 1128, 1144 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 4.41 (m, 2H, NCH_2CF$_3$), 7.14 (t, 1H, NHCH$_2$CF$_3$), 7.28 (d, 1H, 3-*H*), 7.83 (bs, 2H, SO$_2$NH_2), 8.26 (d, 1H, 4-*H*), 8.55 (s, 1H, 6-*H*). Anal. ($C_8H_8F_3N_3O_4S$) C, H, N, S.

4-(2,2,2-Trifluoroethyl)-7-nitro-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (20). 2-(2,2,2-Trifluoroethylamino)-5-nitrobenzenesulfonamide (**19**) (3.5 g, 11.7 mmol) was suspended in triethyl orthoformate (20 mL). The mixture was heated at 160 °C in an open vessel for 2 h. After cooling, the insoluble material that appeared was collected by filtration, washed with diethyl ether, and dried (2.86 g, 79%): mp 243−246 °C; IR (KBr) 1628, 1531, 1353, 1330, 1177, 1132, 1098 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 5,35 (q, 2H, NCH_2CF$_3$), 8.11 (d, 1H, 5-*H*), 8.36 (s, 1H, 8-*H*), 8.59 (s, 1H, 3-*H*), 8.61 (d, 1H, 6-*H*). Anal. ($C_9H_6F_3N_3O_4S$) C, H, N, S.

4-(2,2,2-Trifluoroethyl)-7-nitro-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-Dioxide (21). The title compound was obtained as described for **12a** starting from 4-(2,2,2-trifluoroethyl)-7-nitro-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**20**) (1 g, 3.23 mmol) (0.74 g, 74%): mp 191−193 °C; ^1H NMR (DMSO-d_6, 500 MHz) δ 4.61 (m, 2H, NCH_2CF$_3$), 4.99 (d, 2H, 3-H_2), 7.35 (d, 1H, 5-*H*), 8.27 (d, 1H, 6-*H*), 8.35 (s, 1H, 8-*H*), 8.72 (bs, 1H, N*H*). Anal. ($C_9H_8F_3N_3O_4S$) C, H, N, S.

7-Amino-4-(2,2,2-trifluoroethyl)-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (22). 4-(2,2,2-Trifluoroethyl)-7-nitro-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**20**) (2.5 g, 8.08 mmol) was dispersed in a 1:1 ethanol/water mixture (60 mL). The suspension was heated until the product dissolved. Then ammonium chloride (1.25 g) and powdered iron (5 g) were added. After the mixture was refluxed for 45 min, the insoluble material was removed by filtration and rinsed with a small amount of hot ethanol. The filtrate was concentrated under reduced pressure. The beige precipitate that formed was collected by filtration, washed with water, and dried (1.35 g, 60%): mp 246−248 °C; IR (KBr) 3453, 3366, 1607, 1500, 1420, 197, 1267,

1162, 1137, 1100, 798 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 5.10 (m, 2H, NCH_2CF_3), 5.79 (bs, 2H, 7-NH_2), 6.94 (d, 1H, 6-*H*), 6.97 (s, 1H, 8-*H*), 7.46 (d, 1H, 5-*H*), 7.97 (s, 1H, 3-*H*). Anal. ($C_9H_8F_3N_3O_2S$) C, H, N, S.

7-Chloro-4-(2,2,2-trifluoroethyl)-4*H*-1,2,4-benzothiadiazine 1,1-Dioxide (24). To a suspension of **22** (500 mg, 1.79 mmol) in a HCl/water mixture (1:1, 5 mL) was added an aqueous solution of $NaNO_2$ (200 mg in 5 mL) at 0 °C, affording suspension A. On the other hand, CuCl was collected by filtration after being prepared from an aqueous solution (30 mL) containing $CuSO_4 \cdot 5H_2O$ (14 g) and NaCl (3.68 g) to which was added dropwise an aqueous solution (15 mL) of $Na_2S_2O_5$ (3.36 g). Suspension A was slowly added to a hydrochloric acid (3 mL) solution of the extemporaneously generated CuCl. After the mixture was allowed to warm to ambient temperature, the resulting mixture was extracted 3-fold with ether (3 × 15 mL). The combined organic layers were dried over $MgSO_4$ and filtered. The filtrate was concentrated to dryness under reduced pressure, and the residue of the title compound was recrystallized in methanol at −20 °C (0.21 g, 40%): mp 220−222 °C; IR (KBr) 3235, 1493, 1308, 1338, 1265, 1160, 1081 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 5.26 (s, 2H, NCH_2CF_3), 7.91 (m, 2H, 5-*H*/6-*H*), 8.01 (d, 1H, 8-*H*), 8.23 (s, 1H, 3-*H*). Anal. ($C_9H_6ClF_3N_2O_2S$) C, H, N, S.

