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# Novel 3-Carboxy- and 3-Phosphonopyrazoline Amino Acids as Potent and Selective NMDA Receptor Antagonists: Design, Synthesis, and Pharmacological Characterization

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The design and synthesis of new N1-substituted 3-carboxy- and 3-phosphonopyrazoline and pyrazole amino acids that target the glutamate binding site of NMDA receptors are described. An analysis of the stereochemical requirements for high-affinity interaction with these receptors was performed. We identified two highly potent and selective competitive NMDA receptor antagonists, (5*S*,α*R*)-**1** and (5*S*,α*R*)-**4**, which exhibit good in vitro neuroprotective activity and in vivo anticonvulsant activity by i.p. administration, suggesting that these molecules may have potential use as therapeutic agents.

## Introduction

L-Glutamic acid [(*S*)-Glu] is the most abundant excitatory neurotransmitter in the mammalian central nervous system (CNS), where it is involved in the modulation of many physiological processes such as learning, memory, and synaptic plasticity.[1] Glutamate-activated receptors belong to two families: ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs). The iGluR family comprises three heterogeneous classes, named for the selective agonists *N*-methyl-D-aspartic acid (NMDA), 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA), and kainic acid (KA).[1,2] The iGluRs exist as tetramers; to date, seven NMDA receptor subunits (GluN1, GluN2A–2D and GluNR3A–B), four AMPA receptor subunits (GluA1–4), and five KA receptor subunits (GluK1–5) have been cloned and characterized. In contrast, the mGluRs are a heterogeneous class of G-protein-coupled receptors; to date, eight mGluR subtypes have been cloned and classified in three groups according to their sequence homology, second-messenger coupling, and pharmacology: group I (mGluRs 1 and 5), group II (mGluRs 2 and 3), and group III (mGluRs 4, 6, 7, and 8).[3,4]

It is well known that over-activation of iGluRs generates an uncontrolled influx of calcium, triggering an excitotoxic cascade that leads to neurodegeneration.[5] This phenomenon is associated with several acute and chronic neurodegenerative diseases, including cerebral ischemia, traumatic brain injury, spinal injury, epilepsy, amyotrophic lateral sclerosis, and Parkinson's, Alzheimer's and Huntington's diseases.[6,7] NMDA receptors are the major mediators of excitotoxicity.[8] The blockade of such receptors can be achieved in several ways, including the use of competitive Glu antagonists, glycine$_B$ antagonists, polyamine site antagonists, or open-channel blockers.[9]

Competitive antagonists are typically conformationally constrained Glu homologues in which the spacer between the α- and ω-acidic groups is a 4–6 carbon atom chain; the ω-carboxylate may be replaced by a phosphonate moiety to increase receptor affinity (Figure 1).[10] We previously disclosed the structures of two new competitive NMDA receptor antagonists, **F94b** and **F94c** (Figure 1),[11] which are characterized by the

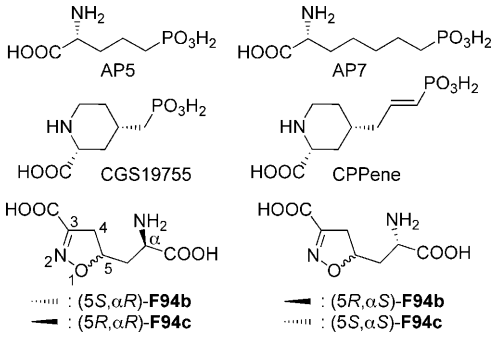


**Figure 1.** Structures of model NMDA antagonists.

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presence of a homologated Glu chain partially locked in an isoxazoline ring. In both cases, the eutomers exhibited *R* configuration at the stereogenic α-center, as observed for a majority of NMDA antagonists [(5*S*,α*R*)-**F94b** $K_i$ = 0.10 μM vs. (5*R*,α*S*)-**F94b** $K_i$ = 1.9 μM; (5*R*,α*R*)-**F94c** $K_i$ = 0.51 μM vs. (5*S*,α*S*)-**F94c** $K_i$ = 2.5 μM].[12] Further pharmacological analysis emphasized the significance of (5*S*,α*R*)-**F94b** and (5*R*,α*R*)-**F94c** as neuroprotective agents, as demonstrated by the results obtained using an in vitro model of cerebral ischemia.[12] Evaluation of the selectivity of these compounds toward different NMDA receptor subtypes revealed a preference for GluN2A and GluN2B vs. GluN2C and GluN2D subtypes.[12]

Based on these results, we decided to pursue the design of new NMDA receptor antagonists with improved affinity and a higher degree of subtype selectivity. According to our previous experience,[13] replacement of the $\Delta^2$-isoxazoline ring with a $\Delta^2$-pyrazoline nucleus may represent an efficient strategy to enhance selectivity for a specific molecular target. This type of substitution potentially improves molecular diversity by functionalizing the nitrogen in position 1, where substituents could be introduced to create additional interactions with the binding site of the target protein, in an attempt to improve both binding affinity and selectivity. Moreover, it has been reported that NMDA antagonists with large side groups have the potential to differentiate between GluN2 subunits.[14] Taking into account the interaction between a tyrosine residue (Y730 in GluN2A) and the isoxazoline oxygen of our most active compound,[12] we envisaged that addition of an aromatic group (i.e. a phenyl moiety) to the nitrogen at position 1 could preserve and possibly strengthen such an interaction, while further exploring the binding pocket in this region.

We initially synthesized and evaluated racemic derivatives (±)-**1** and (±)-**2** (Figure 2). Subsequently, we prepared the pure enantiomers (5*S*,α*R*)-**1**, (5*R*,α*S*)-**1**, (5*R*,α*R*)-**2**, and (5*S*,α*S*)-**2** to examine in detail the relevance of absolute configuration toward biological activity. Furthermore, in an attempt to enhance NMDA antagonist potency, we replaced the distal carboxylate with a phosphonate group, a bioisostere previously used with success in this field [compounds **4**–**5**, Figure 2].[10] Finally, substitution of the pyrazoline moiety with a pyrazole nucleus [compounds (*R*)-**3** and (*S*)-**3**, Figure 2] was used to evaluate the effects of omitting the stereogenic center in position 5 and of using a different ring conformation.

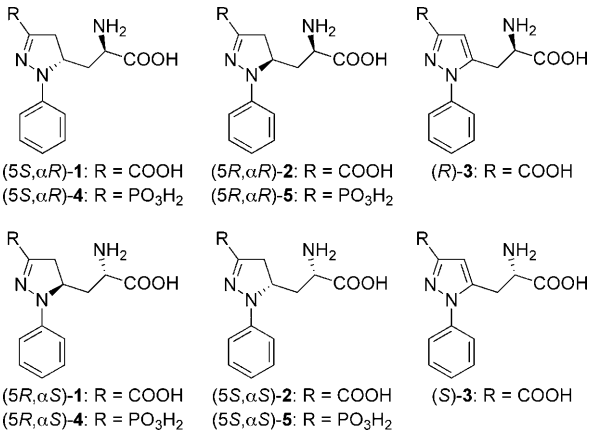
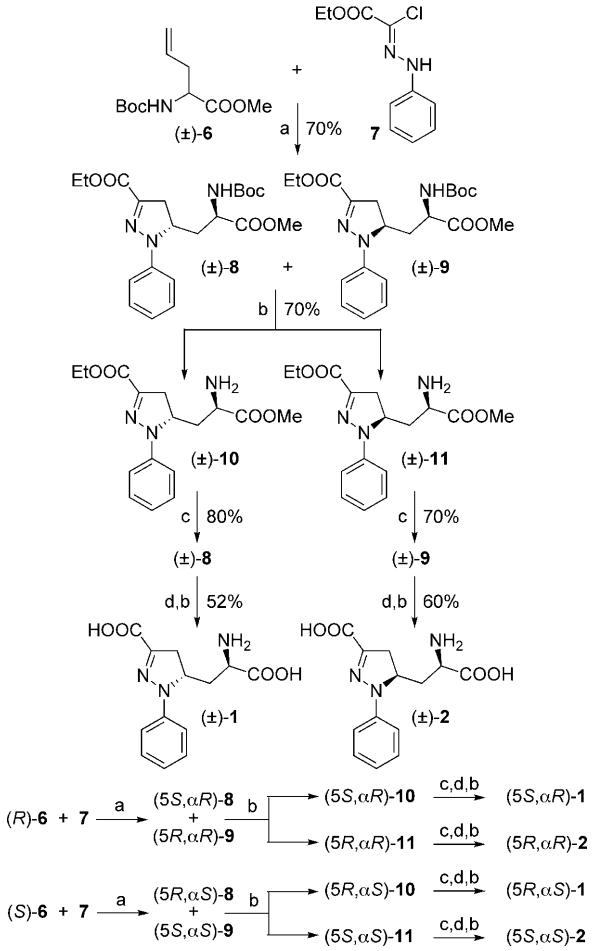



**Figure 2.** Structures of target amino acids.

## Results and Discussion

### Chemistry

The target amino acids (±)-**1** and (±)-**2** were synthesized through 1,3-dipolar cycloaddition of dipolarophile (±)-**6**[11] with *N*-phenyl-ethoxycarbonylformonitrilimine, generated in situ from its stable precursor, ethyl-2-chloro-2-(phenylhydrazono) acetate **7**, in the presence of base (Scheme 1).[13] The cycloaddition reaction produced a mixture of stereoisomers (±)-**8** and (±)-**9** in an approximate 1:1 ratio, as estimated by $^1$H NMR spectroscopy. Chromatographic separation of the two stereoisomers was unsuccessful at this stage; therefore, the mixture was treated with a 30% solution of trifluoroacetic acid (TFA) in dichloromethane to remove the *N*-Boc protecting group. The corresponding primary amines (±)-**10** and (±)-**11** were easily separated by flash chromatography and were then reconverted




**Scheme 1.** Reagents and conditions: a) $NaHCO_3$, EtOAc, 24 h, RT; b) TFA (30%), $CH_2Cl_2$, 3 h, 0 °C→RT; c) $Boc_2O$, $Et_3N$, $CH_2Cl_2$, 12 h, 0 °C→RT; d) NaOH (1 N), MeOH, 3 h, RT.

into (±)-**8** and (±)-**9**, respectively, using standard reaction conditions. Relative configuration of stereoisomers (±)-**8** and (±)-**9** was later established by X-ray analysis carried out on cycloadduct (−)-**8**. Amino acids (±)-**1** and (±)-**2** were ultimately obtained from (±)-**8** and (±)-**9** in two steps by alkaline hydrolysis of the two ester functionalities, followed by TFA removal of the *N*-Boc protecting group, as described above.

