Design, Synthesis, and Pharmacological Characterization of Novel, Potent NMDA Receptor Antagonists

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The two diastereomeric pairs of acidic amino acids 5-(2-amino-2-carboxyethyl)-4,5-dihydroisoxazole-3-carboxylic acid (**8A/8B**) and 4-(2-amino-2-carboxyethyl)-5,5-dimethyl-4,5-dihydroisoxazole-3-carboxylic acid (**10A/10B**) were prepared via a strategy based on a 1,3-dipolar cycloaddition. The four amino acids were tested at ionotropic and metabotropic glutamate receptors. None of the compounds was active, neither as agonists nor as antagonists, at 1 mM on metabotropic receptors (mGluR1, -2, -4, and -5 expressed in CHO cell lines). Conversely, the pair of stereoisomers **8A/8B** showed a remarkable affinity, antagonist potency, and selectivity for NMDA receptors, when tested on ionotropic glutamate receptors. The affinity of **8A** proved to be 5 times higher than that of diastereomer **8B** (K_i values 0.21 and 0.96 μM, respectively). Furthermore, compounds **8A** and **8B** exhibited a noteworthy anticonvulsant activity in in vivo tests on DBA/2 mice. Derivative **10A** was inactive at all ionotropic glutamate receptors, whereas its stereoisomer **10B** displayed a seizable binding to both NMDA and AMPA receptors.

Introduction

Glutamate (Glu) is the main excitatory neurotransmitter in the mammalian central nervous system (CNS) and mediates neurotransmission across most excitatory synapses. Three classes of Glu-gated ion channels, 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA), kainate (KA), and *N*-methyl-D-aspartic acid (NMDA) receptors, transduce the postsynaptic signal, i.e., Na^+ and Ca^{2+} currents.[1] Activation of the NMDA receptors is rather complex, since it requires the binding of both Glu, which plays the neurotransmitter role, and glycine, which acts as a modulator.[1] The correct functioning of NMDA receptors, which are abundant and ubiquitously distributed throughout the brain, is needed to perform physiological effects of utmost importance, i.e., phosphorylation of cAMP response element binding protein, multiple gene activation, and long-term synaptic plasticity.[2] On the other hand, an overstimulation of the NMDA receptors produces an acute neurodegeneration, termed excitotoxicity, which destroys the neurons bearing them.[2]

The role played by NMDA receptors in excitotoxicity has driven the search for antagonists as neuroprotective agents. Their role in synaptic plasticity, on the other hand, has inspired research into receptor potentiators to treat cognitive dysfunctions.[2]

In the past decade, the molecular biology of the NMDA receptor family was defined, and now we know that these receptors are tetramers composed of at least two NR1 subunits in combination with one or more NR2 subunits[3] and, less commonly, an NR3 subunit.[4,5]

From a therapeutic point of view, any neuronal loss caused by Glu-induced excitotoxicity could be treated by blocking NMDA receptors. The NMDA receptor blocking approach could be applied to treat cerebral ischemia, which occurs after stroke or brain trauma, as well as neurodegenerative diseases, e.g. Parkinson's and Huntington's diseases. It could also be used to treat neurological disorders, such as epilepsy and neuropathic pain, which are due to an overactivity of excitatory pathways. Nevertheless, clinical trials with NMDA receptor antagonists have so far failed due to the occurrence of a number of adverse CNS effects, including hallucinations, a centrally mediated increase in blood pressure, and, at high doses, catatonia and anesthesia.[6−8] Current interest largely centers on the development of potent and NR2B subtype-selective antagonists,[9,10] e.g. ifenprodil (**1**) (Figure 1), which have demonstrated therapeutic potential for a number of indications. In preclinical tests such compounds appear to lack the side effects commonly associated with nonselective NMDA antagonists.

