Synthesis, Chiral Resolution, and Enantiopharmacology of a Potent 2,3-Benzodiazepine Derivative as Noncompetitive AMPA Receptor Antagonist

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This paper describes the synthesis of racemic 3,5-dihydro-5-methyl-7,8-methylenedioxy-4*H*-2,3-benzodiazepin-4-one (±)-**5**, attempted stereoselective synthesis of its enantiomers, chiral HPLC resolution of the racemate, and assignment of the absolute configuration. Enantiomer (5*S*)-(−)-**5** is provided with an in vivo anticonvulsant activity 8 times higher than its enantiomer (5*R*)-(+)-**5**. This result is confirmed in the in vitro test by the ability to inhibit the kainate-induced increase of the $[Ca^{2+}]_i$ in a primary culture of rat cerebellar granule cells which express α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors. Binding affinity of compound (±)-**5** at the AMPA and *N*-methyl-D-aspartic acid (NMDA) receptors was also evaluated.

Introduction

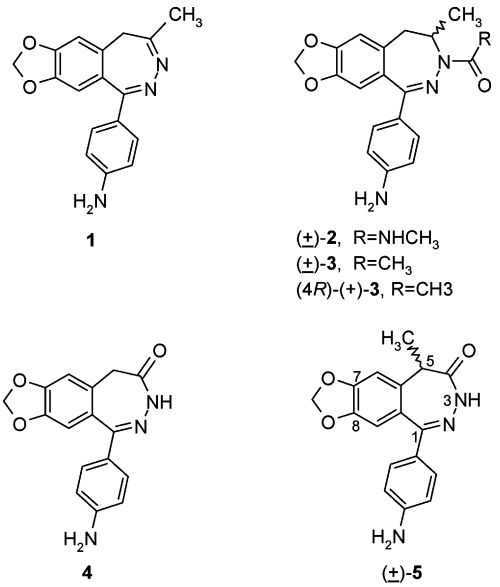
Evidence suggests that glutamate, the major excitatory neurotransmitter in the central nervous system, is involved in several neurodegenerative syndromes, including brain ischemia and epilepsy. In the past few years there has been considerable interest in noncompetitive α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor antagonists since prototype compounds, e.g. 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine (**1**, GYKI 52466) (Chart 1), have demonstrated significant anticonvulsant and neuroprotective action, thus providing the basis for extensive research in this area.[1−3]

Highly active analogues of **1** have been found, e.g. 3,4-dihydro-3-*N*-methylcarbamoyl [(±)-**2**, GYKI 53655] and 3,4-dihydro-3-*N*-acetyl [(±)-**3**, GYKI 53405] derivatives (Chart 1).[4] The enantioselectivity of derivatives (±)-**2** and (±)-**3** has been evaluated, and the eutomer turned out to be the (4*R*)-enantiomer.[3] Subsequently, the eutomer (4*R*)-(+)-**3** was chosen as a drug candidate and is now in clinical investigation as an anticonvulsant and antiischemic (LY 300164, Talampanel).[5]

We have previously investigated[6,7] a series of 1-aryl-3,5-dihydro-7,8-methylenedioxy-4*H*-2,3-benzodiazepin-4-ones, e.g. **4** (Chart 1), which have been shown to possess remarkable anticonvulsant properties and are endowed with a potency higher than that of **1**. In this context we noticed that the substitution of the iminohydrazone portion of the diazepine nucleus of **1** by the iminohydrazide moiety increases the anticonvulsant activity. Moreover, we have recently demonstrated that derivative **4** interacts with the same allosteric AMPA receptor binding site of **1**.[8]

A group of scientists from the IVAX Drug Research Institute, using a set of 2,3-benzodiazepines provided with high affinity to the allosteric AMPA binding site, has recently derived two four-point pharmacophore models.[9] Common features of these models are (i) the electron donor amino group, (ii) the 1-phenyl ring as hydrophobic moiety, and (iii) a second electron-donating group which is the ether moiety attached to C-8. Among the two models, the difference resides in the fourth attachment point. Specifically, while in one model the fourth interaction point involves the lone pair of **1** N-3 atom, in the other one the lone pairs of the C-4 carbonyl oxygen of **4** are taken into account. (4*R*)-(+)-**3** can fit both models due to the rotating 3N−C bond. The authors assume that the second pharmacophore model is more appropriate, since a polar group at C-4, in replacement of the alkyl group, gives a stronger and energetically more pronounced interaction with the binding protein; as a consequence, compounds which meet this requirement, e.g. (4*R*)-(+)-**3** and **4**, possess a higher affinity.

Chart 1



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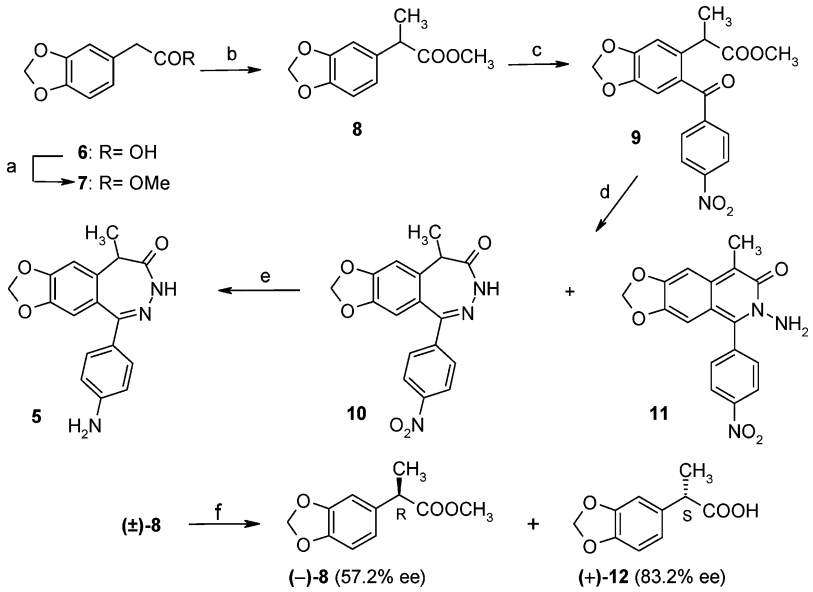
Scheme 1[a]



[a] Reagents and conditions:[21] (a) MeOH, HCl_g, rt, 30 min; (b) KH, THF anhydrous, 30 min, −70 °C, then CH_3I, −70 °C, rt, 16 h; (c) 4-nitrobenzoic acid, P_2O_5, $(CH_2Cl)_2$, rt, 16 h; (d) NH_2NH_2, *n*-BuOH, reflux, 24 h; (e) Raney-Ni, ammonium formate, EtOH, reflux, 2 h; (f) carboxylesterase NP, phosphate, pH 7.0.