The synthetic approach described for the racemates was also applied to the synthesis of enantiomerically pure amino acids (5*S*,α*R*)-**1**, (5*R*,α*R*)-**2**, (5*R*,α*S*)-**1**, and (5*S*,α*S*)-**2**, starting from enantiopure dipolarophile (*R*)-**6**[15] or (*S*)-**6**,[16] respectively (Scheme 1). Intermediate (−)-**8** was crystallized successfully from diisopropyl ether/hexane and, thus, was able to be submitted for X-ray analysis. Due to the absence of anomalous scattering, we were only able to assign relative spatial arrangement and not absolute configuration of the two stereocenters. Nevertheless, because the absolute configuration of the α-amino acid carbon was retained from the starting material (*S*)-**6** and therefore known, the configuration 5*R*,α*S* was assigned to compound (−)-**8** (Figure 3). The absolute configurations of (+)-**8**, (−)-**9** and (+)-**9** were similarly assigned.

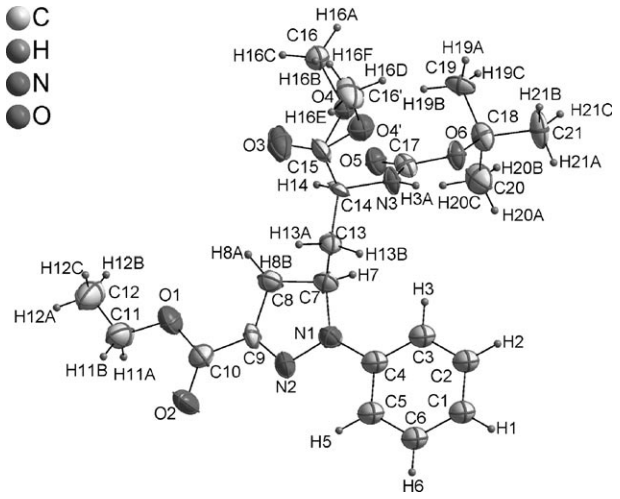
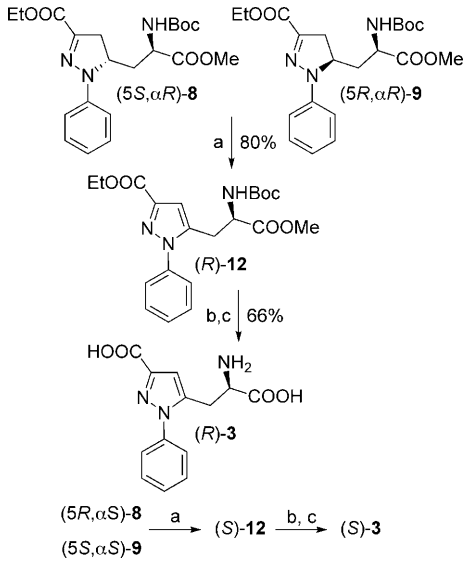


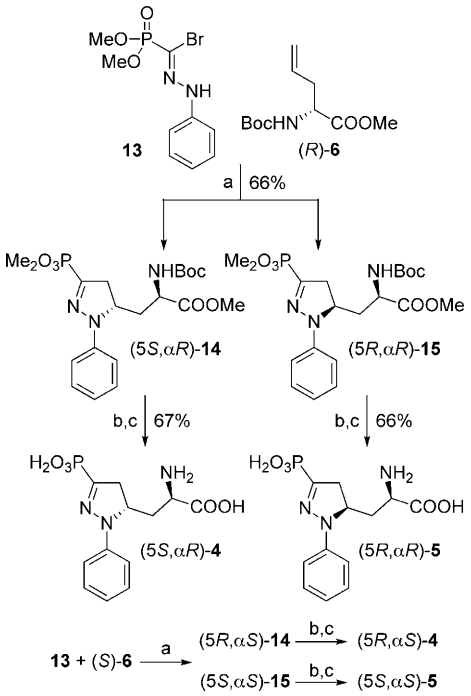

**Figure 3.** Perspective view of molecular structure of (−)-**8**. Displacement ellipsoids are drawn at 20% probability level.

To obtain the corresponding pyrazole derivatives, a mixture of compounds (5*S*,α*R*)-**8** and (5*R*,α*R*)-**9** was oxidized with pyridinium dichromate (PDC) in DMF to yield pyrazole (*R*)-**12**, which was submitted to standard deprotection procedures to yield final amino acid (*R*)-**3** (Scheme 2). The same approach was used to prepare (*S*)-**3**, beginning from a mixture of two diastereomeric pyrazolines exhibiting the *S* configuration at the amino acid stereogenic center (Scheme 2).

Synthesis of the new amino acids (5*S*,α*R*)-**4**, (5*R*,α*R*)-**5**, (5*R*,α*S*)-**4**, and (5*S*,α*S*)-**5** was achieved by using our recently published method for the preparation of 5-substituted 3-phosphono-pyrazolines.[17] The procedure involves in situ generation of dimethoxyphosphorylformonitrilimine from its stable precursor **13** and 1,3-dipolar cycloaddition to chiral dipolarophile (*R*)-**6** or (*S*)-**6**, respectively (Scheme 3). Cycloadducts (5*S*,α*R*)-**14** and (5*R*,α*R*)-**15** were separated by flash chromatography and submitted to hydrolysis with aqueous 1 N sodium hydroxide. Subsequent treatment with bromotrimethylsilane (TMSBr; 10 equiv) afforded the final products (5*S*,α*R*)-**4** and (5*R*,α*R*)-**5** (Scheme 3). The same reaction sequence was applied for the synthesis of (5*R*,α*S*)-**4** and (5*S*,α*S*)-**5**, beginning from alkene (*S*)-**6**.




**Scheme 2.** Reagents and conditions: a) PDC, DMF, 4 h, RT; b) NaOH (1 N), MeOH, 3 h, RT; c) TFA (30%), $CH_2Cl_2$, 3 h, 0 °C→RT.




**Scheme 3.** Reagents and conditions: a) $NaHCO_3$, EtOAc, 24 h, RT; b) NaOH (1 N), MeOH, 3 h, RT; c) TMSBr, $CH_2Cl_2$, 12 h, RT.

### Pharmacology

All new derivatives were subjected to in vitro assays, evaluating receptor binding to rat cortical membranes, using the radioligands [$^3$H]CGP39653, [$^3$H]AMPA, and [$^3$H]KA as representatives for NMDA, AMPA, and KA receptors, respectively (Table 1).[18–20] Amino acid (±)-**2** displayed a fivefold increase in the binding affinity for NMDA receptors, relative to (±)-**F94c** ($K_i$ = 0.20 μM vs. 0.96 μM, Table 1). Additionally, compound

**Table 1.** Receptor binding affinities at native rat iGluRs.[a]

| Compound | $K_i$ [μM] [$^3$H]CGP39653 | $IC_{50}$ [μM] [$^3$H]AMPA | [$^3$H]KAIN |
|---|---|---|---|
| (±)-**F94b**[b] | 0.21 [0.18;0.23] | >100 | >100 |
| (5*R*,α*S*)-**F94b**[b] | 1.9 [1.8;2.0] | >100 | >100 |
| (5*S*,α*R*)-**F94b**[b] | 0.10 [0.09;0.12] | >100 | >100 |
| (±)-**F94c**[b] | 0.96 [0.88;1.1] | >100 | >100 |
| (5*R*,α*R*)-**F94c**[b] | 0.51 [0.49;0.53] | >100 | >100 |
| (5*S*,α*S*)-**F94c**[b] | 2.5 [2.4;2.7] | >100 | >100 |
| (±)-**1** | 0.026 [0.024;0.028] | >100 | >100 |
| (5*S*,α*R*)-**1** | 0.017 [0.016;0.019] | >100 | >100 |
| (5*R*,α*S*)-**1** | 5.5 [4.8;6.3] | >100 | >100 |
| (±)-**2** | 0.20 [0.18;0.22] | >100 | >100 |
| (5*R*,α*R*)-**2** | 5.4 [4.7;6.2] | >100 | >100 |
| (5*S*,α*S*)-**2** | 0.18 [0.15;0.22] | >100 | >100 |
| (*R*)-**3** | 48 [45;51] | >100 | >100 |
| (*S*)-**3** | >100 | >100 | >100 |
| (5*S*,α*R*)-**4** | 0.031 [0.026;0.035] | >100 | >100 |
| (5*R*,α*S*)-**4** | 0.87 [0.76;0.99] | >100 | >100 |
| (5*R*,α*R*)-**5** | 1.1 [1.0;1.3] | >100 | >100 |
| (5*S*,α*S*)-**5** | 2.3 [2.2;2.4] | >100 | >100 |

[a] Values are expressed as the antilog to the log mean of at least three individual experiments; values in brackets [min;max] indicate ±SEM according to a logarithmic distribution of $K_i$. [b] Data from reference [12].

