Synthesis and Evaluation of Pharmacological and Pharmacokinetic Properties of 11*H*-[1,2,4]Triazolo[4,5-*c*][2,3]benzodiazepin-3(2*H*)-ones

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A series of 2,3-benzodiazepine derivatives has been previously described as noncompetitive AMPA-type glutamate receptor antagonists potentially useful for treatment of epilepsy. To further explore the structure−activity relationships of AMPA antagonists, a series of 11*H*-[1,2,4]triazolo[4,5-*c*][2,3]benzodiazepin-3(2*H*)-ones (**6**) was synthesized starting from the corresponding bicyclic 1-aryl-3,5-dihydro-7,8-dimethoxy-4*H*-2,3-benzodiazepin-4-ones (**2**, CFM). The new compounds were found to possess anticonvulsant effects against seizures induced both by means of auditory stimulation in DBA/2 mice and by pentylenetetrazole or maximal electroshock in Swiss mice. In addition, they antagonize the AMPA-induced seizures, and their anticonvulsant activity is reversed by pretreatment with aniracetam, thus suggesting the involvement of AMPA receptors. The pharmacological studies revealed that the 11*H*-[1,2,4]triazolo[4,5-*c*]-[2,3]benzodiazepin-3(2*H*)-ones (**6**) herein reported show anticonvulsant activity comparable to that of their bicyclic precursors. Furthermore, an HPLC study put in evidence that these tricyclic derivatives **6** were converted in vivo into the corresponding **2**, the agents likely to be mainly responsible for the anticonvulsant properties observed.

Introduction

Glutamate (Glu) is the major excitatory neurotransmitter in the vertebrate brain and plays an important role in neuronal activity via different receptor systems. Glutamate receptors (GluRs) are involved in such fundamental processes as neuronal development, learning, and memory. They are also held responsible for the destruction of neural tissue during ischemic states and epileptic seizures and may be involved in a variety of other neurological diseases. This has resulted in an interest in the development of GluR ligands as research tools and potential therapeutic agents.[1,2]

GluRs are divided into metabotropic receptors (mGluRs) and ionotropic receptors (iGluRs), the latter consisting of three primary families: *N*-methyl-D-aspartic acid (NMDA) receptor family and non-NMDA receptor families, composed of the kainic acid (KA) receptor and the 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid (AMPA) receptor.[3] The iGluRs are formed by homo- or hetero-oligomeric assemblies of agonist binding subunits surrounding a central cation-conducting pore. The binding of Glu induces conformational changes which open the channel pore and lead to influx of cations into the postsynaptic cell. At the present time, iGluRs are supposed to be a tetrameric complex of subunits and not a pentameric complex as has hitherto been believed.[4] There are 14 distinct ionotropic GluR subunits with a different distribution in the brain; additionally, some of these subunits are regulated developmentally in an age-related manner. AMPA receptor types are composed of combinations of GluR1−4 subunits, existing as "flip" and "flop" splice variants, which mediate fast excitatory postsynaptic potentials by the flux of Na^+ and Ca^{2+}.[5]

Several AMPA receptor antagonists have been reported in the literature and show promise in terms of their therapeutic potential for the prevention and treatment of a broad range of acute and chronic neurological diseases.[6,7] The AMPA receptor complex has at least three distinct binding sites at which antagonists can act: (a) the Glu binding sites for competitive antagonists, (b) an allosteric site at which noncompetitive antagonists can bind, and (c) a polyamine site, within the ion channel.[8]

A selective and noncompetitive blockade of the AMPA receptor was shown by some 2,3-benzodiazepine derivatives: e.g. 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine (**1**, GYKI 52466) (Chart 1), which possess potent anticonvulsant properties but lack hypnotic activity.[9−19] In particular, some of our previous publications[14−16] reported chemical and biological studies of 1-aryl-3,5-dihydro-7,8-dimethoxy-4*H*-2,3-benzodiazepin-4-ones **2** (CFM) and thiocarbonyl analogues **3** (Chart 1), which have shown marked antiepileptic properties in various seizure models and do not bind to the GABAergic benzodiazepine receptors. Electrophysiological experiments carried out on the lead compound **2f** (CFM-2) and some of its derivatives have confirmed that their anticonvulsant effects, analogous to those of