In a recent paper[11] we reported the synthesis and pharmacological characterization of a new acidic bicyclic amino acid (3a*S**,5*R**,6a*S**)-5-amino-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-3,5-dicarboxylic acid (**2**) (Figure 1). It displayed quite potent antagonism at the NMDA receptors (K_i 0.37 μM) and, concominantly, behaved as an antagonist at mGluR1 and mGluR5 (group I) and as an agonist at mGluR2. This pharmacological profile could suggest it as a promising lead of

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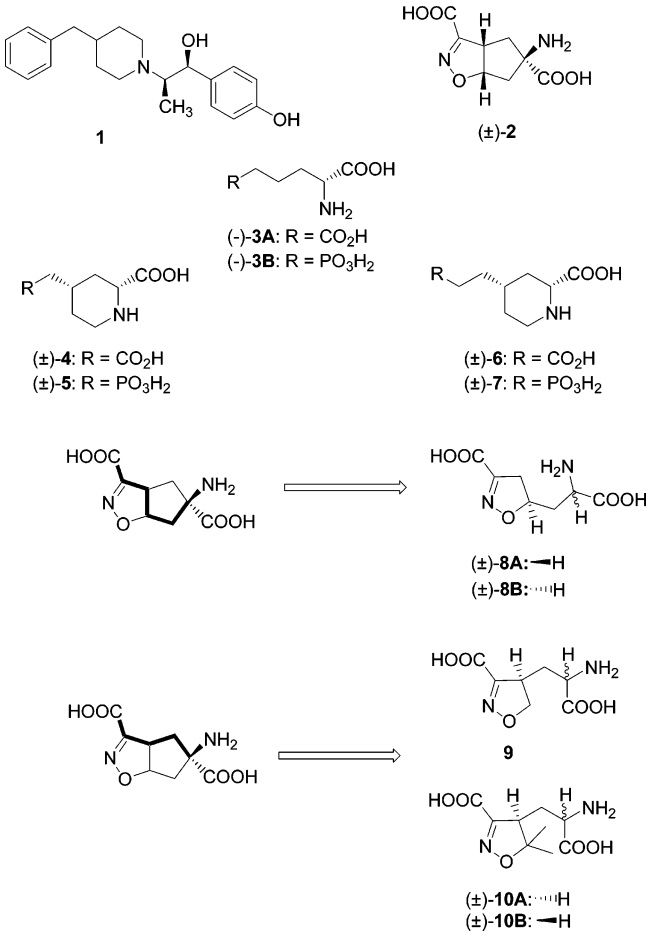


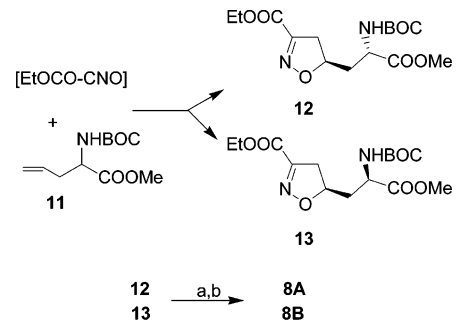
Figure 1. Structure of model and tested compounds.

neuroprotective agents. In in vivo tests on DBA/2 mice, compound **2** showed a noteworthy anticonvulsant activity.[11]

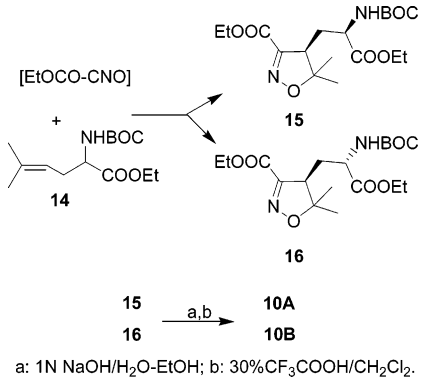
At first sight the structure of derivative **2** can be considered a locked conformation of 2-aminoadipic acid (**3A**) (Figure 1), which is a very weak NMDA receptor antagonist (K_i 13 μM).[12] Subsequently, it was discovered that a substantial enhancement in the NMDA potency could be achieved by exchanging the distal carboxylate group of **3A** with the phosphonate moiety to yield 2-amino-5-phophonopentanoic acid (AP5, **3B**) (K_i 0.29 μM).[13] Since AP5 is devoid of any significant activity following systemic administration in animals, modifications of its structure, e.g., a reduction in the conformational mobility, were thoroughly investigated. Interesting results were achieved by incorporating the required amino acid substructure into a cyclic framework. Whereas derivative **4** was almost inactive (K_i >10 μM),[13] its phosphono analogue **5**, tagged as CGS 19755, turned out to be one of the most potent NMDA antagonist (K_i 0.095 μM)[13] so far discovered. CGS19755 is nowadays commonly used as a radioligand in NMDA assays (Figure 1). It is worth pointing out that the structure of **2** can also be related to that of derivative **6** or its phosphono analogue **7** (Figure 1), which are inactive or marginally active.