7-Chloro-4-(2,2,2-trifluoroethyl)-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-Dioxide (25). The title compound was obtained as described for **12a** starting from 7-chloro-4-(2,2,2-trifluoroethyl)-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**24**) (0.25 g, 0.84 mmol) (0.19 g, 76%): mp 167−169 °C; IR (KBr) 3235, 1493, 1338, 1265, 1160, 1081, 766 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 4.41 (q, 2H, NCH_2CF_3), 4.85 (d, 2H, 3-H_2) 7.16 (d, 1H, 5-*H*), 7.52 (d, 1H, 6-*H*), 7,61 (s, 1H, 8-*H*), 8.40 (t, 1H, N*H*). Anal. ($C_9H_8ClF_3N_2O_2S$) C, H, N, S.

(7-Chloro-1,1-dioxo-4*H*-1,2,4-benzothiadiazin-4-yl)acetic Acid Methyl Ester (26). A mixture of 7-chloro-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (**10b**) (600 mg, 2.77 mmol), potassium carbonate (1.2 g), and methyl bromoacetate (0.6 mL) in acetonitrile (15 mL) was heated at 70 °C for 5 h in a closed vessel. The solvent was removed by distillation under reduced pressure, and the residue was suspended in water (5 mL). The resulting insoluble material was collected by filtration, washed with water, dried, and then suspended in a minimum of ethyl acetate and collected on a filter. The powder thereby obtained was recrystallized from warm acetone to yield the title compound (0.66 g, 82%): mp 214−216 °C; IR (KBr) 1729, 1622, 1484, 1307, 1244, 1166, 783 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 3.73 (s, 3H, NCH_2COOCH_3), 5.11 (s, 2H, NCH_2COOCH_3), 7.46 (d, 1H, 5-*H*), 7.82 (d, 1H, 6-*H*), 7.98 (s, 1H, 8-*H*), 8.12 (s, 1H, 3-*H*). Anal. ($C_{10}H_9ClN_2O_4S$) C, H, N, S.

2-(7-Chloro-1,1-dioxo-3,4-dihydro-2*H*-1,2,4-benzothiadiazin-4-yl)ethanol (27). The title compound was obtained as described for **12b** starting from (7-chloro-1,1-dioxo-4*H*-1,2,4-benzothiadiazin-4-yl)acetic acid methyl ester (**26**) (0.5 g, 1.73 mmol) (0.34 g, 75%): mp 138−140 °C; IR (KBr) 3279, 1600, 1497, 1413, 1154, 1060, 810, 801 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 3.47 (NCH_2CH_2OH), 3.55 (NCH_2CH_2OH), 4.76 (d, 2H, 3-H_2), 4.78 (bs, 1H, NCH_2CH_2OH), 6.98 (d, 1H, 5-H), 7.41 (d, 1H, 6-H), 7.51 (s, 1H, 8-*H*), 7.99 (t, 1H, N*H*). Anal. ($C_9H_{11}ClN_2O_3S$) C, H, N, S.

Biological Evaluations. Effect on AMPA-Evoked Membrane Depolarization. This assay consisted of investigating AMPA-evoked membrane depolarization, measured by fluorescent membrane potential dyes and an imaging based plate reader, on rat primary brain cultures. Dissociated rat primary brain cells were prepared from embryonic rat (E16) and were added to poly-D-lysine coated 96-well culture plates for 18 days at 37 °C/5% CO_2 (density 20 000 cells/well). On the day of the experiment, ground medium was removed from the cells and was replaced by 20 μL/well of membrane potential dye loading solution (Molecular Devices), reconstituted according to the manufacturer's instructions. Plates were incubated for 1 h at room temperature and then directly transferred to the fluorescence imaging based plate reader. Baseline fluorescence was monitored for 10 s followed by the addition of AMPA (3−100 μM) for 3 min and then the compound in the presence of AMPA during 3 min. Subsequent monitoring of fluorescence changes was performed during these two periods of 3 min. Responses were averaged over the last 15 s of each 3 min recording period. AMPA concentration−response curves were calculated in the absence or presence of different concentrations of the compound. Results were expressed as the area under the curve of AMPA-mediated concentration−response effect in the absence or presence of compound. EC_{2X} corresponds to the concentration of compound that evoked a 2-fold increase of all AMPA-mediated responses.