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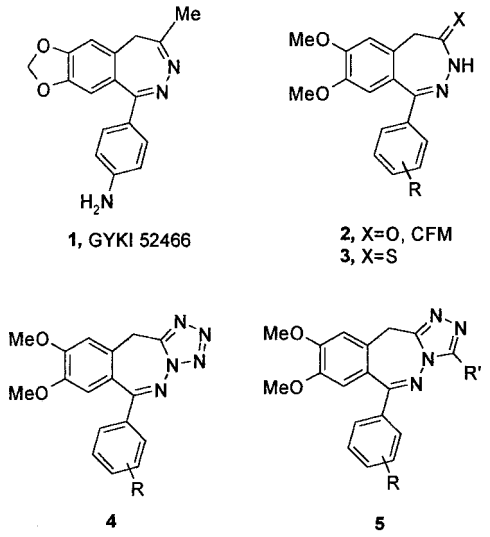
Chart 1



GYKI 52466, are mediated through the AMPA subtype of the iGluR complex in a selective and noncompetitive fashion.

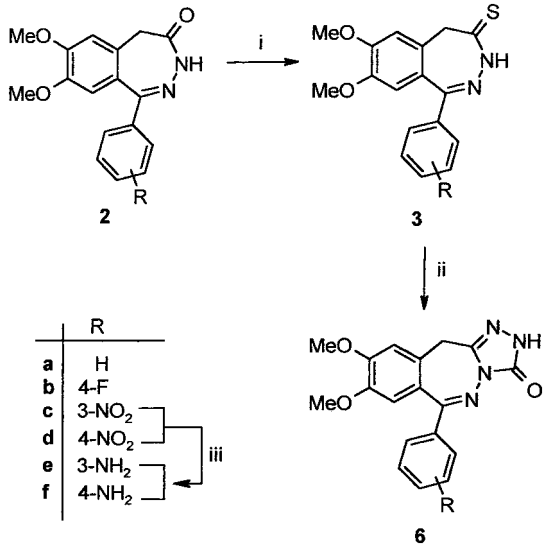
Extensive structure−activity relationship (SARs) studies, aimed at exploring the effects of structural modifications of the heptatomic system of derivatives **2** and at developing AMPA antagonists with increased potency and selectivity, longer-lasting activity, and improved pharmacokinetic features, led to the synthesis of two series of cyclofunctionalized 2,3-benzodiazepines: i.e. 11*H*-tetrazolo[1,5-*c*][2,3]benzodiazepines[19a] **4** and 11*H*-[1,2,4]triazolo[4,5-*c*][2,3]benzodiazepines[19b] **5** (Chart 1), which showed weaker anticonvulsant effects than their parent compounds **2** and **3**.

To determine if the lower potency of the compounds obtained was due to the cyclofunctionalization of the diazepine ring, to the nature of the fused five-membered ring, or to the absence of the lactam moiety, we have now prepared a series of 11*H*-[1,2,4]triazolo[4,5-*c*][2,3]-benzodiazepin-3(2*H*)-ones **6**. The new annelated 2,3-benzodiazepines have been evaluated as anticonvulsant agents in various experimental epilepsy models: i.e. audiogenic seizures in DBA/2 mice and maximal electroshock (MES) and pentylenetetrazole (PTZ)-induced seizures in Swiss mice. In addition, to confirm that their anticonvulsant activity is mediated by interaction with the AMPA receptor system, derivatives **6b**,**f** were also evaluated against sound-induced seizures with concomitant treatment with aniracetam, a positive allosteric modulator of AMPA/KA receptors,[14] and against AMPA-induced seizures. Furthermore, the time course of anticonvulsant activity was also studied to verify if the new compounds had a longer-lasting anticonvulsant activity than GYKI 52466 and CFM derivatives. Another objective of this study was to gain insight into the pharmacokinetic properties of these tricyclic benzodiazepines and to correlate the plasma concentrations of the title compounds and their metabolites with the anticonvulsant effects.

Chemistry

The 11*H*-[1,2,4]triazolo[4,5-*c*][2,3]benzodiazepin-3(2*H*)-ones **6** (Scheme 1) were obtained starting from 3,5-dihydro-4*H*-2,3-benzodiazepin-4-ones **2**, which were activated by transformation into the corresponding thiocarbonyl derivatives **3**, by reaction with Lawesson's reagent. Compounds **2** and **3** were prepared according to a multistep synthetic pathway previously described in our reports.[15,16] By refluxing 3,5-dihydro-4*H*-2,3-benzodiazepine-4-thiones **3a**−**d** with ethyl carbazate, 11*H*-[1,2,4]triazolo[4,5-*c*][2,3]benzodiazepin-3(2*H*)-ones **6a**−**d** were synthesized. Aminophenyl-substituted derivatives **6e,f** were prepared by reduction of the corresponding nitro analogues **6c,d** with tin(II) chloride. The structures of the compounds obtained were supported by elemental analyses and spectroscopic measurements (^{1}H NMR).