Considering that the carbonyl group at C-4, which represents a steady point of our ongoing studies, characterizes the most promising pharmacophore model, we planned an extension of our investigations on the structure−activity relationships of 2,3-benzodiazepin-4-ones by introducing a lipophilic substituent at C-5 of compound **4**. Its effect on the anticonvulsant activity as well as on the affinity for AMPA receptors was consequently checked. Now we report the synthesis of 3,5-dihydro-5-methyl-7,8-methylenedioxy-4*H*-2,3-benzodiazepin-4-one (±)-**5** (Chart 1). Due to the different pharmacological profile previously observed in the enantiopure forms of **3**, we attempted the stereoselective synthesis of enantiomers (+)-**5** and (−)-**5**. Since the chemical approach failed, we resorted to chiral HPLC to isolate sizable quantities of single enantiomers of (±)-**5** and to assign the absolute configuration. In this way, we were able to characterize their in vivo and in vitro pharmacological profile and to evaluate their enantiopharmacology.

Results and Discussion

Chemistry. The synthesis of (±)-**5** was carried out following the reaction sequence reported in Scheme 1. Commercially available 3,4-methylenedioxyphenylacetic acid (**6**) was transformed into methyl ester (**7**) and then converted in high yield into methyl 3,4-methylenedioxy-α-phenylpropionate (**8**) by α-alkylation conducted in THF at −70 °C in the presence of potassium hydride and methyl iodide. Ketoester **9** was prepared via acylation of **8** with 4-nitrobenzoic acid in the presence of an excess of phosphorus pentoxide. The subsequent treatment with hydrazine gave 3,5-dihydro-5-methyl-7,8-methylenedioxy-1-(4-nitrophenyl)-4*H*-2,3-benzodiazepin-4-one (**10**) in low yield. In this reaction, 2-amino-4-methyl-6,7-methylenedioxy-1-(4-nitrophenyl)-2*H*-isoquinolin-3-one (**11**) has also been isolated as byproduct. Reduction of the nitro group of **10**, carried out with Raney-Ni/ammonium formate, afforded derivative (±)-**5**. Physical and spectral data (^1H and ^{13}C NMR) of the synthesized compounds are in agreement with the proposed structures.

To accomplish the synthesis of enantiomers (+)-**5** and (−)-**5**, we planned the enantioselective enzyme-catalyzed hydrolysis of esters (±)-**8** and ketoester (±)-**9**. Several experiments have been performed, at analytical scale, with a number of commercially available enzymes, i.e. lipase A and lipase B from *Candida Antarctica*, papain from *Carica papaya*, acylase I from *Aspergillus melleus*, subtilisin Carlsberg, lipase PS from *Pseudomonas cepacia*, and α-chymotrypsin. Among the tested enzymes, none evidenced any discrimination among the enantiomers of (±)-**9** while carboxylesterase NP gave a partial resolution of substrate (±)-**8**. The progress of the enzymatic hydrolysis was monitored by HPLC using an analytical polysaccharide-derived chiral column (Chiralcel OD-H, cellulose tris-[3,5-dimethylphenylcarbamate]). The best results were obtained when the biocatalyzed hydrolysis was stopped at 40% conversion to yield acid (+)-**12** (e.e. 83.2%) and unreacted ester (−)-**8** (e.e. 57.2%) (Scheme 1). The stereochemistry of (+)-**12** was assigned as *S*, according to literature data.[10] Although the enantiomeric excess of *R*-(−)-**8** and *S*-(+)-**12** was not completely satisfactory, we decided to proceed with the subsequent steps of the synthetic plan (Scheme 1) with the objective to determine the absolute configuration of the enantiomers (+)-**5** and (−)-**5**. Unfortunately the Friedel−Craft acylation and the subsequent cyclization step provoked the complete racemization of the substrate as evidenced by the chiral HPLC analyses (vide infra) carried out on final compounds.

Therefore, to get the pure enantiomers of (±)-**5** we carried out a preparative HPLC analysis using a polysaccharide-derived chiral stationary phase (Chiralpak AS-H, amylose tris[(*S*)-α-methylbenzylcarbamate]). The resolution of (±)-**5** was optimized by varying the amount of the alcohol (the modifier). As shown in Table 1, the separation factor α is slightly affected by the polarity of the mobile phase and the resolution factor R_s remained high in all experiments. Parts a and b of Figure 1 show the HPLC chromatograms of experiments 1 and 5, respectively. On the basis of the chromatographic results, the preparative isolation of the enantiomers of compound (±)-**5** was carried out using a 1:1 mixture of *n*-hexane/ethanol as the mobile phase (experiment 5, Table 1). These experimental conditions ensured, in fact, reasonable elution times and a still good R_s factor. The analytical control performed on the first collected fraction gave an enantiomeric excess of 100% whereas the e.e.

Table 1. Separation in Chiral HPLC of Compound (±)-**5** Using Chiralpak AS-H Column

exp.	*A* (%)[a]	k_1[b]	k_2[c]	α[d]	R_s[e]
1	20	12.7	19.3	1.51	4.2
2	25	8.3	12.7	1.53	4.0
3	35	4.4	6.8	1.53	3.0
4	40	3.3	5.1	1.52	3.2
5[f]	50[f]	2.5	3.9	1.55	2.8

[a] Percentage of ethanol in *n*-hexane. [b] Capacity factor of the first enantiomer. [c] Capacity factor of the second enantiomer. [d] Separation factor. [e] Resolution factor. [f] Experimental conditions used for semipreparative isolation.

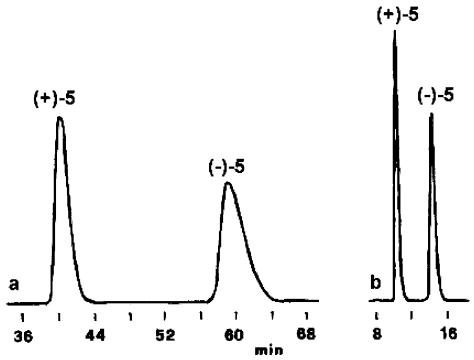


Figure 1. (a) Chromatogram referred to in conditions of experiment 1. (b) chromatogram referred to in conditions of experiment 5, used for semipreparative isolation.