To simplify the structure of **2** and to establish similarities with that of derivatives **4/5** or their homologues **6/7** (Figure 1), we designed regioisomers **9** and **8**, respectively. Since it is well-known that the 1,3-dipolar cycloaddition of nitrile oxides to monosubstituted

Scheme 1



Scheme 2



alkenes is highly regioselective to yield 3,5-disubstituted Δ^2-isoxazolines,[14] we initially planned the synthesis of derivatives **8**. Subsequently, due to the difficulties related to the preparation of the two diastereomers **9**, we designed their analogues **10**, since the 1,3-dipolar cycloaddition of a nitrile oxide to a trisubstituted alkene produces 3,4,5,5-tetrasubstituted Δ^2-isoxazolines, exclusively.[14]

Chemistry

The key step in the synthesis of the target compounds **8A** and **8B** is represented by the 1,3-dipolar cycloaddition of ethoxycarbonylformonitrile oxide, generated in situ by treatment of ethyl 2-chloro-2-(hydroxyimino)-acetate[15] with a base, to the suitably protected racemic allylglycine **11** (Scheme 1). The pericyclic reaction produced a racemic mixture of the two 3,5-disubstituted diastereoisomers **12** and **13** in a 1:1.6 ratio. In analogy, the synthesis of target compounds **10A** and **10B** was carried out by exploiting the cycloaddition reaction of racemic dipolarophile **14** with the same nitrile oxide, which gave a racemic mixture of cycloadducts **15** and **16** in a 1:1 ratio (Scheme 2). Since **14** is a sluggish dipolarophile, the reaction was carried out in a microwave oven.

Dipolarophile (±)-**11**[16] was prepared from commercially available (±)-allylglycine by standard transformations (see the Experimental Section), whereas alkene **14** was synthesized from *N*-(diphenylmethylene)glycine ethyl ester following a procedure reported in the literature.[17] The pairs of racemic stereoisomers **12/13** and **15/16** were separated by silica gel column chromatography and then transformed into final amino acids **8A**, **8B**, **10A**, and **10B** by standard methodologies.

The relative configuration of the stereogenic centers of derivatives **8A** and **8B** (Figure 1) was assigned

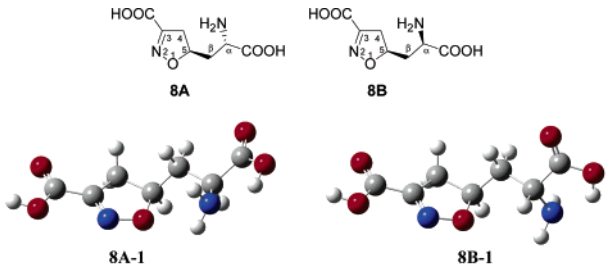


Figure 2. The most populated conformation for compounds **8A** and **8B**.