(±)-**1** showed a NMDA affinity eightfold higher than (±)-**F94b** ($K_i$ = 0.026 μM vs. 0.21 μM, Table 1), therefore behaving as a very potent NMDA receptor ligand. Compounds (±)-**1** and (±)-**2** exhibited no activity at other iGlu receptors (Table 1). Moreover, they were inactive as either agonists or antagonists at up to 1 mM concentration toward rat mGluR1 (group I), mGluR2 (group II), and mGluR4 (group III) receptors expressed in CHO cells.[21]

To confirm that (±)-**1** and (±)-**2** behave as antagonists, and to evaluate their subtype selectivity, the new ligands were tested on *Xenopus* oocytes expressing GluN1, in combination with either GluN2A, GluN2B, GluN2C, or GluN2D subunits.[12] Potential antagonists were evaluated at concentrations of 0.3 and 3 μM for inhibition of current responses to 10 μM Glu (Figure 4). At 3 μM, both compounds antagonized responses from GluN2A, and GluN2B and exhibited no or little antagonistic effects toward GluN2C and GluN2D. Consistent with the binding data, derivative (±)-**1** demonstrated greater inhibition of the Glu response than (±)-**2** at 3 μM. The two antagonists have a preference for GluN2A and GluN2B, as the potency of Glu at the four receptor subtypes is similar (Glu $EC_{50}$ values of GluN1/GluN2A, GluN1/GluN2B, GluN1/GluN2C, and GluN1/GluN2D are 2.9, 1.8, 1.0, and 0.5 μM, respectively).[22] Therefore,

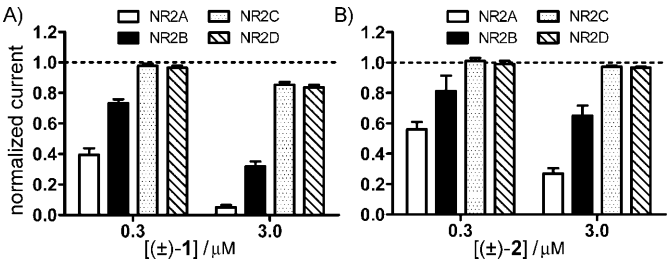



**Figure 4.** Compounds A) (±)-**1** and B) (±)-**2** were evaluated at concentrations of 0.3 and 3 μM for their ability to inhibit current responses to 10 μM Glu in *Xenopus* oocytes expressing cloned rat NMDA receptors, containing GluN1 in combination with either GluN2A, GluN2B, GluN2C, or GluN2D. Responses are normalized to the control response using 10 μM Glu alone. Data represent the mean ±SEM from 3–4 oocytes for each compound toward NMDA receptor subtypes.

the results of this investigation show that (±)-**1** and (±)-**2** share with model compounds **F94b** and **F94c** a similar NMDA receptor subtype preference for GluN2A and GluN2B over GluN2C and GluN2D.

Comparative analysis of the binding affinities obtained for pure enantiomers suggests that the most active antagonists are (5*S*,α*R*)-**1** ($K_i$: 0.017 μM, Table 1) and (5*S*,α*R*)-**4** ($K_i$: 0.031 μM, Table 1), which correlate stereochemically to our model compound (5*S*,α*R*)-**F94b** ($K_i$: 0.10 μM, Table 1). This is in agreement with the general rule that NMDA receptor antagonists possess an *R* configuration at the α-amino acid center. In addition, it appears evident that the presence of a stereocenter in position 5 of the heterocyclic nucleus plays a pivotal role in determining the correct orientation of the pharmacophoric groups to maximize interactions with the target protein. When the ω-acidic group and the side chain at C5 are in the same plane, as observed for pyrazole derivatives (*R*)-**3** or (*S*)-**3**, the affinity for NMDA receptors is no longer seen ($K_i$: 48 and >100 μM, respectively, Table 1). The bioisosteric replacement of the distal carboxylate with a phosphonate group, surprisingly, did not produce the expected increase in affinity; the two compounds exhibited nearly identical activity in this regard.

Due to the high potency of our two antagonists, we decided to extend the investigation of their pharmacological profiles, and to evaluate their potential use as neuroprotectants. Selected compounds (5*S*,α*R*)-**1** and (5*S*,α*R*)-**4** were examined as potential neuroprotective agents in cultured murine cortical cells exposed to oxygen-glucose deprivation (OGD) or NMDA at 300 μM; these assays represent well-established in vitro models of cerebral ischemia and excitotoxicity (Figure 5). As previously described,[23] exposure to OGD for 60 min or to 300 μM NMDA for 10 min produced an intermediate level of neuronal damage, as measured by the release of lactate dehydrogenase (LDH) into the bathing medium, which is approximately 75% (OGD) and 70% (NMDA) of that observed upon exposing the cultures to 1 mM Glu for 24 h (maximal damage).

Upon addition to the incubation medium during OGD or NMDA exposure, and following the subsequent 24 h recovery period, compounds (5*S*,α*R*)-**1** and (5*S*,α*R*)-**4** attenuated OGD and NMDA-induced neuronal death in a dose-dependent manner, with the maximal effect observed at 1 mM. As shown

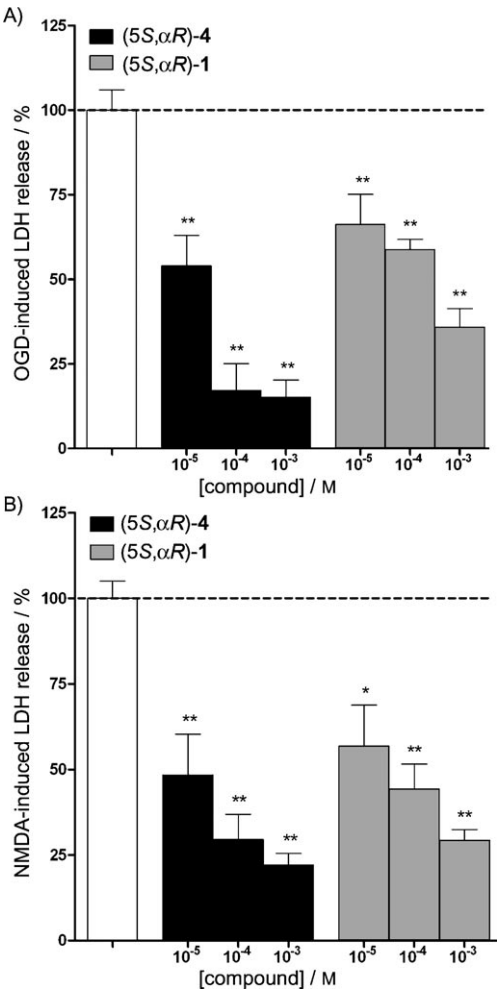



**Figure 5.** Effects of (5*S*,α*R*)-**1** and (5*S*,α*R*)-**4** on mixed mouse cortical cultures exposed to A) OGD or B) 300 μM NMDA. Under basal conditions, drugs were added to the medium, and neuronal death was assessed after 24 h by measuring LDH release in the medium. OGD was applied for 60 min and 300 μM NMDA for 10 min in cortical cultures. For these experiments, drugs were added 15 min before OGD or NMDA and were left in the medium during OGD and NMDA treatment and the subsequent 24 h recovery period. Data are expressed as a percentage of OGD and NMDA-induced neuronal damage. Values are the mean ± SEM of at least six experiments performed in triplicate (*$p < 0.05$, **$p < 0.01$ versus the corresponding control). Statistical analyses was performed using analysis of variance and Tukey's *w* test for multiple comparisons.

in Figure 5, amino acid (5*S*,α*R*)-**4** displayed a greater neuroprotective effect than (5*S*,α*R*)-**1** in both assays. Derivative (5*S*,α*R*)-**4** attenuates NMDA and OGD injury by approximately 78 ± 3.5% and 85 ± 5%, respectively, while (5*S*,α*R*)-**1** does so by 70 ± 3% and 64 ± 5.5%, respectively. Thus, in contrast to the results obtained in binding experiments, the bioisosteric replacement of the ω-carboxylate with a phosphonate moiety yielded a sizable improvement in biological activity in these assays.

The same compounds were also tested in vivo in DBA/2 mice to evaluate their ability to protect the animals against audiogenic seizures. Both compounds were able to produce dose-dependent protection against the tonic and clonic phase of these seizures. The $ED_{50}$ values for (5*S*,α*R*)-**1** and (5*S*,α*R*)-**4** are similar to those of the well-known NMDA receptor antagonist CPPene (Figure 1), with higher potency than those reported for AP7 or certain well-known anticonvulsant drugs (Table 2 and Figure 6). Following i.p. administration, (5*S*,α*R*)-**1** exhibited maximal protection at 15 min, while (5*S*,α*R*)-**4** exerted maximal protection between 30 and 90 min (Figure 7). Again, it was ob-

**Table 2.** Anticonvulsant activity of (5*S*,α*R*)-**1**, (5*S*,α*R*)-**4**, CPPene, AP7, felbamate, valproate, and phenytoin against audiogenic seizures in DBA/2 mice.[a]

| Compound | $ED_{50}$ [μmol kg$^{-1}$] | |
|---|---|---|
| | Clonus | Tonus |
| (5*S*,α*R*)-**1** | 2.7 (1.2–5.8) | 0.5 (0.2–0.9) |
| (5*S*,α*R*)-**4** | 2.8 (1.5–5.5) | 0.3 (0.2–0.7) |
| CPPene | 1.8 (1.2–2.3) | 0.8 (1.4–1.4) |
| AP7 | 30.5 (22.3–41.8) | 23.5 (15.3–36.1) |
| felbamate | 204.0 (149.0–282.0) | 97.0 (51.0–185.0) |
| valproate | 258.0 (196.0–341.0) | 187.0 (127.0–273.0) |
| phenytoin | 9.1 (5.6–14.9) | 7.3 (5.8–9.1) |

[a] All data were calculated according the method of Litchfield and Wilcoxon[32] and represent the 95% confidence limits of $ED_{50}$ values. A minimum of 32 animals (i.p. administration) were used to calculate each $ED_{50}$ value.