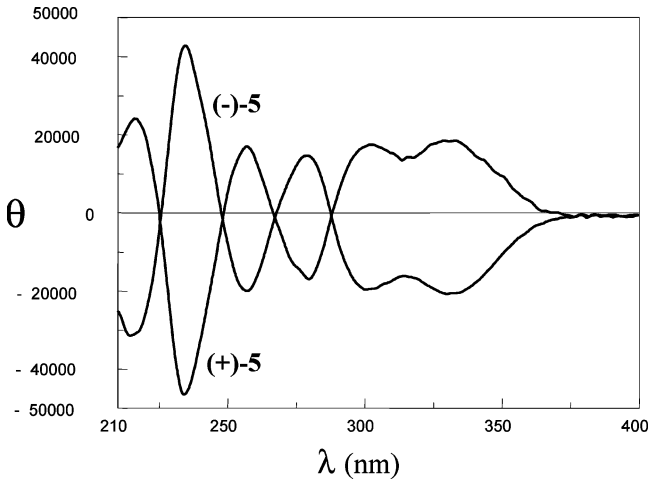


Figure 2. CD spectra of the enantiomers (+)-**5** and (−)-**5**, in ethanol at 22 °C.

of the second fraction amounted to 97.7%. Specific rotation of the first-eluted enantiomer was $[\alpha]^{22}_D = +447.0$ (*c* 0.10, EtOH), while the second eluted enantiomer afforded an experimental $[\alpha]^{22}_D = -439.7$ (*c* 0.10, EtOH).

The quantitative CD spectra of each enantiomer were measured in ethanol (Figure 2). The CD curves of each enantiomer are bisignate, and this experimental evidence indicates a coupling between chromophores.[11] However, the presence of several chromophores in compound **5** and their reciprocal interactions render the CD spectrum quite complex and unhandy to be related to the absolute configuration.

To assign the absolute configuration to the enantiomers, we made several attempts to get crystals suitable for single crystal X-ray analysis. Since this approach failed, we took into account the methodology based on the comparison of the experimental and the calculated optical specific rotation. Examples of assignment of the absolute configuration in conformationally flexible molecules have recently appeared in the literature.[12]

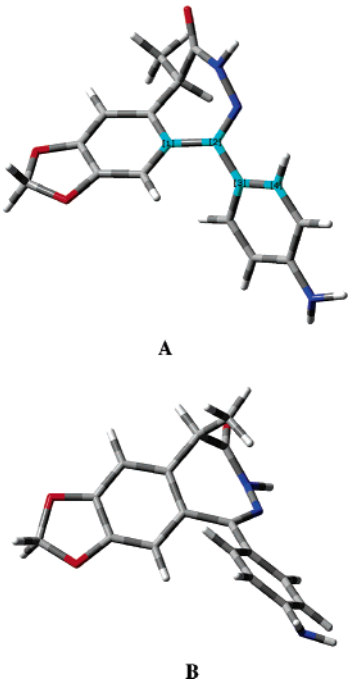


Figure 3. Three-dimensional plots of the global energy minimum conformations of compound (5*R*)-**5** as optimized at the DFT/B3LYP/6−31(d) level and recalculated in ethanol using the PCM model. (A) Conformer (5*R*)-**5**A; the torsion angle optimized by PES is highlighted. (B) Conformer (5*R*)-**5**B.

The $[\alpha]_D$ theoretical prediction of the conformational minimized stereoisomer (5*R*)-**5** was carried out by Gaussian03[13] software by means of TDDFT/B3LYP/6-31G(d) as basis set.[14] The outcome of this research reveals that the 5-methyl- 2,3-benzodiazepine ring exists, at room temperature, in two stable conformations (Figure 3) depending on the equatorial or axial orientation of its methyl group. Moreover, rotation of the 4-aminophenyl moiety linked at position 1 of the 2,3-benzodiazepine ring introduces a further conformational freedom that had to be optimized in order to obtain the most energetically favored conformations. Considering this torsion angle, a 360° potential energy scan (PES) was carried out (Figure 4) on both the ring conformers; the spatial arrangements corresponding to the lowest potential energy were re-optimized by a conjugate gradient method at DFT/b3lyp/ 6-31G(d) level. Only two spatial arrangements, one for each ring conformer, were found to hold the lowest energy potential both in a vacuum and at room temperature. Additionally, to reproduce the experimental conditions used in the $[\alpha]_D$ estimation, the population analysis was completed considering the PCM[15] ethanol solvent model (Table 2). The calculated $[\alpha]_D$ values of the two different spatial arrangements of (5*R*)-**5** were weight averaged to give an $[\alpha]_D = +655.6$, a value in reasonable agreement with the experimental result of +447.0. As a consequence, the *R* configuration has to be assigned to enantiomer (+)-**5**, which is the HPLC first-eluted isomer.

Pharmacology. The anticonvulsant activity of derivatives (±)-**5**, (5*R*)-(+)-**5** and (5*S*)-(−)-**5** against audiogenic seizures was evaluated 30 min after intraperitoneal administration to DBA/2 mice, a strain genetically susceptible to sound-induced seizures. This test has been considered an excellent animal model for generalized epilepsy and for screening new anticonvulsant drugs.[16] The results are compared with those previously reported for derivative **4** and reference compound **1** (Table 3).[6,7]

As shown in Table 3, compound (±)-**5** possesses anticonvulsant properties higher than those of prototype **1** and

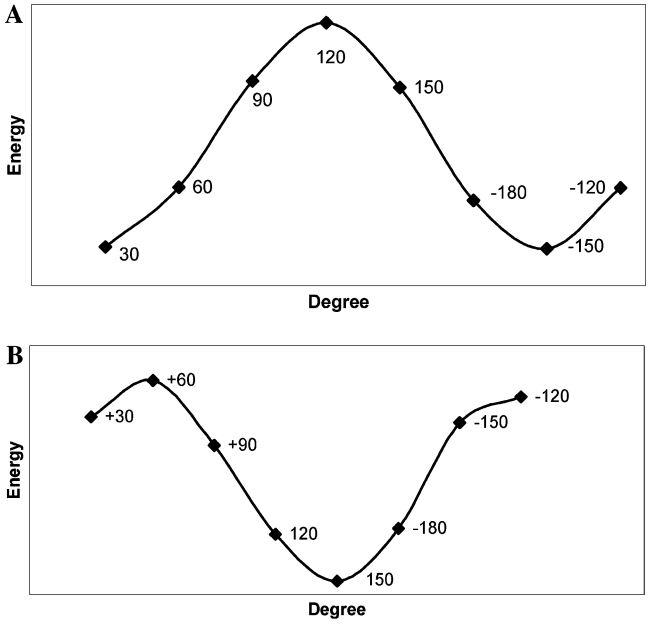


Figure 4. The potential energy scan results. (A) Conformer (5*R*)-**5**A, energy (in hartrees) versus dihedral angle C4a−C5−C1′−C2′. (B) Conformer (5*R*)-**5**B, energy (in hartrees) versus dihedral angle C4a−C5−C1′−C2′.