Scheme 1[a]



[a] Reagents: (i) Lawesson's reagent, dry toluene, △, 90-120 min; (ii) H_2NNHCO_2Et, *n*-BuOH, △, 24 h; (iii) $SnCl_2$, EtOH, 70 °C, 90 min.

Results and Discussion

The anticonvulsant properties of 11*H*-[1,2,4]triazolo-[4,5-*c*][2,3]benzodiazepin-3(2*H*)-ones **6** were evaluated after intraperitoneal (ip) administration against audiogenic seizures in DBA/2 mice (Table 1), which has been considered an excellent animal model for generalized epilepsy and for screening new anticonvulsant drugs.[20] The results were compared with those of GYKI 52466 and bicyclic parent compounds **2**.

As can be seen from the data reported in Table 1, derivatives **6** possess anticonvulsant potency which is sensitive to the nature of the substituent on the phenyl ring at C-6. The introduction of a fluorine atom at position 4′ and an amino group at position 3′ or 4′ affords derivatives which display anticonvulsant activity comparable to (i.e. **6b**) or higher (i.e. **6e,f**) than that of GYKI 52466 and unsubstituted derivative **6a**. In contrast, the presence of a nitro group reduces activity. Moreover, compounds **6** generally induce less motor impairment than GYKI 52466.

A comparison between the biological results of compounds **6a**−**f** and those of the parent bicyclic derivatives **2a**−**f** reveals that the introduction of the triazolone nucleus on the diazepine skeleton leads to compounds with comparable or higher anticonvulsant potency than the corresponding derivatives **2**.

Table 1. Anticonvulsant Activity of Compounds **1**, **2**, and **6** Against Audiogenic Seizures in DBA/2 Mice, TD_{50} Values on Locomotion Assessed by Rotarod Test, and Relative Lipophilicity (R_m)

	ED_{50} μmol/kg[a] (±95% confidence limits)		TD_{50}, μmol/kg[a]	TI[b]	
compd	clonic phase	tonic phase	locomotor deficit	TD_{50}/ED_{50}	R_m
1[c]	35.8 (24.4−52.4)	25.3 (16.0−40.0)	76.1 (47.5−122)	2.1	−0.368
2a[c]	33.9 (26.0−44.2)	31.8 (24.8−40.6)	142 (87.3−231)	4.2	−0.280
2b	78.0 (46.0−132)	57.2 (41.5−78.8)	>150	ND	−0.280
2c[c]	>120	>120	>150	ND	−0.181
2d[c]	>120	>120	>150	ND	−0.145
2e[c]	19.3 (16.9−22.0)	18.3 (16.0−20.8)	51.5 (34.1−77.8)	2.7	−0.619
2f[c]	15.0 (9.01−24.0)	12.6 (8.01−19.0)	56.8 (39.3−82.1)	3.8	−0.647
6a	44.6 (32.5−61.2)	27.5 (15.4−49.4)	123 (73.8−206)	2.8	−0.508
6b	32.1 (16.3−63.0)	21.8 (14.3−31.9)	90.6 (65.1−126)	2.8	−0.402
6c	56.4 (39.2−81.1)	43.2 (32.5−57.4)	>150	ND	−0.402
6d	74.1 (60.3−91.0)	58.3 (41.3−82.3)	>150	ND	−0.368
6e	20.1 (10.5−38.2)	13.1 (8.04−21.3)	76.8 (59.4−99.3)	3.8	−0.740
6f	16.1 (11.8−22.0)	10.2 (8.67−11.93)	65.8 (46.2−99.8)	4.0	−0.837

[a] All data were calculated according to the method of Litchfield and Wilcoxon.[29] At least 32 animals were used to calculate each ED_{50} and TD_{50} value. [b] TI = therapeutic index and represents the ratio between TD_{50} and ED_{50} (from the clonic phase of the audiogenic seizures). [c] Ref 15. ND = not detectable.