Table 1. Experimental ^{1}H NMR Vicinal Coupling Constants for Compounds **8A** and **8B** in Comparison with Their Calculated Values

	$J_{\alpha,\beta a}$	$J_{\alpha,\beta b}$	$J_{\beta a,5}$	$J_{\beta b,5}$	$J_{5,4a}$	$J_{5,4b}$
8A (exp)	8.1	5.3	10.2	3.4	6.6	10.5
8A (calcd)	8.3	4.7	9.0	4.4	4.7	9.6
8B (exp)	6.7	4.4	3.9	9.3	7.3	10.7
8B (calcd)	9.0	3.4	4.3	9.4	5.0	9.4

through an analysis of their ^{1}H NMR spectra in water combined with a theoretical investigation of their conformational properties carried out at the B3LYP/6-31G* level.[18,19] The possible rotamers, deriving from a rotation around the Cα−Cβ and Cβ−C5 single bonds of both **8A** and **8B**, were taken into account and generated nine geometries that were then fully optimized. The relative energies of the various conformations of **8A** and **8B** are strongly influenced by the solvent; as a matter of fact, conformations presenting a hydrogen bond between the amino group and the oxygen atom of the isoxazoline ring show an unrealistic stabilization when calculated in the gas phase. Hence, all energies were recalculated in a polarizable conductor-like continuum solvation model (C-PCM)[20] to obtain values conceivable for water solutions. For both diastereoisomers, the preferred conformation was identified as **8A-1** and **8B-1**, respectively (Figure 2), though several other conformers contribute with a nonnegligible percentage to the overall populations. For each conformer the ^{1}H NMR vicinal coupling constants were calculated by the Haasnoot et al. equation[21] and were weight averaged on the basis of the population percentages. The obtained values are reported in Table 1 together with the experimental coupling constants. As shown in Table 1, coupling constants $J_{\beta a,5}$ and $J_{\beta b,5}$ are highly diagnostic, since their values are quite different in the two diastereomers. The close agreement between the experimental coupling constants and the calculated values ensures that 5*R**,α*S** is the relative configuration of **8A**. Similarly, the agreement between the experimental values of **8B** and the calculated ones allows the assignment of the 5*R**,α*R** configuration to **8B**. The relative stereochemistry of **8B** was then confirmed by an X-ray analysis carried out on cycloadduct **13** (Figure 3).[22]

The same procedure applied to derivatives **10A** and **10B** failed to produce conclusive evidence on the relative configuration of their stereogenic centers. In this case, the values of the hydrogen vicinal coupling constants could not be evaluated, since most of the signals appeared as unresolved multiplets (see the Experimental Section). Thus, the relative stereochemistry of the two diastereoisomers **10A** and **10B** could not be assigned on the basis of their ^{1}H NMR spectra. A strategy

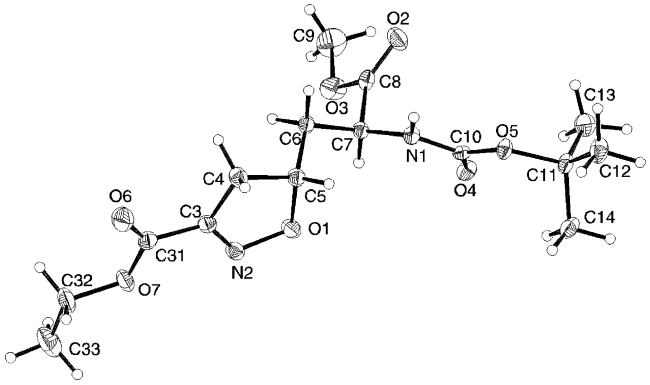
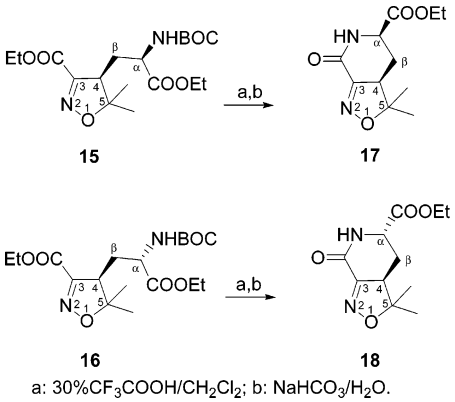


Figure 3. Perspective view of the molecular structure with numbering for compound **13**. Displacement ellipsoids are drawn at 50% of probability level while the size of the hydrogen atom is arbitrary.