Table 2. Calculated and Experimental Specific Rotation of the (5*R*)-**5** Enantiomer

conformer	population %	E_{rel} (kcal/ mol)	$[\alpha]_D$
A	91	0.00	+794.3
B	9	1.38	−746.3

Population Weighted Specific Rotation

$[\alpha]_D$
+655.6

Observed Optical Specific Rotation

isomer	$[\alpha]_D$
first-eluted	+447.0
second-elut ed	−439.7

Table 3. Anticonvulsant Activity of Compounds **1**, **4**, (±)-**5**, (5*R*)-(+)-**5** and (5*S*)-(−)-**5** against Audiogenic Seizures in DBA/2 Mice and TD_{50} Values on Locomotion Assessed by Rotarod Test[a]

	ED_{50}, μmol/kg			PIb
compds	clonic phase	tonic phase	TD_{50}, μmol/kg locomotor deficit	TD_{50}/ED_{50}
1	35.8 (24.4−52.4)	25.3 (16.0−40.0)	76.1 (47.5−122)	2.1
4	15.4 (10.1−23.5)	10.9 (4.60−24.6)	99.1 (72.4−135)	6.3
(±)-5	20.2 (16.7−24.4)	8.30 (13.6−42.7)	48.5 (34.6−67.9)	2.4
(5*R*)-(+)-5	88.4 (58.0−134)	7.36 (3.3−16.6)	>100	>1.1
(5*S*)-(−)-5	11.0 (5.79−21.1)	1.74 (0.87−3.48)	34.2 (25.3−46.3)	3.1

[a] All compounds were given ip (at doses spanning the range 3.3−200 μmol/kg) 30 min before auditory stimulation. All data were calculated according to the method of Litchfield and Wilcoxon;[29] 95% confidence limits are given in parentheses. At least 32 animals were used to calculate each ED_{50} and TD_{50} value. [b] PI, protective index, represents the ratio between TD_{50} and ED_{50} (from the clonic phase of the audiogenic seizures).

comparable to those of its C-5 des-methylated analogue **4**. Noteworthy, (5*S*)-(−)-**5** was approximately twice as potent as racemic (±)-**5**, whereas (5*R*)-(+)-**5** was almost inactive (ED_{50} = 11.0 μmol/kg for (5*S*)-(−)-**5** vs 88.4 μmol/kg for (5*R*)-(+)-**5**, in the clonic phase).

Given that our lead compounds display their anticovulsant activity through the inactivation of AMPA receptors,[8] it was of utmost importance to ascertain if the new derivatives showed

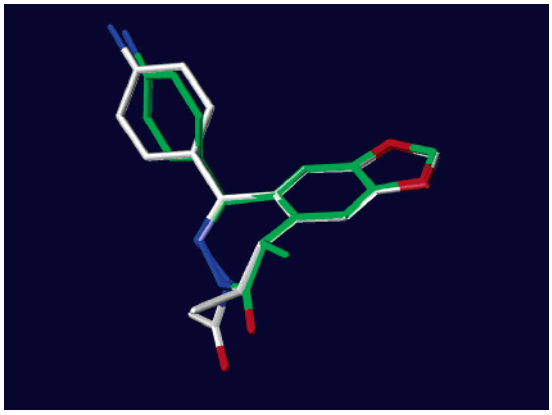


Figure 5. Superimposition of (4*R*)-(+)-**3** (C atoms are colored in white) and (5*S*)-(−)-**5** (C atoms are colored in green).

the same mode of action. Therefore, compound (±)-**5** and its enantiomers (5*R*)-(+)-**5** and (5*S*)-(−)-**5** were examined for their ability to displace [^{3}H]CP-526,427 from the noncompetitive quinoxaline/benzodiazepine binding site of the AMPA receptor complex. In this assay, the quinazolinone CP-465,022 was used as a positive control (IC_{50} = 52 nM). Compound (±)-**5** and (5*R*)-(+)-**5** showed IC_{50} > 10 μM, whereas enantiomer (5*S*)-(−)-**5** showed an IC_{50} = 3.27 μM, a value considerably better than that reported[8] for compound **4** (IC_{50} = 32 μM) and **1** (IC_{50} = 12.6 μM).

Furthermore, compound (±)-**5** and its enantiomers (5*R*)-(+)-**5** and (5*S*)-(−)-**5** were tested for their ability to inhibit the kainate-induced increase of the $[Ca^{2+}]_i$ in a primary culture of rat cerebellar granule cells (CGC) which express AMPA receptors;[17] **1** was used as the control. The results of the CGC test confirmed the data of the in vivo assay. Compound (±)-**5** at 32 μM produced a 94% inhibition of the calcium influx; its IC_{50} is 12 μM, equal to that of derivative **4**. Such an antagonistic activity resides entirely in its enantiomer (5*S*)-(−)-**5** (IC_{50} = 6 μM), whereas (5*R*)-(+)-**5**, up to 32 μM, did not evidence any inhibition of the kainate-induced calcium influx. This outcome is noteworthy since the configuration of the eutomer is opposite to that of (4*R*)-(+)-**3**. Since the noncompetitive binding site of the AMPA receptors, up to now, has not been characterized by X-ray analysis, we performed a superimposition of (4*R*)-(+)-**3** with the most populated conformation of (*S*)-(−)-**5**. This experiment reveals an excellent overlapping of the common functional groups as shown in Figure 5.

The binding affinity of compound (±)-**5** at the AMPA and Gly/NMDA receptors was also evaluated by measuring its ability to displace [^{3}H]AMPA and [^{3}H]glycine, respectively, from rat cerebral cortex synaptic membranes. To assess the functional antagonism at the NMDA receptor−ion channel complex, we tested derivative (±)-**5** for its ability to inhibit the binding of the channel blocking agent [^{3}H]-(+)-MK-801 in rat cortical membranes incubated with 10 μM glutamate and 0.1 μM glycine.[18] The results showed that compound (±)-**5**, similarly to **1**,[8] is devoid of any affinity (IC_{50} > 100 μM) for both the AMPA recognition site and Gly/NMDA receptor site, whereas it possesses a measurable binding affinity (33% binding inhibition at 100 μM) for the MK-801 binding site.

In summary, the enantiomers of 3,5-dihydro-5-methyl-7,8-methylenedioxy-4*H*-2,3-benzodiazepin-4-one (±)-**5** have been obtained by chiral HPLC, and their absolute configuration has been assigned by a comparison of the experimental and the calculated optical specific rotation. The pharmacological assays put in evidence an enantioselective interaction of the enantiomer

(5*S*)-(−)-**5** with the noncompetitive binding site of the AMPA receptors. This result has been rationalized through the overlapping of its most populated conformation with the structure of (4*R*)-(+)-**3**.