Table 2. Anticonvulsant Activity of **1** and **6** Against the MES- and PTZ-Induced Seizures in Swiss Mice

	ED_{50}, μmol/kg[a] (±95% confidence limits)	
compd	MES	PTZ
1[b]	35.7 (29.3−43.4)	68.3 (56.2−83.1)
6a	60.5 (45.6−80.3)	76.5 (51.8−113)
6b	43.4 (33.5−56.0)	60.5 (45.6−80.3)
6d	75.8 (62.4−92.1)	90.7 (72.2−114)
6e	35.1 (26.8−45.8)	58.9 (43.8−79.1)
6f	30.8 (18.6−51.0)	57.2 (41.5−78.8)

[a] All data were calculated according to the method of Litchfield and Wilcoxon.[29] At least 32 animals were used to calculate each ED_{50} value. [b] Ref 15.

The relative lipophilicity (R_m) of 2,3-benzodiazepines **2** and **6** is summarized in Table 1. Although in both series the most active compound is also the most hydrophilic derivative, a direct correlation between lipophilicity and anticonvulsant activity cannot be pointed out. In fact, compounds with the same R_m values (i.e. **2a** and **2b**; **6b** and **6c**) show different potencies, thus suggesting the importance of other parameters.

Compounds **6** were further evaluated against seizures induced by MES and PTZ in Swiss mice (Table 2). As shown in Table 2, the tonic extension of the seizures induced by MES and the clonic phase of the seizures induced by PTZ were significantly reduced 45 min after ip administration of compounds **6**, with the same rank order observed in the audiogenic seizures test.

To establish that the anticonvulsant properties of compounds **6** are mediated by interaction with the AMPA receptor complex, an additional test against AMPA-induced seizures in DBA/2 mice was carried out on the most active derivatives **6b**,**f**. As shown in Table 3, these compounds afforded effective protection against the clonic and tonic phases of the seizures induced by intracerebroventricular (icv) administration of AMPA. Furthermore, the influence of aniracetam, a positive allosteric modulator of the AMPA/KA receptor,[21] on the anticonvulsant activity of derivatives **6b**,**f** in DBA/2 mice was also tested (Table 3). An icv injection of aniracetam (50 nmol/mouse) on its own had no convulsant activity. The administration of aniracetam 30 min before the injection of the tested compounds reduced the anticonvulsant effects of compounds **6b**,**f** and shifted to the right the dose−response curves with a pattern of activity similar to that of **1** and **2**.[15]

Another objective of the present study was to explore a possible metabolic pathway of these new tricyclic 2,3-benzodiazepines and to correlate the plasma concentrations with their anticonvulsant activity. An HPLC method has been developed to detect the most active derivative **6f** and its metabolites and to determine the time profile of plasma concentrations of rats treated ip with **6f**. In the chromatogram from a rat plasma sample obtained after administration of **6f**, the peak corresponding to **2f** was also observed; the formation of **2f** was monitored (Figure 1) and it was observed that as **6f** plasma concentration decreased, the **2f** plasma levels increased, thus suggesting that **6f** was metabolically converted in vivo into **2f**. A comparison between the time course of plasma levels (Figure 1) and that of anticonvulsant activity (Figure 2) showed that compound **6f** had low anticonvulsant activity during the period of peak plasma concentration (15 min), whereas maximal effects of **6f** were observed from 45−120 min after ip administration, that is, after biotransformation into the bicyclic precursor **2f**. On the other hand, when tested at 15 min from administration **6f** was more active than **2f** (ED_{50} 27.8 μmol/kg for **6f** and ED_{50} >50 μmol/kg for **2f**) thus suggesting that **6f** acts both as active compound and after biotransformation. This behavior could also explain its longer-lasting activity compared to analogue **2f** (Figure 2).

The mechanism of biotransformation has not been clarified to date. A possible metabolic pathway might be the cleavage of triazolone ring with subsequent decarboxylation and hydrolytic processes. Because compound **6f** in vivo furnishes the corresponding noncompetitive AMPA receptor antagonist **2f**, we suggest that the anticonvulsant effects observed are attributable to a noncompetitive blockade of the same receptor. In conclusion, a new series of annelated 2,3-benzodiazepines was prepared and characterized. The synthetic approach has been demonstrated to be sufficiently flexible to afford a variety of 6-substituted 11*H*-[1,2,4]-triazolo[4,5-*c*][2,3]benzodiazepin-3(2*H*)-ones (**6**).