Effect on AMPA-Evoked Currents in *Xenopus laevis* Oocytes. Electrophysiological recordings were performed at room temperature on *Xenopus laevis* oocytes injected with either rat cortex or human hippocampus poly(A^+) mRNA in a Plexiglas recording chamber continuously superfused with "OR2" solution. Under anesthesia, a cluster of oocytes was removed from the abdomen of *Xenopus laevis* and placed in Barth's solution. Oocytes were gently isolated with pincers under a stereodynascope and were then left overnight in order to detect and eliminate oocytes that were impaired by the manipulation. Only oocytes presenting regular pigmentation were injected with 50.6 nL of an aqueous solution containing rat cortex or human hippocampus poly(A^+) mRNA (1 mg/mL), by using an automatic microinjector. Injected oocytes were then incubated at 18 °C in Barth's solution for 3 days to provide expression. They were then stored at 4 °C until use. Rat cortex poly(A^+) mRNA was prepared from the cerebral cortex of male Wistar rats (15 days old) by the guanidium thiocyanate/cesium chloride method. Human hippocampus poly(A^+) mRNA was purchased from Clontech. AMPA-evoked current was recorded at a holding potential of −60 mV, using a standard two-electrodes voltage clamp system. An amount of 10 μM (*S*)-AMPA was bath-applied during 30 s each 5 min with a constant flow rate of 3 mL/min, and the amplitude of the evoked current was measured at the peak of the current. Tested compound was bath-applied at successively increasing concentrations 45 s before, 30 s during, and 30 s after the application of 10 μM (*S*)-AMPA each 5 min on the same oocyte. The amplitude of the AMPA-evoked current in the presence of compound was expressed as a percentage of that induced on the same oocyte in the absence of compound, taken as 100%. EC_{2X} corresponds to the concentration of compound that evoked a 2-fold increase in the amplitude of the AMPA-evoked current, EC_{5X} corresponds to the concentration of compound that evoked for a 5-fold increase in the amplitude of the AMPA-evoked current.

Effect on Object Recognition Test in Wistar Rats. The one-trial object recognition paradigm measures a form of episodic memory in the rat.[20] Recognition is measured by the time spent by rats in exploring two different objects, one familiar and the other new. With an intertrial interval of 1 h, normal rats spend more time exploring the new object, which demonstrates that they recognize the familiar one. After a retention interval of 24 h, the rats do not discriminate between the two objects, as indicated by similar times spent in exploring them. Male rats (220−330 g) were always submitted to three sessions of the test. The first one was a session of habituation (2 min) to the experimental conditions. During the acquisition session, the rats were presented with the two similar objects. The third session (3 min) consisted of the recognition test between the familiar and the new object, one object presented during the acquisition session was replaced by a new object. The times spent in exploring the familiar and new objects during the last session were recorded separately, and the difference between the two exploration times was taken as the discrimination index (Δ new-fam). Compound or vehicle was orally administered 1 h

before each session of the test. Statistical analyses (ANOVA following by Dunnett's test) were performed both on individual exploration of familiar and new objects and on the difference in duration of exploration of the two objects.