Experimental Section

Lipase A from *Candida Antarctica*, papain from *Carica papaya*, acylase I from *Aspergillus melleus*, lipase PS from *Pseudomonas cepacia*, and α-chymotripsin were purchased from Fluka. Subtilisin Carlsberg (Proleather) was obtained from Amano Pharmaceuticals Co. Lipase B from *Candida Antarctica* was bought from Roche Diagnostics (CHIRAZYME L-2). Carboxylesterase NP (Biofine Esterase) was purchased from International Bio-Synthetics (Rijswick, The Netherlands). The enzyme activity is expressed in ECU/g using (*S*)-naproxen methyl ester as the substrate. One esterase cleavage unit (ECU) is defined as the quantity of enzyme that will form 1 μmol of (*S*)-naproxen acid per liter per minute at pH 7.5 and 25 °C. Melting points were determined on a Kofler hot stage apparatus and are uncorrected. Elemental analyses were carried out on a C. Erba Model 1106 elemental analyzer for C, H, and N, 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 and ^{13}C NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 by means of a Varian Gemini 300 spectrometer (see Supporting Information). The HPLC system consisted of a PU 980 pump, a LG-1580−02 low pressure mixer, and a DG-1580-54 in line degasser, all from Jasco, with a Rheodyne injection valve equipped with 20 or 100 μL sample loops, a Varian DU-50 spectrophotometer, and a Varian 4400 integrator or Houston Omniscribe recorder for fraction collecting. The polysaccharide derived columns (250 mm × 4.6 mm) were Chiralcel OD-H (cellulose tris[3,5-dimethylphenylcarbamate]) and Chiralpak AS-H (amylose tris[(S)-α-methylbenzylcarbamate]) coated on 5 μm silica gel from Daicel. Column void volume (t_0) was measured by injection of tri-*tert*-butylbenzene as a nonretained sample.[19] The HPLC parameters (k, α, and R_s) were those typically employed.[20] CD spectra were recorded in ethanol on a Jasco 810 spectropolarimeter using a 1 mm cell and scanning five times from 400 to 200 nm at 50 nm/min. Optical rotations were measured in ethanol with a Jasco DIP-730 digital polarimeter using a 10 cm microcell.

Synthesis of Methyl 2-(3,4-Methylenedioxyphenyl)propionate (8). To a stirred solution of 3,4-methylenedioxyphenylacetic acid methyl ester (**7**) (730 mg, 3.76 mmol) in anhydrous THF (40 mL) at −70 °C was added potassium hydride (166 mg, 4.14 mmol). After 30 min was added methyl iodide (587 mg, 4.14 mmol), keeping the temperature at −70 °C. The cooling bath was removed, and the reaction mixture was stirred for further 16 h, then diluted with 50% acetic acid, poured into water, and extracted with diethyl ether (2 × 150 mL). The combined organic layers were washed with a saturated solution of Na_2CO_3 and dried over Na_2SO_4. The solvent was then removed to give compound **8** (681 mg, 86.8%) (lit.[21] 63%) as a colorless oil: R_f = 0.59 (diethyl ether/cyclohexane 40/60). Anal. ($C_{11}H_{12}O_2$) C, H.

Synthesis of Methyl 2-[2-(4-Nitrobenzoyl)-4,5-methylenedioxy-phenyl]propionate (9). 4-Nitrobenzoic acid (711 mg, 4.25 mmol) and phosphorus pentoxide (2.0 g) were added to a stirred 1,2-dichloroethane solution (50 mL) of **8** (681 mg, 3.27 mmol). The mixture was further stirred at room temperature overnight, then water (30 mL) was cautiously added, and the mixture was extracted with chloroform (2 × 30 mL). The organic layer was separated and sequentially treated with 10% NaOH (30 mL), brine (30 mL), and water (2 × 30 mL). The organic phase was dried (Na_2SO_4) and the solvent removed under reduced pressure to yield crude **9** which was purified by a silica gel column chromatography using diethyl ether/light petroleum (40/60) as eluant: mp 118−120 °C (387 mg, 33%) (lit.[21] 12.3%); R_f = 0.35 (diethyl ether/light petroleum 40/60). Anal. ($C_{18}H_{15}NO_7$) C, H, N.

Synthesis of 3,5-Dihydro-5-methyl-7,8-methylenedioxy-1-(4-nitrophenyl)-4*H*-2,3-benzodiazepin-4-one (10) and 2-Amino-4-methyl-6,7-methylenedioxy-1-(4-nitrophenyl)-2*H*-isoquinolin-3-one (11). A solution of compound **9** (387 mg, 1.08 mmol) and hydrazine hydrate (0.058 mL, 1.19 mmol) in *n*-butanol (40 mL) was heated at reflux. After 20 h, the expected compound **10** was collected by hot filtration and recrystallized from methanol: mp > 300 °C (75 mg, 20%): R_f = 0.72 (EtOAc/cyclohexane 60/40). Anal. ($C_{17}H_{13}N_3O_5$) C, H, N. By cooling, the remaining butanol solution afforded compound **11** which was filtered off and recrystallized from methanol: mp > 300 °C (67.5 mg, 18%); R_f = 0.10 (EtOAc/cyclohexane, 60/40). Anal. ($C_{17}H_{13}N_3O_5$) C, H, N.

Synthesis of 1-(4-Aminophenyl)-5-methyl-7,8-methylenedioxy-3,5-dihydro-4*H*-2,3-benzodia zepin-4-one (±)-5. A suspension of **10** (75 mg, 0.22 mmol) and Raney-Ni (45 mg) in EtOH (30 mL) was stirred with ammonium formate (250 mg, 4.10 mmol). The mixture was refluxed for 2 h and then filtered through Celite. The solvent was evaporated under reduced pressure, and the residue, dissolved in $CHCl_3$, was washed with saturated NaCl to remove ammonium formate. The organic layer, dried over Na_2SO_4, was evaporated under reduced pressure, and the residue was purified by a silica gel column chromatography eluting with EtOAc/cyclohexane: mp 142−144 °C (17 mg, 25%) (lit.[21] 2.75% global yield starting from **9**); R_f = 0.74 (EtOAc). Anal. ($C_{17}H_{15}N_3O_3$) C, H, N.