In addition, the present study demonstrated that compounds **6** are slightly more potent and slightly less toxic but have a significant improvement in terms of

Table 3. ED_{50} Values of **1** and **6b,f** Against AMPA-Induced Seizures and Against Audiogenic Seizures after Pretreatment with Aniracetam in DBA/2 Mice

	ED_{50}, μmol/kg[a] (±95% confidence limits)			
	AMPA[b]		pretreatment with aniracetam[c]	
compd	clonic phase	tonic phase	clonic phase	tonic phase
1[d]	57.5 (43.5−76.0)	40.5 (26.3−60.8)	134 (88.8−203)*	100 (63.4−158)*
6b	42.1 (27.5−64.3)	32.2 (20.6−50.3)	50.9 (28.3−91.5)	38.4 (23.5−62.7)*
6f	22.1 (13.8−37.2)	18.1 (11.4−28.7)	38.4 (20.5−71.9)*	24.1 (12.7−45.5)*

[a] All data were calculated according to the method of Litchfield and Wilcoxon.[29] At least 32 animals were used to calculate each ED_{50} value. [b] AMPA was administered icv at the CD_{97} for either clonus (9.7 nmol) or forelimb tonic extension (11.7 nmol) 30 min after injection of tested compounds. [c] Significant differences between ED_{50} values of the group treated with aniracetam + 2,3-benzodiazepine and the group treated with 2,3-benzodiazepine alone (Table 1) are denoted: $*p < 0.01$. [d] Ref 15.

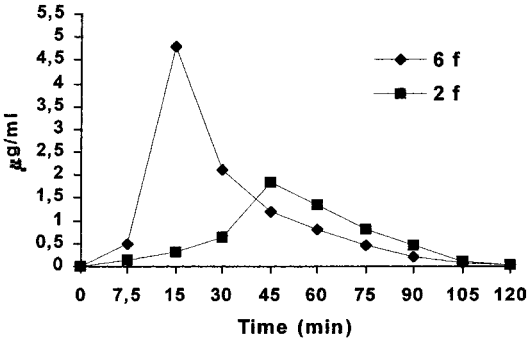


Figure 1. Time profiles of plasma levels after ip administration of **6f** in rats. The ordinate shows the plasma level; the abscissa shows the time after ip administration of drug in minutes.

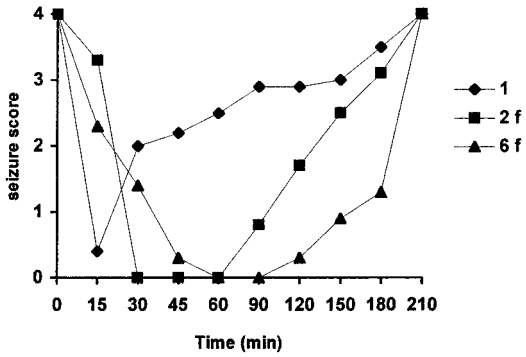


Figure 2. Anticonvulsant effects of **2f**, **6f**, and **1** (33 μmol/kg) against audiogenic seizures in DBA/2 mice. The ordinate shows seizure score; the abscissa shows the time after ip administration of drug in minutes. Ten animals were used for the determination of each point.

time of action than **1** and **2**. They undergo biotransformation into the corresponding bicyclic derivatives **2** which are proposed to be the main agents responsible for the anticonvulsant activity of the tricyclic derivatives in various seizure models. Also in this series of AMPA antagonists the trend that the 4-aminophenyl derivative is more potent than other analogues has been confirmed.

Experimental Section

Chemistry. Melting points were determined on a Kofler hot stage apparatus and are uncorrected. Elemental analyses (C, H, N) were carried out on a Carlo Erba model 1106 elemental analyzer and the results are within 0.4% of the theoretical values. Merck silica gel 60 F_{254} plates were used for analytical TLC; column chromatography was performed on Merck silica gel 60 (70−230 mesh). ^{1}H NMR spectra were measured in $CDCl_3$ with a Varian Gemini 300 spectrometer; chemical shifts are expressed in δ (ppm) relative to TMS as internal standard and coupling constants (*J*) in Hz. All exchangeable protons were confirmed by addition of D_2O.

Compounds **2a**−**d** and **3a**−**d** were prepared according to a procedure previously described;[15,16] derivatives **2b** and **3b** are new products.

3,5-Dihydro-7,8-dimethoxy-1-(4-fluorophenyl)-4*H*-2,3-benzodiazepin-4-one (2b). Mp: 208−211 °C, yield 51%. ^{1}H NMR: 3.50 (s, 2H, CH_2), 3.73 (s, 3H, MeO-7), 3.97 (s, 3H, MeO-8), 6.64 (s, 1H, H-6), 6.85 (s, 1H, H-9), 7.12 (dd, 2H, $J_{HH} = 8.5$ and $J_{HF} = 8.7$, H-3′,5′), 7.63 (dd, 2H, $J_{HF} = 5.4$ and $J_{HH} = 8.5$, H-2′,6′), 8.45 (bs, 1H, NH). Anal. ($C_{17}H_{15}FN_2O_3$) C, H, N.