Effect on Excitatory Postsynaptic Response Evoked in CA1 Area on Rat Hippocampal Slices in Vitro. After light anesthesia with isoflurane, the male Wistar rat (200−300 g) was decapitated; the brain was rapidly removed and placed in cold artificial cerebrospinal fluid (ACSF) solution (4 °C). The two hemispheres were separated, and both hippocampi were carefully isolated. Transversal hippocampal slices of 400 μm thickness were cut parallel to the long axis with a tissue chopper. Slices were transferred to a rapid-flow rectangular recording chamber, continuously superfused with ACSF solution (30 ± 1 °C). The recording microelectrodes were filled with 2 M NaCl and had a resistance between 3 and 8 MΩ. The stimulating electrode consisted of a 200 mm bipolar tungsten electrode. Extracellular excitatory postsynaptic field potentials were elicited in the dendritic field of the CA1 area by stimulating the Schaeffer collaterals every 30 s with a stimulus intensity that evoked approximately 50% of the maximum evoked response. This synaptic response resulted from the direct activation of postsynaptic AMPA receptors. The compound was bath-applied during 10 min at successively increasing concentrations. A wash period without drug during two applications was performed until a recovery of the synaptic response was obtained. The amplitude of the response (mV) was determined by measuring the amplitude of the peak voltage of the negative set with respect to the baseline voltage. The area of the response (mV/ms) was determined by measuring the area under the negative set with respect to the baseline voltage. The half-width (ms) was determined by measuring the width of the signal at half of the maximal amplitude. The amplitude, area, and half-width of the synaptic response were averaged on five consecutive pulses and were normalized against the average of the amplitude, area, and half-width of five consecutive synaptic responses recorded before the application of the compound (control value taken as 100%). The effect of the compound on the synaptic response was compared to the effect of the vehicle (two-way ANOVA followed by Dunnett's test).

Effect on Long-Term Potentiation (LTP) of the Postsynaptic Response Evoked in the Dentate Gyrus on Anesthetized Rats. Extracellular excitatory postsynaptic field potentials were recorded in the dentate gyrus following stimulation of the perforant path on anesthetised rats. A potentiation of the response was induced by a short high frequent stimulation or tetanus. Long-term potentiation (LTP) of the synaptic response is considered to be one of the synaptic plastic mechanisms underlying learning and memory processes.[21]

Male Wistar rats (300−400 g) were anaesthetised by ip injection of 400 mg/kg chloral hydrate and were fixed in a stereotaxic frame. Body temperature was monitored continuously by a rectal probe and was maintained at 36.6−37 °C. The recording microelectrode was an epoxy-coated tungsten electrode with a tip diameter of 15 mm and a resistance of 9−12 MΩ. The stimulating electrode was a bipolar concentric tungsten-stimulating electrode (diameter: 200 mm, insulated except for the tips). Both electrodes were lowered stereotaxically using the atlas of Paxinos and Watson (bregma served as reference). The recording electrode was placed in the dentate gyrus at the following coordinates: AP = −2.8−3 mm, ML = 1.6−1.8 mm, and DV = 3−4 mm from the surface of the brain. The stimulating electrode was placed in the perforant path in the same hemisphere (AP = −7.3−7.8 mm, ML = 4.1−4.3 mm, and DV = 2.0−3.0 mm from the surface of the brain). A 60 min baseline period was first completed, during which single pulse stimuli (0.1 ms) were delivered every 15 s with an intensity that evoked 40% of the maximal response. Rats received then ip injection of the compound or vehicle. After 60 min, a tetanus was delivered (four bursts (400 Hz, 20 ms) given at 0.1 Hz; stimulus intensity that evoked 70% of the maximal postsynaptic response). Recording continued for 3 h at the same rate and intensity of stimulation as during the baseline recording period. The amplitude of the response (mV) was determined by measuring the amplitude of the peak voltage of the negative set with respect to the baseline voltage. The amplitude was calculated from an average of eight consecutive pulses and was normalized against the average amplitude of the response obtained during the baseline period (control value taken as 100%). First, the effect of the compound on the amplitude of the synaptic response prior the tetanus was compared to the effect of the vehicle with a two-way ANOVA followed by Dunnett's test. Second, a two-way ANOVA followed by Dunnett's test compared the effect of the compound on the potentiation of the response evoked by the tetanus to the effect of the vehicle.

In Vitro Metabolism Study. The parent compounds were dissolved in methanol and added directly to the incubation medium in order to reach, in both cases, a final substrate concentration of 200 μM and a final percentage in methanol lower than 1%. The incubations were performed at 37 °C in a water shacking bath with a final protein content of 1 mg/mL in a total volume of 1 mL. The reactions were initiated by addition of 100 μL of a NADPH generating system consisting of 1 mM NADP, 1 mM NAD, 5 mM G6P, 1 IU/mL G6PDH, and 0.05 M $MgCl_2$ in a 100 mM potassium phosphate buffer at pH 7.4. The reactions were stopped after an incubation time of 60 min by addition of 1 volume of methanol and 2 volumes of acetonitrile and by a subsequent vortexing step. Precipitated proteins were removed by centrifugation at 2000*g* for 5 min. The supernatant was further decanted into a glass tube, and organic solvents were evaporated under an inert nitrogen flux in order to concentrate the samples. The residue was finally redissolved in 300 μL of HPLC mobile phase consisting of a mixture of 10 mM ammonium formate buffer, pH 3.0, and acetonitrile (50/50, v/v). Nonincubated samples (T_0) were also realized for comparison. The latter samples were prepared by addition of 1 volume of methanol and 2 volumes of acetonitrile before supplementing the incubation medium with the NADPH generating system.