Preparative Enzymatic Hydrolysis of Methyl 2-(3,4-Methylenedioxyphenyl)propionate [(±)-8]. To a suspension of (±)-**8** (1.5 g, 7.21 mmol) in 0.1 M phosphate buffer pH 7.0 (80 mL) was added carboxylesterase NP (2 mL; 200 U/mL), and the mixture was vigorously stirred at room temperature for 8 h. The progress of the reaction was monitored by HPLC (column: Chirlacel OD-H; eluant: petroleum ether/2-propanol 100:0.5; flow rate: 0.5 mL/min; λ = 254 nm). The reaction was stopped at 40% conversion to yield acid (+)-**12** (e.e. 83.2%) and residual ester (−)-**8** (e.e. 57.2%). Solid $NaHCO_3$ was added, the reaction mixture was extracted with ethyl acetate, and the pooled organic layers were evaporated to recover the residual ester (−)-**8** (890 mg, 59%). The aqueous phase was made acid with 2 N HCl, and the monoacid (+)-**12** (540 mg, 39%) was obtained after extraction with ethyl acetate, drying over anhydrous Na_2SO_4, and evaporation of the solvent. (−)-**8**: $[\alpha]^{20}_D$: −26.1 (*c* 1.03, $CHCl_3$). Colorless oil. R_f = 0.78 (CH_2Cl_2/MeOH, 95:5). HPLC retention time: (−)-**8**, 29.56 min (78.6%); (+)-**8**, 33.46 min (21.4%) (column: Chiralcel OD-H; eluant: petroleum ether/ 2-propanol 100:0.5; flow rate: 0.5 mL/min; λ = 254 nm); e.e. 57.2%. (+)-**12**: $[\alpha]^{20}_D$: +25.5 (*c* 1.07, $CHCl_3$). Colorless oil. R_f = 0.27 (CH_2Cl_2/MeOH, 95:5). HPLC retention time: (+)-**12**, 81.02 min (91.6%); (−)-**12**, 63.96 min (8.4%) (column: Chiralcel OD-H; eluant: petroleum ether/2-propanol 100:0.5; flow rate: 0.5 mL/min; λ = 254 nm); e.e. 83.2%. Anal. ($C_{10}H_{10}O_4$) C, H.

Preparative HPLC Separation of (+)-5 and (−)-5 and Their Chiroptical Measurements. The resolution of (±)-**5** was carried out using a Chiralpak AS-H column (amylose tris[(*S*)-α-metilbenzylcarbamate]). Eluent: *n*-hexane/ethanol 1:1. Flow rate at 1 mL/min; t_0 = 2.90; UV detector λ = 240 nm. The isolation was accomplished by 50 μL repeated injections (0.2−0.3 mg) of a solution of (±)-**5** (68 mg in 10 mL *n*-hexane/ethanol 1:1). Collection of the eluates corresponding to the two chromatographic peaks gave, after filtration and evaporation, 17 mg of each enantiomer. The analytical control performed on the first collected fraction gave an enantiomeric excess of 100% whereas the e.e. of the second fraction amounted to 97.7%. Specific rotation of the first-eluted enantiomer was $[\alpha]^{22}_D$ = +447.0 (*c* 0.10, EtOH), while the second eluted enantiomer afforded an experimental $[\alpha]^{22}_D$ = − 439.7 (*c* 0.10, EtOH). Due to the limited amount of the individual enantiomers of **5**, first ethanolic solutions of them (about 34 × 10^{-4} M) were made for optical rotation measurements, then these solutions were diluted 1:10 with ethanol, and CD spectra were measured. After chiroptical measurements, the solutions of the individual enantiomers were taken to dryness in a drying pistol in a vacuum and in the presence of P_2O_5 as desiccant and the powder residues used for pharmacological tests.

Assignment of the Absolute Configurations. Molecular structures were built by GView implemented in the Gaussian03[13]

Windows package. Both 5-methyl-benzodiazepine ring conformers, following the preliminary optimization by semiempirical/PM3[22] methods, were submitted to the 360° PES analysis (step size = 30°) at B3LYP/6-31G(d) level considering the torsion angle among the 4-aminophenyl group and the benzodiazepine ring (highlighted in Figure 2A). The lowest potential energy was found at a torsion angle value of −150° (or +30° due to symmetry of the molecules) and +150° for **5A** and **5B**, respectively. These values of the torsion angle were then used for a re-optimization in vacuo of the entire structures (*R*)-**5A** and (*R*)-**5B**. Furthermore, a single point calculation, using the PCM ethanol solvent model, permitted us to estimate the conformers population in the experimental conditions. Theoretical values of the $[\alpha]_D$ were achieved specifying appropriate keyword (*polar* and *cphf*) into the Gaussian input.

Audiogenic Seizures Test in DBA/2 Mice.[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 randomly assigned to controls and drug-treatment groups and 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% DMSO and 50% sterile saline (0.9% NaCl). Individual mice were placed under a hemispheric Perspex dome (diameter 58 cm), and 60 s were 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]

[^{3}H]CP-526,427 Binding.[17] The binding of [^{3}H]CP-526,427 was characterized in rat forebrain membranes. Forebrains of adult male Sprague−Dawley rats were homogenized in 0.32 M sucrose at 4 °C. The crude nuclear pellet was removed by centrifugation at 1000*g* for 10 min, and the supernatant centrifuged at 17 000*g* for 25 min. The resulting pellet was re-suspended in 5 mM Tris acetate, pH 7.4, at 4 °C for 10 min to lyse cellular particles and again centrifuged at 17 000*g*. The resulting pellet was washed twice in Tris acetate, re-suspended at 10 mg of protein/mL and stored at −20 °C until use. Immediately before binding assays, membranes were thawed, homogenized, and diluted to 0.5 mg of protein/mL with 50 mM Tris·HCl, pH 7.4. Scatchard analyses were performed by incubating different concentrations of radioligand with membranes. For competition assays, compounds were added at various concentrations followed by 3 nM [^{3}H]CP-526,427 (specific activity, 24.36 Ci/mmol). After incubation for 20 min at 30 °C in a shaking water bath, samples were filtered onto Whatman GFB glass fiber filters using a MB-48R cell harvester (Brandel Research and Development Laboratories, Gaithersburg, MD). Filters were washed for 10 s with ice-cold Tris·HCl buffer, and the radioactivity trapped on the filter was quantified by liquid scintillation counting. Nonspecific binding for [^{3}H]CP-526,427 was determined in parallel incubations containing 10 μM unlabeled CP-526,427 or CP-465,-022. Specific binding was defined as total binding minus nonspecific binding.

Primary Cultures of Rat Cerebellar Granule Neurons. Primary cultures of rat cerebellar granule neurons were prepared as described previously.[25]

$^{45}Ca^{2+}$ Uptake.[17] Neurons in poly-D-lysine-coated 96-well plates were preincubated for 30 min with different concentrations of compounds in balanced salt solution (BSS; 115 mM NaCl, 5.4 mM KCl, 0.96 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 11 mM *d*-glucose, and 25 mM HEPES, pH 7.3). They were then exposed at room temperature to 100 μM kainate or NMDA in BSS containing 10 μM glycine, 0.5 mM dithiothreitol, and 0.5 μCi $^{45}Ca^{2+}$ (final specific activity, 2.78 μCi/μmol) in a volume of 100 μL/well. After 10 min, the neurons were then rapidly washed five times with 200 μL/well of ice-cold BSS containing 5 mM EGTA. Neurons were then lysed in 30 μL/well of 0.6% Triton X-100, and radioactivity in aliquots of the lysate were measured with a TopCount microtiter scintillation counter (Packard Instrument Co., Downers Grove, IL).