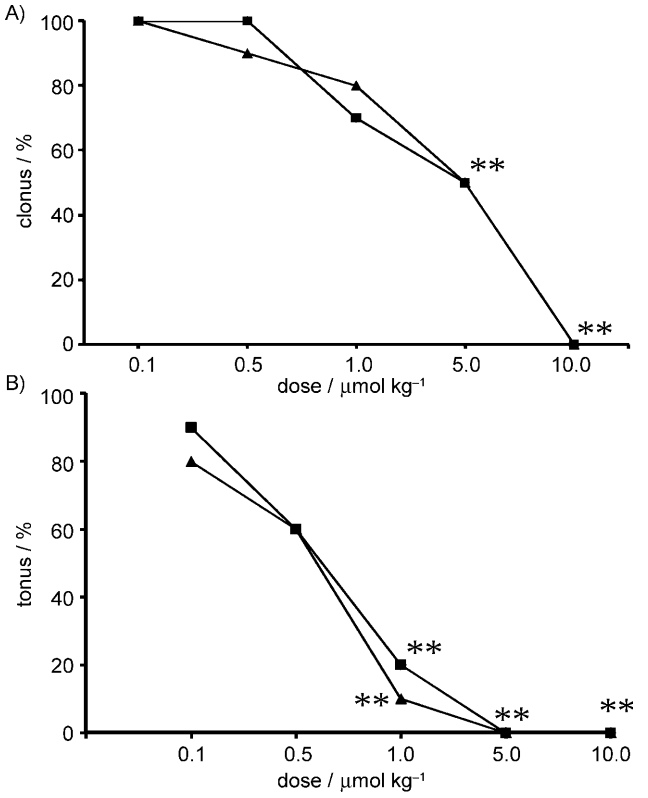



**Figure 6.** Dose–response curves for the anticonvulsant effects of (5*S*,α*R*)-**1** and (5*S*,α*R*)-**4** following i.p. administration. Abscissae correspond to dosage, and ordinates show: A) percent of clonic seizures and B) percent of tonic seizures. Mice (in groups of 10 per dose) were pretreated with increasing doses of (5*S*,α*R*)-**1** (■) or (5*S*,α*R*)-**4** (▲), and auditory stimulation was applied for 30 min following drug administration (**$p < 0.01$ versus the corresponding control). Statistical analysis was performed using Fisher's exact probability test.

served that replacement of the ω-carboxylate with a phosphonate group produces significant modification of the pharmacological profile. In fact, although we obtained nearly identical $ED_{50}$ values for anticonvulsant activity, a result that seems to parallel the data of binding experiments, the two compounds displayed appreciable differences in pharmacokinetic profiles with a significant impact on overall pharmacological activity. Indeed, (5*S*,α*R*)-**4** had a slower onset of action than (5*S*,α*R*)-**1**, a delay that may be attributed to a different absorption rate into the CNS.

Additionally, Figure 7 clearly shows that (5*S*,α*R*)-**4** offers a longer period of protection against seizures, a result that, together with the notable neuroprotective activity described above, suggests this compound may be a promising neuroprotective agent. Further pharmacological investigations, as well as evaluation of pharmacokinetic and toxicological parameters, will be pursued and the results reported in due course.

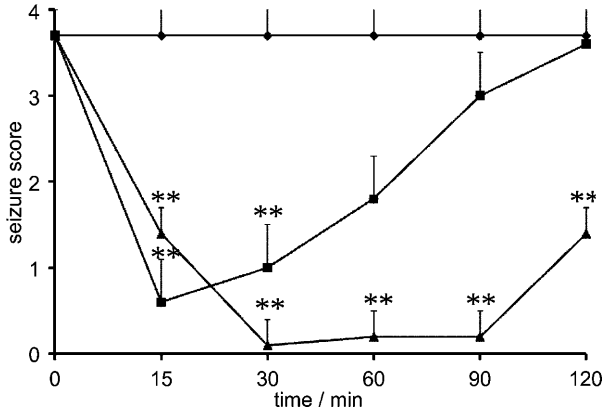



**Figure 7.** Anticonvulsant effects following i.p. administration of 10 μmol kg$^{-1}$ of (5*S*,α*R*)-**1** (■) and (5*S*,α*R*)-**4** (▲), plotted against audiogenic seizures in DBA/2 mice; ◆: vehicle. Abscissa corresponds to time following i.p. administration of drug. Groups of 10 mice were used for determination of each point, which represents the mean seizure score ±SEM against audiogenic seizures in DBA/2 mice (**$p < 0.01$ versus the corresponding control). Statistical analysis was performed using Fisher's exact probability test.

## Conclusions

In the present work we have described the development of new, highly potent, NMDA receptor antagonists with promising pharmacological activity as neuroprotective agents. These agents may potentially be used to counteract damages induced by cerebral ischemia, and are characterized by good in vivo anticonvulsant activity. The rationale for their design was based on the structural modification of previously described NMDA antagonists that we decided to further modify in order to strengthen interactions with the receptor binding site. To this end, we replaced the original $\Delta^2$-isoxazoline ring with an N1-substituted pyrazoline and, subsequently, replaced the distal carboxylate with a bioisosteric phosphonate group. Finally, we investigated the relevance of stereochemistry toward biological activity by preparing and evaluating all possible stereoisomers, as well as by abolishing the stereocenter associated with the heterocycle. Our goal to improve the potency of lead compounds **F94b** and **F94c** was successfully accomplished. Moreover, the introduction of a phosphonic acid group has emphasized the possibility for modification of not only the pharmacodynamic but also, interestingly, the pharmacokinetic profile of the molecule.

The ligands described are able to differentiate between the GluN2A/B subtypes over the GluN2C/D subtypes; however, higher selectivity toward a single receptor subtype is desired. Work is underway to identify suitable substituents for the N1 position in order to further enhance interactions with an amino acid residue of the active site, which is predicted to increase the affinity for a specific receptor subunit, particularly the GluN2B subtype.

## Experimental Section

### *Materials and methods*

$^1$H NMR and $^{13}$C NMR spectra were recorded with a Varian Mercury 300 (300 MHz) spectrometer. Chemical shifts ($\delta$) are expressed in ppm, and coupling constants ($J$) are expressed in Hz. Rotary power determinations were carried out using a Jasco P-1010 spectropolarimeter, coupled with a Haake N3-B thermostat. TLC analyses were performed on commercial silica gel 60 $F_{254}$ aluminum sheets; spots were further evidenced by spraying with a dilute alkaline potassium permanganate solution or ninhydrin. Melting points were determined on a model B 540 Büchi apparatus and are uncorrected. Microanalyses (C, H, N) of new compounds were within ±0.4% of theoretical values.

**Ethyl (5*R**,α*S**)-5-(2-*tert*-butoxycarbonylamino-2-methoxycarbonylethyl)-4,5-dihydro-1-phenyl-1*H*-pyrazole-3-carboxylate [(±)-8] and ethyl (5*R**,α*R**)-5-(2-*tert*-butoxycarbonylamino-2-methoxycarbonylethyl)-4,5-dihydro-1-phenyl-1*H*-pyrazole-3-carboxylate [(±)-9].** Ethyl-2-chloro-2-(phenylhydrazone)acetate **7**[13] (2.22 g, 9.81 mmol) and solid $NaHCO_3$ (2.75 g, 32.7 mmol) were added to a solution of methyl 2-*tert*-butoxycarbonylaminopent-4-enoate (±)-**6**[11] (1.50 g, 6.54 mmol) in EtOAc (25 mL). The mixture was stirred vigorously for 24 h; progress of the reaction was followed by TLC [petroleum ether (PE)/EtOAc, 7:3]. $H_2O$ (5 mL) was added, and the organic layer was separated and dried over anhydrous $Na_2SO_4$. The crude material obtained upon evaporation of the solvent was purified by column chromatography on silica gel (PE/EtOAc, 85:15) to give an inseparable mixture of two diastereomers: (±)-**8** and (±)-**9** (1.92 g, 70%). $R_f = 0.28$ (PE/EtOAc, 4:1).

**Ethyl (5*R**,α*S**)-5-(2-amino-2-methoxycarbonylethyl)-4,5-dihydro-1-phenyl-1*H*-pyrazole-3-carboxylate [(±)-10] and ethyl (5*R**,α*R**)-5-(2-amino-2-methoxycarbonylethyl)-4,5-dihydro-1-phenyl-1*H*-pyrazole-3-carboxylate [(±)-11].** The mixture of compounds (±)-**8** and (±)-**9** (1.92 g, 4.57 mmol) was treated with a 30% $CH_2Cl_2$ solution of TFA (11.6 mL) at 0 °C. The solution stirred at room temperature for 3 h, and the reaction was followed by TLC (PE/EtOAc, 7:3). A saturated solution of $NaHCO_3$ (50 mL) and $CH_2Cl_2$ (35 mL) were then added, and the organic layer was separated, dried over anhydrous $Na_2SO_4$, and evaporated under reduced pressure. The crude material was purified on silica gel (PE/EtOAc, 3:7) to give (±)-**10** (0.49 g, 34%) and (±)-**11** (0.53 g, 36%) as yellow oils. The amines were used directly in the subsequent synthetic step without further purification. (±)-**10**: $R_f = 0.64$ (EtOAc); (±)-**11** $R_f = 0.50$ (EtOAc).