3,5-Dihydro-7,8-dimethoxy-1-(4-fluorophenyl)-4*H*-2,3-benzodiazepine-4-thione (3b). Mp: 176−179 °C, yield 89%. ^{1}H NMR: 3.73 (s, 3H, MeO-7), 3.95 (bs, 2H, CH_2), 3.97 (s, 3H, MeO-8), 6.60 (s, 1H, H-6), 6.87 (s, 1H, H-9), 7.14 (dd, 2H, $J_{HH} = 8.5$ and $J_{HF} = 8.8$, H-3′,5′), 7.63 (dd, 2H, $J_{HF} = 5.5$ and $J_{HH} = 8.5$, H-2′,6′), 9.93 (bs, 1H, NH). Anal. ($C_{17}H_{15}FN_2O_2S$) C, H, N.

General Procedure for Synthesis of 11*H*-[1,2,4]Triazolo[4,5-*c*][2,3]benzodiazepin-3(2*H*)-ones 6a−d. A solution of 2,3-benzodiazepine-4-thione **3a**−**d** (1 mmol) and ethyl carbazate (1.5 mmol) in *n*-BuOH (20 mL) was refluxed for 24−30 h. The solution was evaporated to dryness, the oil crystallized by adding a small amount of acetone, and the products **6a**−**d** were recrystallized from EtOAc.

8,9-Dimethoxy-6-phenyl-11*H*-[1,2,4]triazolo[4,5-*c*][2,3]-benzodiazepin-3(2*H*)-one (6a). Mp: >300 °C, yield 62%. ^{1}H NMR: 3.68 (s, 3H, MeO-8), 3.84 (s, 2H, CH_2), 3.97 (s, 3H, MeO-9), 6.65 (s, 1H, H-7), 6.85 (s, 1H, H-10), 7.41−7.81 (m, 5H, ArH), 9.58 (bs, 1H, NH). Anal. ($C_{18}H_{16}N_4O_3$) C, H, N.

8,9-Dimethoxy-6-(4-fluorophenyl)-11*H*-[1,2,4]triazolo-[4,5-*c*][2,3]benzodiazepin-3(2*H*)-one (6b). Mp: >300 °C, yield 64%. ^{1}H NMR: 3.69 (s, 3H, MeO-8), 3.83 (s, 2H, CH_2), 3.97 (s, 3H, MeO-9), 6.61 (s, 1H, H-7), 6.85 (s, 1H, H-10), 7.13 (dd, 2H, $J_{HH} = 8.5$ and $J_{HF} = 8.8$, H-3′,5′), 7.82 (dd, 2H, $J_{HF} = 5.3$ and $J_{HH} = 8.5$, H-2′,6′), 9.77 (bs, 1H, NH). Anal. ($C_{18}H_{15}FN_4O_3$) C, H, N.

8,9-Dimethoxy-6-(3-nitrophenyl)-11*H*-[1,2,4]triazolo-[4,5-*c*][2,3]benzodiazepin-3(2*H*)-one (6c). Mp: >300 °C, yield 58%. ^{1}H NMR: 3.69 (s, 3H, MeO-8), 3.81 (s, 2H, CH_2), 4.00 (s, 3H, MeO-9), 6.56 (s, 1H, H-7), 6.99 (s, 1H, H-10), 7.70 (t, 1H, $J = 8.0$, H-5′), 8.38 (m, 2H, H-4′,6′), 8.49 (d, 2H, $J = 1.8$, H-2′), 8.80 (bs, 1H, NH). Anal. ($C_{18}H_{15}N_5O_5$) C, H, N.

8,9-Dimethoxy-6-(4-nitrophenyl)-11*H*-[1,2,4]triazolo-[4,5-*c*][2,3]benzodiazepin-3(2*H*)-one (6d). Mp: >300 °C, yield 54%. ^{1}H NMR: 3.69 (s, 3H, MeO-8), 3.87 (s, 2H, CH_2), 3.99 (s, 3H, MeO-9), 6.53 (s, 1H, H-7), 6.87 (s, 1H, H-10), 8.07 (d, 2H, $J = 8.9$, H-2′,6′), 8.30 (d, 2H, $J = 8.9$, H-3′,5′), 8.65 (bs, 1H, NH). Anal. ($C_{18}H_{15}N_5O_5$) C, H, N.