Scheme 3



to overcome this difficulty could be envisaged in a restriction of the conformational flexibility of the structures. Thus, racemic cycloadducts **15** and **16**, precursors of **10A** and **10B**, respectively, were transformed into the corresponding bicyclic lactams **17** and **18** (Scheme 3), which are characterized by a much more rigid structure. The ^{1}H NMR spectra of derivatives **17** and **18** showed resolved signals and, as expected, significant differences in the coupling pattern of the protons present in the lactam ring, i.e., H$_\alpha$, H$_{\beta a}$, H$_{\beta b}$, and H$_4$ (Figure 4). As shown in Table 2, the $J_{\beta b,\alpha}$ coupling constant is quite different in the two distereoisomers and becomes diagnostic in the structure assignment.

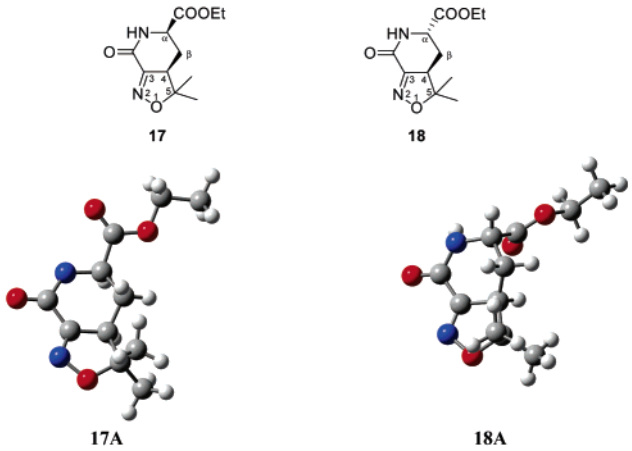


Figure 4. The most populated conformation for bicyclic derivatives **17** and **18**.

Table 2. Experimental ^{1}H NMR Vicinal Coupling Constants for Compounds **17** and **18** in Comparison with Their Calculated Values

	$J_{4,\beta a}$	$J_{4,\beta b}$	$J_{\beta a,\alpha}$	$J_{\beta b,\alpha}$
17 (exp)	4.4	12.8	3.7	12.8
17 (calcd)	3.6	12.4	3.6	11.8
18 (exp)	5.1	13.5	1.1	5.1
18 (calcd)	3.7	12.3	1.9	4.8

The conformational analysis of **17** and **18** located only two minima for each compound, confirming that the bicyclic skeleton is quite rigid. The only degree of conformational freedom resides in the ethoxycarbonyl moiety, which, however, has only a slight influence on the geometry of the bicyclic skeletons. The preferred conformations **17A** and **18A**, depicted in Figure 4, account for 86% and 81%, respectively, of their overall populations. The values of the coupling constants, calculated according to the equation of Haasnoot et al.,[21] are reported in Table 2 together with the corresponding experimental ones. The close agreement between experimental and theoretical data allows the attribution of the 4*R**,α*R** configuration to the stereogenic centers of **17** and, consequently, to the monocyclic compounds **15** and **10A**. Thus, the configuration 4*R**,α*S** is assigned to the diastereoisomers **18**, **16**, and **10B**.

Results and Discussion

The two pairs of acidic amino acids **8A/8B** and **10A/10B** were assayed in vitro by means of receptor binding techniques, second messenger assays, and electrophysiological studies. The mGluR activity of the new compounds was evaluated at rat mGluR1 and mGluR5 (group I), mGluR2 (representative of group II), and mGluR4 (representative of group III), all expressed in CHO cells.[23] The compounds were tested at 1 mM for both agonist and antagonistic activity and showed no activity at any of the mGluRs.