**Ethyl (5*R**,α*S**)-5-(2-*tert*-butoxycarbonylamino-2-methoxycarbonylethyl)-4,5-dihydro-1-phenyl-1*H*-pyrazole-3-carboxylate [(±)-8] from [(±)-10].** $Et_3N$ (0.32 mL, 2.25 mmol) and a solution of di-*tert*-butyl dicarbonate (425 mg, 1.95 mmol) in $CH_2Cl_2$ (3 mL) were added to a stirred solution of (±)-**10** (0.49 g, 1.50 mmol) in $CH_2Cl_2$ (10 mL) at 0 °C. The reaction mixture stirred at room temperature overnight and was monitored by TLC (EtOAc). The solvent was evaporated under reduced pressure, and the crude material was purified by column chromatography on silica gel (PE/EtOAc, 85:15) to give (±)-**8** (0.50 g, 80%) as a pale-yellow oil; $R_f = 0.28$ (PE/EtOAc, 4:1); $^1$H NMR ([D$_6$]DMSO, 100 °C): $\delta = 1.28$ (t, $J = 7.0$, 3H); 1.42 (s, 9H); 1.83 (ddd, $J = 4.1$, 10.2, 14.0, 1H); 2.20 (ddd, $J = 2.6$, 10.7, 14.0, 1H); 2.96 (dd, $J = 5.6$, 17.7, 1H); 3.23 (dd, $J = 11.7$, 17.7, 1H); 3.60 (s, 3H); 4.10 (ddd, $J = 4.1$, 8.0, 10.7, 1H); 4.24 (q, $J = 7.0$, 2H); 4.60 (dddd, $J = 2.6$, 5.6, 10.2, 11.7, 1H); 6.92 (t, $J = 7.2$, 1H); 7.12 (d, $J = 8.0$, 1H); 7.18 (d, $J = 9.0$, 2H); 7.27 (dd, $J = 7.2$, 9.0, 2H); $^{13}$C NMR ([D$_6$]DMSO): $\delta = 14.93$, 28.82, 31.80, 36.38, 50.55, 52.69, 58.57, 61.11, 79.25, 114.99, 121.52, 129.92, 139.75, 142.41, 156.34, 162.76, 173.10; Anal. calcd for $C_{21}H_{29}N_3O_6$: C 60.13, H 6.97, N 10.02, found: C 60.18, H 7.04, N 9.90.

**(5*R**,α*S**)-5-(2-Amino-2-carboxyethyl)-4,5-dihydro-1-phenyl-1*H*-pyrazole-3-carboxylic acid [(±)-1].** A) Compound (±)-**8** (0.50 g, 1.20 mmol) was dissolved in MeOH (5 mL) and treated with 1 N aqueous NaOH (3.6 mL). Disappearance of the starting material was monitored by TLC (PE/EtOAc, 7:3). Upon evaporation of the MeOH, the aqueous layer was washed with $Et_2O$, acidified with 2 N aqueous HCl, and extracted with EtOAc. The organic layer was dried over anhydrous $Na_2SO_4$ and, upon evaporation of the solvent, the diacid derivative (0.44 g, 98%) was obtained as a yellow solid. B) The diacid derivative (0.44 g, 1.18 mmol) was treated with a 30% $CH_2Cl_2$ solution of TFA (3 mL) at 0 °C. The solution stirred at room temperature for 3 h, and the reaction was followed by TLC ($CHCl_3$/MeOH, 9:1 + 1% AcOH). Volatiles were removed under reduced pressure and the residue was crystallized from EtOH/$Et_2O$ to give (±)-**1** (0.17 g, 53%) as a yellow powder; mp: >158 °C (dec.); $R_f = 0.42$ (BuOH/$H_2O$/AcOH, 4:2:1); $^1$H NMR ($D_2O$ +1 drop $CF_3COOD$): $\delta = 1.96$ (ddd, $J = 6.3$, 9.3, 15.0, 1H); 2.04 (ddd, $J = 3.0$, 7.7, 15.0, 1H); 2.77 (dd, $J = 5.5$, 17.9, 1H); 3.09 (dd, $J = 12.1$, 17.9, 1H); 3.92 (dd, $J = 6.3$, 7.7, 1H); 4.65 (m, 1H); 6.85 (t, $J = 7.7$, 1H); 6.96 (d, $J = 7.7$, 2H); 7.18 (t, $J = 7.7$, 2H); $^{13}$C NMR ($D_2O$ + 1 drop $CF_3COOD$): $\delta = 32.14$, 35.80, 49.84, 58.48, 115.61, 122.66, 129.76, 139.71, 141.37, 165.65, 171.14; Anal. calcd for $C_{13}H_{15}N_3O_4$: C 56.31, H 5.45, N 15.15, found: C 56.26, H 5.55, N 14.96.

**Ethyl (5*R**,α*R**)-5-(2-*tert*-butoxycarbonylamino-2-methoxycarbonylethyl)-4,5-dihydro-1-phenyl-1*H*-pyrazole-3-carboxylate (±)-9 from (±)-11.** $Et_3N$ (0.37 mL, 2.49 mmol) and a solution of di-*tert*-butyl dicarbonate (0.47 g, 2.16 mmol) in $CH_2Cl_2$ (5 mL) were added to a stirred solution of (±)-**11** (0.53 g, 1.66 mmol) in $CH_2Cl_2$ (15 mL) at 0 °C. The reaction mixture stirred at room temperature overnight and was followed by TLC (EtOAc). The solvent was evaporated under reduced pressure, and the crude material was purified by column chromatography on silica gel (PE/EtOAc, 85:15) to give (±)-**9** (0.49 g, 70%) as a pale-yellow oil. $R_f = 0.28$ (PE/EtOAc, 4:1); $^1$H NMR ([D$_6$]DMSO, 100 °C): $\delta = 1.28$ (t, $J = 7.0$, 3H); 1.40 (s, 9H); 1.74 (ddd, $J = 7.0$, 9.7, 14.0, 1H); 2.24 (ddd, $J = 3.0$, 6.7, 14.0, 1H); 2.97 (dd, $J = 5.5$,17.9, 1H); 3.23 (dd, $J = 11.7$, 17.9, 1H); 3.67 (s, 3H); 4.13 (ddd, $J = 6.7$, 7.0, 7.0, 1H); 4.24 (q, $J = 7.0$, 2H); 4.64 (dddd, $J = 3.0$, 5.5, 9.7, 11.7, 1H); 6.92 (d, $J = 7.3$, 1H); 6.92 (d, $J = 7.0$, 1H) 7.14 (d, $J = 8.8$, 2H); 7.30 (dd, $J = 7.3$, 8.8, 2H); $^{13}$C NMR ([D$_6$]DMSO): $\delta = 14.91$, 28.83, 33.57, 37.57, 51.56, 52.76, 59.24, 61.13, 79.23, 114.89, 121.60, 130.03, 140.19, 142.55, 155.97, 162.75, 173.08; Anal. calcd for $C_{21}H_{29}N_3O_6$: C 60.13, H 6.97, N 10.02, found: C 60.22, H 7.13, N 9.77.

**(5*R**,α*R**)-5-(2-Amino-2-carboxyethyl)-4,5-dihydro-1-phenyl-1*H*-pyrazole-3-carboxylic acid [(±)-2].** A) Compound (±)-**9** (0.49 g, 1.16 mmol) was dissolved in MeOH (10 mL) and treated with 1 N aqueous NaOH (3.5 mL). Disappearance of the starting material was monitored by TLC (PE/EtOAc, 7:3). Upon evaporation of the MeOH, the aqueous layer was washed with $Et_2O$, acidified with 2 N aqueous HCl, and extracted with EtOAc. The organic layer was dried over anhydrous $Na_2SO_4$ and, upon evaporation of the solvent, the diacid derivative (0.37 g, 86%) was obtained as a yellow solid. B) The diacid (0.37 g, 1.00 mmol) was treated with a 30% $CH_2Cl_2$ solution of TFA (0.77 mL, 10.00 mmol) at 0 °C. The solution stirred at room temperature for 3 h, and the reaction was followed by TLC ($CHCl_3$/MeOH, 9:1 + 1% AcOH). Volatiles were removed under reduced pressure, and the residue was crystallized from EtOH/$Et_2O$ to give (±)-**2** (0.16 g, 70%) as a yellow powder; mp: >188 °C (dec.); $R_f = 0.44$ (BuOH/$H_2O$/AcOH, 4:2:1); $^1$H NMR ($D_2O$ + 1 drop $CF_3COOD$): $\delta = 1.66$ (ddd, $J = 3.8$, 10.2, 14.0, 1H); 2.13 (ddd, $J = 3.0$, 11.0, 14.0, 1H); 2.76 (dd, $J = 5.1$, 17.6, 1H); 3.09 (dd, $J = 12.1$, 17.6, 1H); 3.80 (dd, $J = 3.8$, 11.0, 1H); 4.60 (m, 1H); 6.80 (t, $J = 7.7$, 1H); 6.93 (d, $J = 7.7$, 2H); 7.18 (t, $J = 7.7$, 2H); $^{13}$C NMR ($D_2O$ + 1 drop $CF_3COOD$): $\delta = 31.55$, 35.52, 49.02, 58.72, 115.14, 122.41, 129.72, 139.56, 141.23, 165.65, 171.17; Anal. calcd for $C_{13}H_{15}N_3O_4$: C 56.31, H 5.45, N 15.15, found: C 56.19, H 5.63, N 14.95.

**Synthesis of (5*S*,α*R*)-1, (5*R*,α*S*)-1, (5*R*,α*R*)-2, and (5*S*,α*S*)-2.** The synthetic procedure described for racemates (±)-**1** and (±)-**2** was also used to obtain the pure enantiomers (5*S*,α*R*)-**1**, (5*R*,α*R*)-**2**, (5*R*,α*S*)-**1** and (5*S*,α*S*)-**2**, starting from alkenes (*R*)-**6**[15] and (*S*)-**6**,[16] respectively.