LC−MS/MS Conditions. The liquid chromatography analyses were carried out on an HPLC Alliance 2695 system obtained from Waters (Milford, MA). The samples (10 μL) were injected with an autosampler, and the analytes were separated using an Omnispher C18 (100 mm × 3 mm; particle size, 3 μm) from Varian (Middelburgh, The Netherlands) with a linear gradient consisting of buffer A (0.2% formic acid in water/methanol (95/5)) and buffer B (acetonitrile (ACN)), with a flow rate of 0.30 mL/min at 50 °C.

The gradient schedule was as follows: 5−95% B linear (0−20 min), 95% B (20−21 min). The column was re-equilibrated with 5% solvent B for a 10 min period with initial conditions prior to the next injection. Mass spectral analyses were performed in a positive electrospray ionization mode on a hybrid analyzer Q-Tof II mass spectrometer from Micromass (Manchester, U.K.) directly coupled to the liquid chromatography device. The metabolite detection was performed by monitoring the total ionic current (TIC) with 0.5 s regular intervals and a range m/z extending from 90 to 650. The optimum cones voltages, capillary probe voltages, and ionization energy were respectively 21 V, 3 V, and 10 eV. Collision induced dissociation (CID) was performed using a collision energy ramp between 20 and 25 eV with Ar as collision gas. For accurate mass measurements the mass spectrometer was calibrated with a 0.1% phosphoric acid/ACN solution (50/50) to give a resolution of about 9000. Leukine eukephaline (m/z 556.227) was used for lock-mass correction during accurate mass measurements of detected metabolites and their productions resulting from CID. All the data acquisitions were achieved using MassLynx, version 4.1, software.

LC−SPE−NMR Conditions. The liquid chromatography separations were carried out on an Agilent 1100 series liquid

chromatography system from Agilent (Waldbronn, Germany) equipped with a quaternary pump, a column thermostat, an autosampler, and a variable set-length detector. The analyte separations were performed on a Alltech Hypersil BDS C18 (150 mm × 4.6 mm; particle size, 3 μm) from Alltech (Breda, The Netherlands) using mobile phases A (0.2% formic acid in water) and B (acetonitrile (ACN)) with a flow rate of 0.8 mL/min, at 40 °C and the following linear gradient: 0 min, 20% ACN; 20 min, 40% ACN; 25 min, 60% ACN; 30 min, 20% ACN.

The eluate followed the flow path to a Prospekt II automated solid phase extraction (SPE) unit from Bruker/Spark Holland (Emmen, The Netherlands) under the control of Bruker Hystar 3.0 software, and the peaks of interest (detected using the UV response at 254 nm) were trapped on Hysphere GP cartridges (10 mm × 2 mm) from Spark Holland (Emmen, The Netherlands) by addition of water at a 2 mL/min flow rate to the postcolumn eluate. Three consecutive injections (80 μL per injection) were trapped on the same cartridge in order to enhance the metabolite concentration. After the trapping process, the cartridges were dried under a constant nitrogen flow to remove residual nondeuterated solvents. The collected fractions were further directly transferred from the SPE cartridges to 1.7 mm NMR tubes (Match System, Bruker) with 180 μL of acetonitrile-d_3 through the use of a liquid handler (Bruker-Gilson). The NMR measurements were carried out using an AV500 MHz spectrometer from Bruker BioSpin (Rheinstetten, Germany) equipped with a 5 mm BBI probe with z-gradient flow probe.

Determination of pK_a Values. Determination of pK_a values was achieved at 25 °C using the UV method (Perkin-Elmer UV−visible 554 spectrophotometer) with buffers ranging from pH 6.0 to pH 11.0. The pK_a values were calculated by the Debye−Hückel equation at the wavelength giving the maximum absorbance.[22]

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Supporting Information Available: Elemental analysis results for the new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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