The affinity for NMDA, AMPA, and KA receptors was determined by using the radioligands [^{3}H]CGP 39653, [^{3}H]AMPA, and [^{3}H]KA, respectively.[24−26] As reported in Table 3, the pairs of racemic stereoisomers **8A/8B** and **10A/10B** showed no significant affinity for AMPA and KA receptors (IC_{50} > 100 μM), except for **10B**, which possesses a weak affinity for AMPA receptors (IC_{50} 12.0 μM). In contrast to this, **8A** and **8B** showed a remarkable affinity for NMDA receptors. Worth noting is that compound **8A** displays a highly selective affinity for the NMDA receptors (K_i 0.21 μM) that is only slightly lower than that displayed by the phosphono amino acid CGS 19755 (**5**) (K_i 0.095 μM), one of the most potent NMDA antagonists,[13] and significantly higher than that of derivative **7** (K_i 6.6 μM), the phosphono derivative with the same bond connection (Figure 1). The above-reported pharmacological data were confirmed in the rat cortical slice model,[27] where **8A** antagonized the responses induced by 10 μM NMDA (IC_{50} 14.2 μM) and left the responses induced by either AMPA (5 μM) or KA (5 μM) unaffected. Generally, the activities observed in the rat cortical slice model are significantly lower than the affinities observed in binding assays.[27] Taking into account the excellent antagonist activity of **8A** at NMDA receptors, we decided to test compounds **8A** and **8B** as anticonvulsant agents. Their ability to block seizures induced by audiogenic stimuli was evaluated in vivo in DBA/2 mice after ip administration; their potency, expressed as ED_{50} value (μM/Kg), is reported in Table 4 and compared with that of compound **2**,[11] CPPene, a highly potent NMDA antagonist, and phenytoin, a typical anticonvulsant agent. The anticonvulsant potency of test compounds **8A** and **8B** is only 5−10 times lower than that of CPPene, which is characterized by a very high NMDA receptor affinity (K_i 0.04 μM).[13] Compound **8B** at all tested doses did not show either a reduction in body temperature or behavior changes, whereas **8A** at the highest dose (100 mmol/kg, ip) induced sedation, reduction of locomotor activity, and a slight ataxia in three out of 10 mice. It is worth noting that acidic amino acids **8A** and **8B** are active after ip administration, thus indicating their ability to pass the blood−brain barrier.

An analysis of the biological data gathered in Table 3 puts in evidence that on passing from bicyclic derivative **2** to the monocyclic analogues, either **8A/8B** or **10A/10B**, we lose completely the activity at all the mGluR subtypes, whereas in some derivatives, i.e., **8A/8B**, the affinity at NMDA receptors is maintained or even increased. If we take into account the bond connection among the pharmacophoric groups of compounds **8A/8B**, we can find an analogy with derivative **6** or with the phosphono analogue **7** (Figure 1), which are inactive or marginally active.

Analysis of the structure−activity relationship can be carried out by considering the model for the antagonist-preferring state of the NMDA receptors reported by

Table 3. Rat Cortical Membrane Receptor Binding and in Vitro Electrophysiological Data

	[^{3}H]CGP 39653	IC_{50} (μM)		
compd[a]	K_i (μM)	[^{3}H]AMPA	[^{3}H]KA	electrophysiology
8A	0.21 [0.18; 0.23][b]	>100	>100	14.2 [12.9; 15.7][b,c]
8B	0.96 [0.88; 1.1][b]	>100	>100	30.5 [24.9; 37.4][b,c]
10A	>100	>100	>100	nd[i]
10B	79 [73; 86][b]	12.0 [11.7; 12.4][a]	>100	nd
(±)-**2**	0.37 [0.34; 0.40][b]	>100	>100	13 [12; 15][b,c]
(−)-**3A**	13[d,e]			
(±)-**4**	>10[f,g]			
(±)-**5**	0.095 ± 0.028[f,h]			
(±)-**6**	>10[f,h]			
(±)-**7**	6.6 ± 1.3[f,h]			
(−)-CPPene	0.04 ± 0.01[f,h]			

[a] Derivatives **8A/8B** and **10A/10B** have been assayed as racemates. [b] Values are expressed as the antilog to the log mean of at least three individual experiments. The number in brackets [min; max] indicates ± SEM according to a logaritmic distribution. [c] Antagonism of NMDA (10 μM) induced responses. [d] Data from the literature.[12] [e] the radioligand used is [^{3}H]-D-APV. [f] Data from the literature.[13] [g] The radioligand used is [^{3}H]CGS19755. [h] The radioligand used is [^{3}H]CPP. [i] nd, not determined.