(5*S*,α*R*)-**8** was crystallized from hexane/diisopropyl ether as white prisms; mp: 63–65 °C; $[\alpha]_D^{20} = +313.1$ ($c = 0.91$; $CHCl_3$); Anal. calcd for $C_{21}H_{29}N_3O_6$: C 60.13, H 6.97, N 10.02, found: C 60.08, H 6.85, N 10.11.

(5*R*,α*S*)-**8** was crystallized from hexane/diisopropyl ether as white prisms; mp: 63–65 °C; $[\alpha]_D^{20} = -315.0$ ($c = 1.00$; $CHCl_3$); Anal. calcd for $C_{21}H_{29}N_3O_6$: C 60.13, H 6.97, N 10.02, found: C 60.23, H 6.94, N 10.00.

(5*R*,α*R*)-**9** was obtained as a pale-yellow oil; $[\alpha]_D^{20} = -398.1$ ($c = 0.93$; $CHCl_3$); Anal. calcd for $C_{21}H_{29}N_3O_6$: C 60.13, H 6.97, N 10.02, found: C 60.30, H 6.79, N 10.15.

(5*S*,α*S*)-**9** was obtained as a pale-yellow oil; $[\alpha]_D^{20} = +395.1$ ($c = 0.95$; $CHCl_3$); Anal. calcd for $C_{21}H_{29}N_3O_6$: C 60.13, H 6.97, N 10.02, found: C 60.21, H 6.83, N 9.89.

(5*S*,α*R*)-**1** was crystallized from EtOH/$Et_2O$ as yellow prisms; mp: >192 °C (dec.); $[\alpha]_D^{20} = +334.7$ ($c = 0.10$; $H_2O$); Anal. calcd for $C_{13}H_{15}N_3O_4$: C 56.31, H 5.45, N 15.15, found: C 56.51, H 5.55, N 14.90.

(5*R*,α*S*)-**1** was crystallized from EtOH/$Et_2O$ as yellow prisms; mp: >192 °C (dec.); $[\alpha]_D^{20} = -336.2$ ($c = 0.11$; $H_2O$); Anal. calcd for $C_{13}H_{15}N_3O_4$: C 56.31, H 5.45, N 15.15, found: C 56.48, H 5.50, N 15.23.

(5*R*,α*R*)-**2** was crystallized from EtOH/$Et_2O$ as yellow prisms; mp: >218 °C (dec.); $[\alpha]_D^{20} = -394.0$ ($c = 0.10$; $H_2O$); Anal. calcd for $C_{13}H_{15}N_3O_4$: C 56.31, H 5.45, N 15.15, found: C 56.57, H 5.23, N 15.31.

(5*S*,α*S*)-**2** was crystallized from EtOH/$Et_2O$ as yellow prisms; mp: >218 °C (dec.); $[\alpha]_D^{20} = +396.2$ ($c = 0.10$; $H_2O$); Anal. calcd for

$C_{13}H_{15}N_3O_4$: C 56.31, H 5.45, N 15.15, found: C 56.49, H 5.58, N 14.92.

**Ethyl (*R*)-5-(2-*tert*-butoxycarbonylamino-2-methoxycarbonylethyl)-1-phenyl-1*H*-pyrazole-3-carboxylate [(*R*)-12].** PDC (6.71 g, 17.85 mmol) was added to a mixture of (5*S*,α*R*)-**8** and (5*R*,α*R*)-**9** (0.50 g, 1.19 mmol) in DMF (7 mL). The solution stirred at room temperature for 4 h and the reaction was followed by TLC (PE/EtOAc, 4:1). $H_2O$ (10 mL) was added, and the solution was extracted with EtOAc (3×30 mL). The combined organic layers were washed with brine, dried over anhydrous $Na_2SO_4$, and evaporated under reduced pressure. The crude material was purified on silica gel (PE/EtOAc, 4:1) to give (*R*)-**12** (0.39 g, 80%), which was crystallized from diisopropyl ether as white prisms; mp: >110 °C (dec.); $R_f$=0.28 (PE/EtOAc, 4:1); $[\alpha]_D^{20}=-35.2$ ($c$=0.22; $CHCl_3$); $^1$H NMR ([D$_6$]DMSO, 100 °C): $\delta$=1.30 (t, $J$=7.0, 3H); 1.34 (s, 9H); 3.03 (dd, $J$=9.0, 15.7, 1H); 3.13 (dd, $J$=5.4, 15.7, 1H); 3.58 (s, 3H); 4.32 (m, 1H); 4.35 (q, $J$=7.0, 2H); 6.75 (s, 1H); 6.92 (d, $J$=7.0, 1H); 7.42–7.60 (m, 5H); $^{13}$C NMR ([D$_6$]DMSO): $\delta$=14.90, 28.14, 28.73, 52.58, 52.80, 60.97, 79.50, 109.29, 126.41, 129.74, 130.10, 139.28, 142.04, 143.57, 155.98, 162.33, 172.23; Anal. calcd for $C_{21}H_{27}N_3O_6$: C 60.42, H 6.52, N 10.07, found: C 60.69, H 6.63, N 10.18.

**(*R*)-5-(2-Amino-2-carboxyethyl)-1-phenyl-1*H*-pyrazole-3-carboxylic acid [(*R*)-3].** A) Compound (*R*)-**12** (0.39 g, 0.95 mmol) was dissolved in MeOH (10 mL) and treated with 1 N aqueous NaOH (2.85 mL). Disappearance of the starting material was monitored by TLC (PE/EtOAc, 7:3). Upon evaporation of MeOH, the aqueous layer was washed with $Et_2O$, acidified with 2 N aqueous HCl, and extracted with EtOAc. The organic layer was dried over anhydrous $Na_2SO_4$ and, upon evaporation of the solvent, the diacid derivative (0.32 g, yield 90%) was obtained as a white solid. B) The diacid (0.32 g, 0.85 mmol) was treated with a 30% $CH_2Cl_2$ solution of TFA (2.2 mL) at 0 °C. The solution stirred at room temperature for 3 h, and the reaction was followed by TLC ($CHCl_3$/MeOH, 9:1 + 1% AcOH). Volatiles were removed under reduced pressure, and the residue was crystallized from EtOH/$Et_2O$ to give (*R*)-**3** (0.14 g, 73%) as white prisms; mp: >230 °C (dec.); $R_f$=0.20 (BuOH/$H_2O$/AcOH 4:2:1); $[\alpha]_D^{20}=+3.7$ ($c$=0.10; $H_2O$); $^1$H NMR ($D_2O$ + 1 drop $CF_3COOD$): $\delta$=3.13 (dd, $J$=6.8, 16.0, 1H); 3.23 (dd, $J$=6.8, 16.0, 1H); 3.90 (t, $J$=6.8, 1H); 6.76 (s, 1H); 7.22 (m, 2H); 7.35 (m, 3H); $^{13}$C NMR ($D_2O$ + 1 drop $CF_3COOD$): $\delta$=26.21, 51.53, 110.02, 126.03, 129.90, 130.23, 137.35, 139.36, 143.50, 164.88, 170.12; Anal. calcd for $C_{13}H_{13}N_3O_4$: C 56.72, H 4.76, N 15.27, found: C 56.52, H 4.64, N 15.35.

**(*S*)-5-(2-Amino-2-carboxyethyl)-1-phenyl-1*H*-pyrazole-3-carboxylic acid [(*S*)-3].** The same procedure described for the synthesis of (*R*)-**3** was used to prepare (*S*)-**3**, starting from a mixture of the two diastereomeric pyrazolines (5*R*,α*S*)-**8** and (5*S*,α*S*)-**9**.

(*S*)-**12** was crystallized from diisopropyl ether as white prisms; mp: >110 °C (dec.); $[\alpha]_D^{20}=+34.5$ ($c$=0.25; $CHCl_3$); Anal. calcd for $C_{21}H_{27}N_3O_6$: C 60.42, H 6.52, N 10.07, found: C 60.52, H 6.44, N 10.00.

(*S*)-**3** was crystallized from EtOH/$Et_2O$ as white prisms; mp: >230 °C (dec.); $[\alpha]_D^{20}=-4.4$ ($c$=0.10; $H_2O$); Anal. calcd for $C_{13}H_{13}N_3O_4$: C 56.72, H 4.76, N 15.27, found: C 56.89, H 4.71, N 15.44.

**Dimethyl (5*S*,α*R*)-5-(2-*tert*-butoxycarbonylamino-2-methoxycarbonylethyl)-4,5-dihydro-1-phenyl-1*H*-pyrazole-3-phosphonate [(5*S*,α*R*)-14] and dimethyl (5*R*,α*R*)-5-(2-*tert*-butoxycarbonylamino-2-methoxycarbonylethyl)-4,5-dihydro-1-phenyl-1*H*-pyrazole-3-phosphonate [(5*R*,α*R*)-15].** Dimethyl [bromo(phenylhydrazono)-methyl]-phosphonate **13**[17] (2.61 g, 8.50 mmol) and solid $NaHCO_3$ (2.40 g, 28.57 mmol) were added to a solution of (*R*)-**6** (1.30 g, 5.70 mmol) in EtOAc (25 mL). The reaction mixture stirred vigorously for 24 h and was monitored by TLC (PE/EtOAc, 3:7). $H_2O$ (2 mL) was added, and the organic layer was isolated and dried over anhydrous $Na_2SO_4$. Upon evaporation of the solvent, the crude material was purified by flash chromatography on silica gel (PE/EtOAc, 2:3) to give pure (5*R*,α*R*)-**15** (690 mg, 27%) and (5*S*,α*R*)-**14** (1.02 g, 39%).