General Procedure for Synthesis of Aminophenyl Derivatives 6e,f. A mixture of nitro derivative **6c** or **6d** (0.2 mmol) and $SnCl_2 \cdot 2H_2O$ (1 mmol) in EtOH (20 mL) was heated on a boiling water bath for 90 min. The mixture was cooled, poured in water, neutralized with a solution of $NaHCO_3$ and extracted with EtOAc. The organic phase was dried over Na_2SO_4, the solvent was evaporated and from the crude residue compounds **6e**,**f** were purified by column chromatography using $CHCl_3$/MeOH (90:10) as eluant.

6-(3-Aminophenyl)-8,9-dimethoxy-11*H*-[1,2,4]triazolo-[4,5-*c*][2,3]benzodiazepin-3(2*H*)-one (6e). Mp: >300 °C,

yield 65.3%. ^{1}H NMR: 3.70 (s, 3H, MeO-8), 3.80 (bs, 2H, NH_2), 3.81 (s, 2H, CH_2), 3.96 (s, 3H, MeO-9), 6.69 (s, 1H, H-7), 6.80 (m, 1H, H-4′), 6.82 (s, 1H, H-10), 6.89 (m, 1H, H-6′), 7.17 (t, 1H, J = 7.8, H-5′), 7.34 (m, 2H, H-2′). Anal. ($C_{18}H_{17}N_5O_3$) C, H, N.

6-(4-Aminophenyl)-8,9-dimethoxy-11*H*-[1,2,4]triazolo-[4,5-*c*][2,3]benzodiazepin-3(2*H*)-one (6f). Mp: >300 °C, yield 40.5%. ^{1}H NMR: 3.72 (s, 3H, MeO-8), 3.79 (s, 2H, CH_2), 3.97 (s, 3H, MeO-9), 4.01(bs, 2H, NH_2), 6.74 (s, 1H, H-7), 6.69 (d, 2H, J = 8.6, H-3′,5′), 6.82 (s, 1H, H-10), 7.64 (d, 2H, J = 8.6, H-2′,6′), 8.41 (bs, 1H, NH). Anal. ($C_{18}H_{17}N_5O_3$) C, H, N.

Lipophilicity Measurements. The relative lipophilicity (R_m) of the compounds was measured by reversed-phase high-performance thin-layer chromatography (RP-HPTLC) according to the method previously described.[22] Briefly, Whatman KC18F plates were used as the nonpolar stationary phase. The plates were dried at 105 °C for 1 h before use. The polar mobile phase was a 2:1 (v/v) mixture of acetone and water. Each compound was dissolved in $CHCl_3$ (3 mg/mL), and 1 μL of solution was applied onto the plate. The experiments were repeated five times with a different disposition of the compounds on the plate. The R_f values were expressed as the mean values of the five determinations. The R_m values were calculated from the experimental R_f values according to the formula: $R_m = \log[(1/R_f) - 1]$. Higher R_m values indicate higher lipophilicity.

Testing of Anticonvulsant Activity. 1. Audiogenic Seizures in DBA/2 Mice. All experiments were performed with DBA/2 mice which are genetically susceptible to sound-induced seizures.[23] DBA/2 mice (8−12 g, 22−25 days old) were purchased from Charles River (Calco, Como, Italy). Groups of 10 mice of either sex were exposed to auditory stimulation 30 min following administration of vehicle or each dose of drugs studied. The compounds were given ip (0.1 mL/10 g of body weight of the mouse) as a freshly prepared solution in 50% dimethyl sulfoxide (DMSO) and 50% sterile saline (0.9% NaCl). Individual mice were placed under a hemispheric Perspex dome (diameter 58 cm), and 60 s was allowed for habituation and assessment of locomotor activity. Auditory stimulation (12−16 kHz, 109 dB) was applied for 60 s or until tonic extension occurred and induced a sequential seizure response in control DBA/2 mice, consisting of an early wild running phase, followed by generalized myoclonus and tonic flexion and extension sometimes followed by respiratory arrest. The control and drug-treated mice were scored for latency to and incidence of the different phases of the seizures.[24] The time course of the anticonvulsant action of **6f** (33 μmol/kg) was determined following administration to groups of 10 mice that were tested for sound-induced seizure responses at 5−210 min after drug administration.