Measurement of $[Ca^{2+}]_i$.[17] Neurons in 96-well, black/clear, poly-D-lysine-coated tissue culture plates were rinsed once with BSS then incubated for 1 h in BSS containing 4 μM Fluo-4/AM (Molecular Probes, Inc., Eugene, OR). Fluo-4/AM was prepared immediately before use as a 1 mM stock solution in dimethyl sulfoxide with 10% (w/v) pluronic acid. Cells were then washed three times and held in BSS at room temperature and used within 1 h. A fluorescent imaging plate reader (FLIPR; Molecular Devices, Sunnyvale, CA) was used for simultaneous imaging and fluid addition. Cells were preincubated with test compounds for approximately 6 min, then stimulated with 32 μM AMPA. Changes in fluorescent intensity were measured at a frequency of 1 sample/2 s after AMPA addition. Raw data are expressed in relative fluorescent units (RFUs) after the background fluorescence was subtracted.

[^{3}H]AMPA Binding. Affinity for the competitive AMPA receptor binding site was determined with 5 nM [^{3}H]AMPA as previously described.[26]

[^{3}H]Glycine Binding. These experiments were carried out in rat cortical synaptic membrane preparations with 8 nM [^{3}H]glycine as ligand, according to the method described.[27]

[^{3}H]-(+)-MK-801 Binding. [^{3}H]-(+)-MK-801 ((+)-5-methyl-10,11-dihydro-5*H*-dibenzo-[a,d]cyclohepten-5,10-imine maleate) binding assays were carried out according to Yoneda et al.[28] with slight modifications. Preparations of frozen rat cortical membranes were resuspended (0.5 mg protein/mL) in ice-cold 5 mM Tris-HCl buffer, pH 7.4, containing 0.08% v/v Triton X-100 and stirred for 10 min at 0−2 °C. They were then collected by centrifugation (48 000*g* for 10 min) and submitted to four additional resuspension and centrifugation cycles before finally being resuspended in the appropriate volume of buffer (0.2−0.3 mg protein/tube) for the binding assay. The assay incubations were carried out at room temperature for 120 min with 2.5 nM [^{3}H]-(+)-MK-801 (22.5 Ci/mmol), 10 mM glutamic acid, and 0.1 mM glycine in the presence and absence of the test compound in a total volume of 0.5 mL. Bound radioactivity was separated by filtration through GF/C filters presoaked in 0.05% polyethylenimmine and washed with ice-cold buffer (3 × 5 mL). Nonspecific binding was determined in the presence of 100 mM phencyclidine hydrochloride.

Statistical Analysis. The ED_{50} values of each phase of the audiogenic seizure were determined for each dose of compound administered, and dose−response curves were fitted using a computer program by the method of Litchfield and Wilcoxon.[29] The median toxic dose (TD_{50}) values were estimated using the method of Litchfield and Wilcoxon.[29]

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Supporting Information Available: Analytical and spectral data of all the synthesized compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Bräuner-Osborne, H.; Egebjerg, J.; Nielsen, E. Ø.; Madsen, U.; Krogsgaard-Larsen, P. Ligands for glutamate receptors: design and therapeutic prospects. *J. Med. Chem.* **2000**, *43*, 2609−2645.
(2) Zappalà, M.; Grasso, S.; Micale, N.; Polimeni, S.; De Micheli, C. Synthesis and structure−activity relationship of 2,3-benzodiazepines AMPA receptor antagonists. *Mini-Rev. Med. Chem.* **2001**, *1*, 243−253.
(3) Sólyom, S.; Tarnawa, I. Noncompetitive AMPA antagonists of 2,3-benzodiazepine type. *Curr. Pharm. Design* **2002**, 913−939.
(4) Szabados, T.; Gigler, G.; Gacsalyi, I.; Gyertan, I.; Levay, G. Comparison of anticonvulsive and acute neuroprotective activity of