(5*S*,α*R*)-**14** was obtained as a pale-yellow oil. $R_f$=0.26 (PE/EtOAc, 3:7); $[\alpha]_D^{20}=+149.5$ ($c$=0.85; $CHCl_3$); $^1$H NMR ([D$_6$]DMSO, 100 °C): $\delta$=1.40 (s, 9H); 1.75 (ddd, $J$=4.4, 10.4, 14.8, 1H); 2.20 (dddd, $J$=1.1, 2.9, 10.7, 14.8, 1H); 2.91 (ddd, $J$=1.4, 5.8, 17.6, 1H); 3.23 (ddd, $J$=1,6, 11.3, 17.6, 1H); 3.61 (s, 3H); 3.74 (d, $J$=11.0, 1H); 3.75 (d, $J$=11.0, 1H); 4.10 (ddd, $J$=4.4, 8.2, 10.7, 1H); 4.49 (dddd, $J$=2.9, 5.8, 10.4, 11.3, 1H); 6.90 (m, 1H); 6.92 (m, 1H); 7.16 (d, 2H); 7.27 (d, 2H); $^{13}$C NMR ([D$_6$]DMSO): $\delta$=28.82, 31.69, 39.79 (d, $J_{C-P}$=19 Hz), 50.72, 52.69, 53.72, 53.80, 57.39, 79.28, 114.81, 121.24, 129.87, 139.24 (d, $J_{C-P}$=231 Hz), 142.80, 156.38, 173.03; $^{31}$P NMR ($CDCl_3$): $\delta$=12.89; Anal. calcd for $C_{20}H_{30}N_3O_7P$: C 52.74, H 6.64, N 9.23, found: C 52.90, H 6.72, N 9.17.

(5*R*,α*R*)-**15** was obtained as a pale-yellow oil. $R_f$=0.28 (PE/EtOAc, 3:7); $[\alpha]_D^{20}=-195.0$ ($c$=0.98; $CHCl_3$); $^1$H NMR ([D$_6$]DMSO, 100 °C): $\delta$=1.40 (s, 9H); 1.68 (ddd, $J$=7.0, 10.0, 14.0, 1H); 2.23 (dddd, $J$=1.1, 3.0, 7.0, 14.0, 1H); 2.94 (ddd, $J$=1.4, 5.5, 17.6, 1H); 3.23 (ddd, $J$=1,6, 11.3, 17.6, 1H); 3.67 (s, 3H); 3.74 (d, $J$=11.3, 1H); 3.75 (d, $J$=11.3, 1H); 4.12 (ddd, $J$=7.0, 1H); 4.52 (dddd, $J$=3.0, 5.5, 10.0, 11.3, 1H); 6.90 (t, 1H); 6.92 (m, 1H); 7.10 (d, 2H), 7.28 (d, 2H); $^{13}$C NMR ([D$_6$]DMSO): $\delta$ 28.82, 33.56, 39.57 (d, $J_{C-P}$=19 Hz) 51.62, 52.76, 53.72, 53.79, 57.92, 79.25, 114.68, 121.31, 130.00, 140.02 (d, $J_{C-P}$=231 Hz), 142.96, 156.01, 173.07; $^{31}$P NMR ($CDCl_3$): $\delta$=12.86; Anal. calcd for $C_{20}H_{30}N_3O_7P$: C 52.74, H 6.64, N 9.23, found: C 52.86, H 6.75, N 9.08.

**(5*S*,α*R*)-5-(2-Amino-2-carboxyethyl)-4,5-dihydro-1-phenyl-1*H*-pyrazole-3-phosphonic acid [(5*S*,α*R*)-4].** A) Compound (5*S*,α*R*)-**14** (1.02 g, 2.24 mmol) was dissolved in MeOH (5 mL) and treated with 1 N aqueous NaOH (5 mL). Disappearance of the starting material was monitored by TLC (PE/EtOAc, 7:3). Upon evaporation of the MeOH, the aqueous layer was washed with $Et_2O$, acidified with 2 N aqueous HCl, and extracted with EtOAc. The organic layer was dried over anhydrous $Na_2SO_4$, and, upon evaporation of the solvent, the carboxylic acid intermediate was obtained as a colorless oil. B) Bromotrimethylsilane (TMSBr; 3 mL, 22.5 mmol) was added to a solution of the carboxylic acid derivative in $CH_2Cl_2$ (10 mL). The reaction stirred at room temperature overnight. The solvent was evaporated under reduced pressure, and MeOH (5 mL) was added. The reaction mixture stirred for 1 h, followed by evaporation of the solvent to give pure (5*S*,α*R*)-**4** (480 mg, 67%) as a white hygroscopic powder; mp: >210 °C (dec.); $R_f$=0.34 (BuOH/$H_2O$/AcOH 4:2:1); $[\alpha]_D^{20}=+116.5$ ($c$=0.13; $H_2O$); $^1$H NMR ($D_2O$): $\delta$=1.90–2.10 (m, 2H); 2.80 (dd, $J$=4.7, 17.9, 1H); 3.16 (dd, $J$=10.7, 17.9, 1H); 3.92 (dd, $J$=6.0, 7.7, 1H); 4.42 (dddd, $J$=4.7, 5.0, 10.0, 10.7, 1H); 6.84 (t, $J$=7.7, 1H); 7.01 (d, $J$=7.7, 2H); 7.20 (t, $J$=7.7, 2H); $^{13}$C NMR ($D_2O$): $\delta$=31.92, 39.85 (d, $J_{C-P}$=20 Hz), 50.16, 57.28, 115.65, 121.68, 129.58, 143.26, 148.12 (d, $J_{C-P}$=215 Hz), 171.42; $^{31}$P NMR ($D_2O$): $\delta$=4.80; Anal. calcd for $C_{12}H_{16}N_3O_5P$: C 46.01, H 5.15, N 13.41, found: C 45.95, H 5.23, N 13.26.

**(5*R*,α*R*)-5-(2-Amino-2-carboxyethyl)-4,5-dihydro-1-phenyl-1*H*-pyrazole-3-phosphonic acid [(5*R*,α*R*)-5].** A) Compound (5*R*,α*R*)-**15** (690 mg, 1.52 mmol) was dissolved in MeOH (5 mL) and treated with 1 N aqueous NaOH (5 mL). Disappearance of the starting ma-

terial was monitored by TLC (PE/EtOAc, 7:3). Upon evaporation of the MeOH, the aqueous layer was washed with $Et_2O$, acidified with 2 N aqueous HCl, and extracted with EtOAc. The organic layer was dried over anhydrous $Na_2SO_4$ and, upon evaporation of the solvent, the carboxylic acid intermediate was obtained as a colorless oil. B) TMSBr (2 mL, 15.0 mmol) was added to a solution of the carboxylic acid derivative in $CH_2Cl_2$ (10 mL). The reaction stirred at room temperature overnight. The solvent was evaporated under reduced pressure, and MeOH (5 mL) was added. The reaction mixture stirred for 1 h, followed by evaporation of the solvent to give pure (5*R*,α*R*)-**5** (320 mg, 66%) as a white hygroscopic powder; mp: >215 °C (dec.); $R_f$ = 0.37 (BuOH/$H_2O$/AcOH 4:2:1); $[\alpha]_D^{20} = -219.2$ (*c* = 0.11; $H_2O$); $^1$H NMR ($D_2O$): $\delta$ = 1.70 (ddd, *J* = 4.0, 10.7, 14,5, 1H); 2.16 (ddd, *J* = 2.2, 11.0, 14.5, 1H); 2.83 (ddd, *J* = 1.4, 4.7, 17.6, 1H); 3.15 (ddd, *J* = 1.4, 11.0, 17.6, 1H); 3.91 (dd, *J* = 4.0, 11.0, 1H); 4.52 (dddd, *J* = 2.2, 4.7, 10.7, 11.0, 1H); 6.83 (t, *J* = 7.4, 1H); 6.98 (d, *J* = 7.7, 2H); 7.18 (dd, *J* = 7.4, 7.7, 2H); $^{13}$C NMR ($D_2O$): $\delta$ = 31.26, 39.57 (d, $J_{C-P}$ = 19 Hz), 49.57, 57.55, 115.22, 121.45, 129.77, 143.35, 148.62 (d, $J_{C-P}$ = 214 Hz), 171.60; $^{31}$P NMR ($D_2O$): $\delta$ = 5.16; Anal. calcd for $C_{12}H_{16}N_3O_5P$: C 46.01, H 5.15, N 13.41, found: C 46.29, H 4.97, N 13.36.

**Synthesis of (5*R*,α*S*)-4 and (5*S*,α*S*)-5.** The synthetic procedures described for (5*S*,α*R*)-**4** and (5*R*,α*R*)-**5** were also used for synthesis of enantiomers (5*R*,α*S*)-**4** and (5*S*,α*S*)-**5**, starting from alkene (*S*)-**6**.

(5*R*,α*S*)-**14** was obtained as a pale-yellow oil; $[\alpha]_D^{20} = -149.0$ (*c* = 0.90; $CHCl_3$); Anal. calcd for $C_{20}H_{30}N_3O_7P$: C 52.74, H 6.64, N 9.23, found: C 52.61, H 6.84, N 9.19.

(5*R*,α*S*)-**4** was obtained as a white hygroscopic powder; mp: >210 °C (dec.); $[\alpha]_D^{20} = -115.3$ (*c* = 0.11; $H_2O$); Anal. calcd for $C_{12}H_{16}N_3O_5P$: C 46.01, H 5.15, N 13.41, found: C 46.31, H 5.33, N 13.27.

(5*S*,α*S*)-**15** was obtained as a pale-yellow oil; $[\alpha]_D^{20} = +194.5$ (*c* = 0.95; $CHCl_3$); Anal. calcd for $C_{20}H_{30}N_3O_7P$: C 52.74, H 6.64, N 9.23, found: C 52.90, H 6.48, N 9.11.