Table 4. ED_{50} Values of Test Compounds against Audiogenic Seizures Induced in DBA/2 Mice[a]

compd	ED_{50} values (95% confidence limits) clonus	tonus
8A	17.9 (11.6−27.6)	13.9 (8.5−22.9)
8B	29.9 (21.4−41.8)	19.7 (12.5−31.3)
(±)-**2**	28 (19−41)	22 (14−35)
(±)-CPPene	3.5 (2.1−5.6)	1.3 (0.7−2.6)
phenytoin	9.1 (5.6−14.9)	7.3 (5.8−9.1)

[a] All data are expressed as μmol/kg for ip administration.

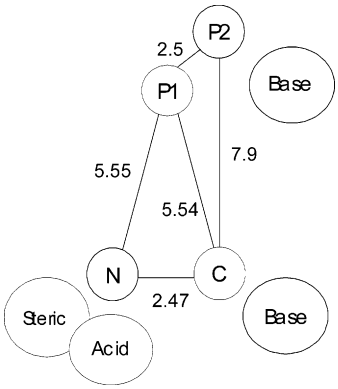


Figure 5. Hutchison's model for the antagonist-preferring state of the NMDA receptors.[28]

Table 5. Conformational Studies of Compounds **2**, **8A/8B**, and **10A/10B**

	ΔE_{rel} (kcal/mol)	$d_{N/C\omega}$ (Å)	$d_{C\alpha/C\omega}$ (Å)
Hutchison's model		5.55	5.54
2-1	0.00	5.58	5.63
2-2	1.71	5.58	4.19
8A-1	0.00	6.21	7.28
8A-2	0.19	6.70	6.83
8A-3	0.28	6.83	6.94
8A-4	1.02	7.34	6.19
8A-5	1.51	6.71	6.10
8B-1	0.00	6.83	7.10
8B-2	0.81	6.18	6.84
8B-3	1.00	5.83	7.33
8B-4	1.28	6.70	6.62
8B-5	1.97	6.65	5.68
10A-1	0.00	3.72	3.16
10A-2	0.19	3.93	5.35
10A-3	0.33	4.70	5.05
10A-4	0.66	5.16	5.26
10A-5	0.69	3.42	5.18
10B-1	0.00	3.75	4.82
10B-2	1.01	3.86	5.06
10B-3	1.77	5.33	5.04
10B-4	2.01	4.85	3.36
10B-5	2.12	3.30	5.18

Hutchison et al.[28] (Figure 5). According to this model, the optimal distances amine/ω-carboxylate and α-carboxylate/ω-carboxylate are almost identical, i.e., 5.55 and 5.54 Å, respectively, and correspond to the values found in the active conformation of compound **5**. On this ground, we performed a conformational analysis of compound **2** and found that it is characterized by only two populated conformations. For each conformer we measured the distances among the ionizable groups; as shown in Table 5, the values of the most populated conformation (**2**−**1**) are in close agreement with those of Hutchison's model. This result might explain the high binding affinity of compound **2** for the NMDA receptors.

As far as compounds **8A** and **8B** are concerned, the higher mobility of their unconstrained side chain generated several low-energy conformations; among them, five conformations that gave significant contributions to the overall population are reported in Table 5. Examination of the distances among the ionizable groups of each conformer (Table 5) shows that none of them matches the optimal values found for compound **2**. The distances appear somewhat longer than the values of Hutchison's model for derivatives structurally related to **5**. Since this model accounts for the biological activity of a series of derivatives related to **5** as well as a number of homologues related to 2-amino-7-phosphonoheptanoic acid, a distance of 7.9 Å between the α and ω carboxylate (phosphonate) groups appears to be optimal for this set of compounds. The values found for **8A** and **8B** are between the values proposed for the two models, and as a consequence, the range 5.5−7.9 Å may be considered compatible with a productive interaction of the ligand pharmacophoric groups with the complementary binding sites of the NMDA receptors.