three 2,3-benzodiazepine compounds, GYKI 52466, GYKI 53405, and GYKI 53655. *Brain Res. Bull.* **2001**, *55*, 387−391.
(5) Chappell, A. S.; Sander, J. W.; Brodie, M. J.; Chadwick, D.; Liedo, A.; Zhang, D.; Bjerke, J.; Kiesler, G. M.; Arroyo, S. A crossover, add-on trial of talampanel in patients with refractory partial seizures. *Neurology* **2002**, *58*, 1680−1682.
(6) De Sarro, A.; De Sarro, G.; Gitto, R.; Grasso, S.; Micale, N.; Quartarone, S.; Zappalà, M. 7,8-Methylenedioxy-4*H*-2,3-benzodiazepin-4-ones as novel AMPA receptor antagonists. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 971−976.
(7) De Sarro, A.; De Sarro, G.; Gitto, R.; Grasso, S.; Micale, N.; Zappalà, M. Synthesis and anticonvulsant activity of new 2,3-benzodiazepines as AMPA receptor antagonists. *Farmaco* **1999**, *54*, 179−189.
(8) Grasso, S.; Micale, N.; Zappalà, M.; Galli, A.; Costagli, C.; Menniti, F. S.; De Micheli, C. Characterization of the mechanism of anticonvulsant activity for a selected set of putative AMPA receptor antagonists. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 443−446.
(9) Rezessy, B.; Sólyom, S. Advanced pharmacophore model of non-competitive AMPA antagonist 2,3-benzodiazepines. *Lett. Drug Des. Discovery* **2004**, *1*, 217−223.
(10) (a) Smeets, J. W. H.; Kieboom, A. P. G. Enzymic enantioselective ester hydrolysis by carboxylesterase NP. *Recl. Trav. Chim. Pays−Bas* **1992**, *111*, 490−495. (b) Quax, W. J.; Broekhuizen, C. P. Development of a new Bacillus carboxyl esterase for use in the resolution of chiral drugs. *Appl. Microbiol. Biotechnol.* **1994**, *41*, 425−431.
(11) Nakanishi, K.; Berova, N. In *Circular Dichroism: Principles and Applications*; Berova, N., Nakanishi, K., Woody, R. W., Eds.; Wiley-VCH Publishers: New York, 2000; Chapter 12.
(12) (a) Stephens, P. J.; Devlin, F. J.; Cheeseman, J. R.; Frisch, M. J.; Rosini, C. Determination of absolute configuration using optical rotation calculated using density functional theory. *Org. Lett.* **2002**, *4*, 4595−4598. (b) Mennucci, B.; Tomasi, J.; Cammi, R.; Cheeseman, J. R.; Frisch, M. J.; Devlin, F. J.; Gabriel, S.; Stephens, P. J. Polarizable Continuum model (PCM) calculations of solvent effects on optical rotations of chiral molecules *J. Phys. Chem. A* **2002**, *106*, 6102−6113. (c) Giorgio, E.; Roje, M.; Tanaka, K.; Hamersak, Z.; Sunjic, V.; Nakanishi, K.; Rosini, C.; Berova, N. Determination of the absolute configuration of flexible molecules by ab initio ORD calculations: a case study with cytoxazones and isocytoxazones. *J. Org. Chem.* **2005**, *70*, 6557−6563. (d) Petrovic, A. C.; He, J.; Polavarapu, P. L.; Xiao, L. S.; Armstrong, D. W. Absolute configuration and predominant conformations of 1,1-dimethyl-2-phenylethyl phenyl sulfoxide. *Org. Biomol. Chem.* **2005**, *3*, 1977−1981.
(13) Gaussian 03, Revision C.02, Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Montgomery, J. A., Jr.; Vreven, T.; Kudin, K. N.; Burant, J. C.; Millam, J. M.; Iyengar, S. S.; Tomasi, J.; Barone, V.; Mennucci, B.; Cossi, M.; Scalmani, G.; Rega, N.; Petersson, G. A.; Nakatsuji, H.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Klene, M.; Li, X.; Knox, J. E.; Hratchian, H. P.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Ayala, P. Y.; Morokuma, K.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Zakrzewski, V. G.; Dapprich, S.; Daniels, A. D.; Strain, M. C.; Farkas, O.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Ortiz, J. V.; Cui, Q.; Baboul, A. G.; Clifford, S.; Cioslowski, J.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen, W.; Wong, M. W.; Gonzalez, C.; Pople, J. A.; Gaussian, Inc., Wallingford, CT, 2004.
(14) (a) Becke, A. D. A new mixing of Hartree−Fock and local-density-functional theories. *J. Chem. Phys.* **1993**, *98*, 1372−1377. (b) Becke, A. D. Density-functional thermochemistry. III. The role of exact exchange. *J. Chem. Phys.* **1993**, *98*, 5648−5652.
(15) Miertus, S.; Scrocco, E.; Tomasi, J. Electrostatic interaction of a solute with a continuum − A direct utilization of *ab initio* molecular potentials for the provision of solvent effects. *Chem. Phys.* **1981**, *55*, 117−129.
(16) (a) Chapman, A. G.; Croucher, M. J.; Meldrum, B. S. Evaluation of anticonvulsant drugs in DBA/2 mice with sound-induced seizures. *Arzneim. Forsch.* **1984**, *34*, 1261−1264. (b) Engstrom, F. L.; Woodbury, D. M. Seizure susceptibility in DBA/2 and C57 Mice: the effects of various convulsants. *Epilepsia* **1988**, *29*, 389−395.
(17) Menniti, F. S.; Chenard, M. B.; Collins, M. F.; Ducat, M. F.; Elliot, M. L.; Ewing, F. E.; Huang, J. I.; Kelly, K. A.; Lazzaro, J. T.; Pagnozzi, M. J.; Weeks, J. L.; Welch, W. M.; White, W. F Characterization of the binding site for a novel class of noncompetitive alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor antagonists. *Mol. Pharmacol.* **2000**, *58*, 1310−1317.
(18) Wong, E. H. F.; Knight, A. R.; Ranson, R. Glycine modulated [^{3}H]-MK-801 binding to the NMDA receptor in rat brain. *Eur. J. Pharmacol.* **1987**, *142*, 487−488.
(19) Koller, H.; Rimböck, K.-E.; Mannschreck, A. High-pressure liquid chromatography on triacetylcellulose: characterization of a sorbent for the separation of enantiomers *J. Chromatogr. A* **1983**, *282*, 89−94.
(20) Allenmark, S. *Chromatographic enantioseparation: methods and applications*, 2nd ed.; Ellis Horwood: Chichester, 1991; p 82.
(21) This approach is similar to that previously reported: Xia, H.; Cai, S. X.; Field, G.; Lan, N. C.; Wang, Y. Substituted 2,3-benzodiazepin-4-ones and the use thereof. US 5891871, 1999.
(22) (a) Stewart, J. J. P. Optimisation of parameters for semiempirical methods I. Method. *J. Comput. Chem.* **1989**, *10*, 209−220. (b) Stewart, J. J. P. Optimisation of parameters for semiempirical methods II. Applications. *J. Comput. Chem.* **1989**, *10*, 221−264.
(23) Collins, R. L. Audiogenic Seizures. In *Experimental Models of Epilepsy*; Purpura, P., Penry, J. K., Tower, D., Woodbury, D. M., Walter, R., Eds.; Raven Press: New York, 1972; pp 347−372.
(24) De Sarro, G. B.; Croucher, M. J.; Meldrum, B. S. Anticonvulsant actions of DS 103−282: pharmacological studies in rodents and the baboon *papio papio*. *Neuropharmacology* **1984**, *23*, 526−530.
(25) Parks, T. N.; Artman, L. D.; Alasti, N.; Nemeth, E. F Modulation of N-methyl-D-aspartate receptor-mediated increases in cytosolic calcium in cultured rat cerebellar granule cells. *Brain Res.* **1991**, *552*, 13−22.
(26) Nielsen, E. O.; Madsen, U.; Shaumburg, K.; Brehm, L.; Krogsgaard-Larsen, P. Studies on receptor active conformations of excitatory amino acid agonists and antagonists. *Eur. J. Chem.-Chim. Ther.* **1986**, *118*, 433−437.
(27) Colotta, V.; Catarzi, D.; Varano, F.; Cecchi, L.; Filacchioni, G.; Galli, A.; Costagli, C. Synthesis and biological evaluation of a series of quinazoline-2-carboxylic acids and quinazoline-2,4-diones as glycine-NMDA antagonists: a pharmacophore model based approach. *Arch. Pharm. Med. Chem.* **1997**, *330*, 129−134.
(28) Yoneda, Y.; Ogita, K.; Suzuku, T. Interaction of strychnine-insensitive glycine binding with MK-801 binding in brain synaptic membranes. *J. Neurochem.* **1990**, *55*, 237−244.
(29) Litchfield, J. T.; Wilcoxon, F. A simplified method of evaluating dose−effects experiments. *J. Pharmacol. Exp. Ther.* **1949**, *96*, 99−113.

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