(5*S*,α*S*)-**5** was obtained as a white hygroscopic powder; mp: >215 °C (dec.); $[\alpha]_D^{20} = +220.5$ (*c* = 0.10; $H_2O$); Anal. calcd for $C_{12}H_{16}N_3O_5P$: C 46.01, H 5.15, N 13.41, found: C 46.18, H 5.24, N 13.53.

### *X-ray analysis of ethyl (5R,αS)-5-(2-tert-butoxycarbonylamino-2-methoxycarbonylethyl)-4,5-dihydro-1-phenyl-1H-pyrazole-3-carboxylate [(−)-(5R,αS)-8]*

Crystals suitable for X-ray diffraction studies were grown by slow evaporation at 4 °C from a 1:1 mixture of diisopropyl ether and hexane. A very thin crystal (0.200 × 0.075 × 0.025 mm$^3$) was chosen for an experiment directed toward solving the structure.

#### Crystal data

Title compound: $C_{21}H_{29}N_3O_6$, $M_r$ = 419.5 Da; orthorhombic; space group: $P2_12_12_1$ (No 19); unit cell (Å, Å$^3$): *a* = 5.4027 (11), *b* = 19.2240 (38), *c* = 22.3810 (45), *V* = 2325 (1); *Z*: 4; Dc: 1.199 g cm$^{-3}$; *F*(000): 896; m: 0.09 mm$^{-1}$.

#### Data collection and reduction

A total of 34927 reflections within an entire sphere (below; $\sin\theta/\lambda_{max}$ = 0.65 Å$^{-1}$) were collected using a graphite-monochromated Mo $K_\alpha$ radiation source (*l* = 0.71073 Å) on a Bruker AXS Smart Apex diffractometer, equipped with an Apex II CCD detector. Integration of the diffraction frames was performed using the SAINT-NT program (SAINT v. 7.23A User's Manual (2003), Bruker AXS Inc., Madison, WI, USA), while the SORTAV program was employed to analyze and merge the raw data.[24,25] Due to the low absorption coefficient, an absorption correction was deemed unnecessary. Notably, due to the small dimensions and poor scattering ability of the sample, the majority (~80%) of the final, merged, 5310 independent data was weak, with $I < 2\sigma(I)$.

#### *Structure solution and refinement*

The structure was solved using direct methods and was subsequently refined against the final set of 5310 independent reflections. Due to the lack of anomalous scattering in the title compound, the absolute configuration of the C7 stereogenic center (*R*) was inferred, taking into consideration the fact that the stereochemistry of the C14 carbon (*S*) was precisely known and the title compound crystallized in a non-centrosymmetric space group. The program SHELX97[26] was used throughout as a component of the WinGX program package.[27] Positions of the hydrogen atoms were determined by applying suitable riding motion constraints. The terminal methoxy moiety was determined to be disordered between two accessible conformations, with estimated occupation probabilities as high as 70 and 30%, respectively. The thermal motion of C and O atoms belonging to disordered chains was considered isotropic. A list of relevant final least-squares parameters follows (more information can be found in the deposited Crystallographic Information File): $R(F) = 0.0941$ (for 1093 independent reflections; $R(F) = 0.3470$ (all independent data); $wR(F^2) = 0.2970$ (all independent data); goodness-of-fit: 0.831; residual Fourier density in the unit cell, $\Delta\rho_{max/min} = +0.22$ (4)/−0.16 (4) *e* Å$^{-3}$. CCDC 781612 [(−)-(5*R*,α*S*)-**8**] contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

### *Pharmacology*

#### iGlu receptor binding assays

All binding assays were performed using rat brain synaptic membranes from male Sprague–Dawley rats, and tissue preparations were prepared as previously described.[28] Affinities for NMDA,[18] AMPA,[19] and KA[20] receptors were determined using 2 nM [$^3$H]CGP39653 (50 Ci mmol$^{-1}$, $K_d$ = 6 nM), 5 nM [$^3$H]AMPA (45.5 Ci mmol$^{-1}$), and 5 nM [$^3$H]KA (58 Ci mmol$^{-1}$), respectively, with minor modifications as previously described.[29] Nonspecific binding was determined using 1 mM (*S*)-Glu.

#### Electrophysiology of NMDA receptors in *Xenopus* oocytes

For expression of NMDA receptors, cRNA for rat GluN1—1A, GluN2A, GluN2B, GluN2C, and GluN2D were transcribed in vitro and injected into *Xenopus* oocytes as previously described.[12] Recordings were performed 2–4 days after injection, using whole-cell two-electrode voltage clamp from −40 mV to −80 mV in extracellular solution containing 115 mM NaCl, 2.5 mM KCl, 1.9 mM $BaCl_2$, and 10 mM HEPES (pH 7.6). During recording, 10 μM glycine was included in all agonist and/or antagonist applications. Electrophysiological data were acquired and processed as previously described.[12]

### Determination of mGluR activity

The mGluR subtypes mGluR1A, mGluR2 and mGluR4A were expressed in Chinese hamster ovary (CHO) cell lines. Cells were maintained and tested as previously described by measurement of intracellular calcium (mGluR1) or cyclic AMP (mGluR2, 4) levels.[21] Compounds (±)-**1** and (±)-**2** were evaluated at concentrations up to 1 mM.

### Oxygen-glucose deprivation (OGD) and NMDA neurotoxicity in cortical cell cultures

Primary cultures of mixed cortical cells containing both neuronal and glial elements were prepared as previously described.[23] Briefly, cerebral cortices were dissected from fetal mice at 14–15 days of gestation, minced using medium stock [MS, composed of Eagle's minimal essential medium (MEM, with Earle's salts, glutamine- and $NaHCO_3$-free) supplemented with 38 mM $NaHCO_3$, 22 mM glucose, 100 U mL$^{-1}$ penicillin, and 100 μg mL$^{-1}$ streptomycin], and incubated for 10 min at 37 °C in MS with 0.25% trypsin and 0.05% DNAse. Enzymatic digestion was terminated by a second incubation (10 min at 37 °C) in MS, supplemented with 10% heat-inactivated horse serum and 10% fetal bovine serum, after which the cells were mechanically disrupted and counted. After brief centrifugation, cells were resuspended (approximately $4\times10^5$ cells mL$^{-1}$) and plated in 15 mm multi-well vessels on a layer of confluent astrocytes using a plating medium of MS supplemented with 10% heat-inactivated horse serum, 10% fetal bovine serum, and 2 mM glutamine. Cultures were stored at 37 °C with 100% humidity and a 95% air/5% $CO_2$ atmosphere. Following 4–5 days in vitro (DIV), non-neuronal cell division was halted by the application of 3 μM cytosine arabinoside for 24 h. Cultures were then shifted to a maintenance medium, identical to the plating medium but lacking fetal bovine serum, which was partially replaced twice weekly. Experiments were performed using mature cultures (14–15 DIV).

OGD was induced as previously described.[23] Briefly, the culture medium was replaced by thorough exchange with a glucose-free balanced salt solution (116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1 mM $NaH_2PO_4$, 26 mM $NaHCO_3$, 1.8 mM $CaCl_2$, and 10 mg mL$^{-1}$ phenol red), previously saturated with 95% $N_2$/5% $CO_2$ and heated at 37 °C. Multi-well plates were then placed in an airtight incubation chamber, equipped with inlet and outlet valves, and 95% $N_2$/5% $CO_2$ was blown through the chamber for 10 min to ensure maximal removal of oxygen. The chamber was then sealed and incubated at 37 °C for 60 min. OGD was terminated by removing the cultures from the chamber, replacing the exposure solution with oxygenated MS, and returning the multi-well plates to the incubator under normoxic conditions. NMDA neurotoxicity was obtained by exposing cultures to 300 μM NMDA for 10 min (mild insult). During this exposure, the original culture medium was replaced by thorough exchange with a HEPES controlled salt solution (HCSS: 120 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 20 mM HEPES, 15 mM glucose, 10 mM NaOH and 10 mg mL$^{-1}$ phenol red). Upon exposure to NMDA, cultures were rinsed with HCSS, original medium was restored, and cells were stored at 37 °C in 100% humidity and a 95% air/5% $CO_2$ atmosphere for 24 h, followed by assessment of the extent of neuronal death. To achieve maximal neuronal injury, the cultures were exposed for 24 h to 1 mM Glu in MS at 37 °C, with 100% humidity and a 95% air/5% $CO_2$ atmosphere. Cell damage was measured by the amount of lactate dehydrogenase (LDH) released from injured cells into the extracellular fluid 24 h after exposure to OGD or Glu.[23] Background LDH release was determined for control cultures not exposed to OGD and was subtracted from all experimental values.

### *In vivo pharmacology*

DBA/2 mice (8–12 g, 22–25 days old) were purchased from Charles River (Calco, Como, Italy). Procedures involving animals and their care were conducted in conformity with international and national laws and policies (European Communities Council Directive of November 24, 1986, 86/609EEC). Groups of 10 mice of either gender were exposed to auditory stimulation for 15, 30, 60, 90 and 120 min following administration of vehicle or each dose of drugs. Compounds were administered i.p. (0.1 mL per 10 g body weight) as a freshly prepared solution in 50% DMSO and 50% sterile 0.9% NaCl solution. This latter protocol has been validated several times for systemic and focal intracerebral microinjections for in vitro electrophysiological studies.[30] Individual mice were placed under a hemispheric perspex dome (∅: 58 cm); 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.[31] Behavioral changes were observed and recorded during the period between drug administration and auditory testing. The $ED_{50}$ values for each phase of the audiogenic seizure were determined for each dose of compound administered, and dose–response curves were fitted using a computer method of Litchfield and Wilcoxon.[32]

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