2. MES Test in Swiss Mice. Male Swiss mice (20−26 g, 42−48 days old) were purchased from Charles River (Calco, Como, Italy). Electrical stimuli were applied via ear-clip electrodes to the mice (rectangular constant current impulses, amplitude 50 mA, width 20 ms, frequency 35 Hz, duration 400 ms) according to the method of Swinyard et al.[25] Abolition of tonic hindlimb extension after drug treatment was considered as the endpoint of protection. In general, the dose−response curves were estimated by testing 4−5 doses using 8−10 mice for each dose.

3. PTZ-Induced Seizures in Swiss Mice. Male Swiss mice were pretreated with vehicle or drug 45 min before the subcutaneous (sc) administration of pentylenetetrazole. For systemic injections, all tested compounds were given ip (0.1 mL/10 g of body weight of the mouse) as a freshly prepared solution in 50% DMSO and 50% sterile saline (0.9% NaCl). The convulsive dose 97 (CD_{97}) of PTZ (85 mg/kg) was applied and the animals were observed for 30 min. A threshold convulsion was an episode of clonic spasms lasting for at least 5 s. The absence of this threshold convulsion over 30 min indicated that the tested substance had the ability to elevate PTZ seizure threshold.[26]

4. AMPA-Induced Seizures in DBA/2 Mice. Seizures were also induced by icv injection of AMPA. The CD_{50} of AMPA for clonus was 1.76 nmol (1.06−3.07), while that for tonus was 2.90 (1.83−4.58) nmol. For icv injection, the mice were anesthetized with diethyl ether, and injections were made in the left or right lateral ventricle (coordinates 1 mm posterior and 1 mm lateral to the bregma; depth 2.4 mm) using a 10-μL Hamilton microsyringe (type 701N) fitted with a nylon cuff on the needle as previously described.[27] Injections of drugs by this procedure led to a uniform distribution throughout the ventricular system within 10 min. The animals were placed singly in a 30- × 30- × 30-cm box, and the observation time was 30 min after the administration of AMPA.

5. Pretreatment with Aniracetam. The icv microinjection of aniracetam was performed in accordance with experimental procedures previously described for AMPA microinjection.[27] The dose of aniracetam (50 nmol icv) was administered 60 min before auditory stimulation or 30 min before each compound in DBA/2 mice.

Effects on Motor Movements. Groups of 10 mice were trained to make coordinated motor movements continuously for 2 min on a rotarod, 3-cm diameter, at 8 rpm (U. Basile, Comerio, Varese, Italy). Impairment of coordinated motor movements was defined as the inability of the mice to remain on the rotarod for a 2-min test period.[28] The ability of the mice to remain on the rotarod was tested 30 min after administration of various compounds.

HPLC Assay. Plasma levels of **6f** were quantified by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection, according to a suitable modified method recently described by Rizzo et al.[18] Briefly, the method included a single-step extraction and the use of internal standard for quantitation. The HPLC system consisted of a Jasco LG-980-02 ternary gradient unit with a Jasco PU-980 pump and a 20-μL loop injection valve; the detector used was a Jasco UV-975 set at 240 nm; all data were elaborated by a Borwin chromatography software, version 1.21. A Hypersil ODS 5-μm (250-× 4.6-mm) column with an ODS guard column was used. The column was eluted isocratically at room temperature with phosphate buffer/MeOH/MeCN (57:17:26, v/v), pH 5.8, at a flow rate of 1 mL/min. Stock solution (1 mg/mL) of **6f** was prepared in MeOH and refrigerated at 4 °C. Working solutions were made by appropriate dilution in MeOH and used to prepare plasma standards for the calibration curves. For the extraction procedures an aliquot of 0.5 mL of rat plasma was mixed with 0.5 mL of carbonate buffer, pH 9.0. The sample was applied to Extrelut NT 1 (Merck, Germany), a prepacked glass column. After 10 min, Et_2O (6 mL) was added to the column. The eluate was collected and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 100 μL of mobile phase and a 20-μL aliquot was injected into the chromatographic system. A linear response was observed over the examined concentration range (0.5−5 μg/mL). The lower limit of detection was 70 ng/mL for **6f**.

Statistical Analysis. Statistical comparisons between groups of control and drug-treated animals were made using Fisher's exact probability test (incidence of the seizure phases). The ED_{50} value of each phase of the audiogenic seizure or seizures induced by MES or PTZ was determined for each dose of compound administered, and dose−response curves were fitted using a computer program by the Litchfield and Wilcoxon's method.[29] The relative anticonvulsant activities were determined by comparison of respective ED_{50} values. Statistical significance between control and test groups of data means was tested using a two-tailed Student's t-test.

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