Similar computational studies carried out on compounds **10A** and **10B** also showed the presence of several minimum energy conformations (Table 5). However, most of them are characterized by too short distances among the ionic groups. The tendency to assume folded conformations could be the reason for the inactivity of both isomers at the NMDA receptors.

In summary, we herein report the synthesis of four new acidic amino acids, and two of them (**8A** and **8B**) behaved in vitro as potent antagonists at NMDA receptors and in vivo, after ip administration in DBA/2 mice, as effective anticonvulsants. Their biological activity compares very well with that of phosphono derivative **5** and can be accounted for on the basis of the distances among the three pharmacophoric groups according to the model proposed by Hutchison for NMDA antagonists.[28] Since the bioisosteric replacement of the ω-carboxylate group with the ω-phosphonate moiety strongly increases the potency of an NMDA antagonist,[13] the synthesis of the phosphono analogues of **8A/8B** will be undertaken and the pharmacological results will further support the analogy with derivative **5**.

Experimental Section

Materials and Methods. All reagents were purchased from Aldrich. Ethyl 2-chloro-2-(hydroxyimino)acetate was prepared according to a literature procedure.[15]

^1H and ^{13}C NMR spectra were recorded on a Varian Mercury 300 (300 MHz) spectrometer in $CDCl_3$ or D_2O solution at 20 °C. Chemical shifts (δ) are expressed in ppm and coupling constants (*J*) in hertz. Potentiometric titrations were performed with the GLpKa apparatus (Sirius Analytical Instruments Ltd, Forrest Row, East Sussex, UK) equipped with a pH electrode, a temperature probe, an overhead stirrer, and precision dispensers for automated distribution of the diluent (0.15 M KCl in water) and titrants (0.5 M HCl, 0.5 M KOH). Four separate 15 mL aqueous solutions were added to weighted samples (1−10 mg) and acidified to pH 1.8 with HCl. The solutions were then titrated with standardized KOH to pH 8. The titrations were conducted under nitrogen at 25.0 ± 0.1 °C. The initial estimates of the pK_a values were obtained by difference plots (Bjerrum plots).[29] These values were then refined by a weighted nonlinear least-squares procedure. Microwave reactions were performed with a Ethos Microsynth Milestone apparatus. 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 with ninhydrin. Melting points were determined on a Büchi B-540 apparatus and are uncorrected. Microanalyses of new compounds agreed with theoretical values ±0.3%.

Methyl 2-*tert*-Butoxycarbonylaminopent-4-enoate 11.[16] **Step A.** To a stirred suspension of (±)-allylglycine (2 g, 13.3 mmol) in CH_2Cl_2 (20 mL) was added triethylamine (3 mL, 21.5 mmol) and a solution of di-*tert*-butyl dicarbonate (3.77 g, 17.3 mmol) in CH_2Cl_2 (10 mL) cooled to 0 °C. The reaction mixture was stirred at room temperature overnight and the progress of the reaction was monitored by TLC (*n*-butanol/water/acetic acid 4:2:1). The mixture was washed with 2 N HCl (2 × 10 mL) and dried over anhydrous Na_2SO_4 and the solvent evaporated. The yield was quantitative

Step B. The crude material obtained from the previous transformation (13.3 mmol) was dissolved in acetone (20 mL) and then treated with solid K_2CO_3 (3.7 g, 26.6 mmol) and excess methyl iodide (1.7 mL, 26.6 mmol). The mixture was refluxed for 4 h, and the progress of the reaction was monitored by TLC ($CHCl_3$/MeOH 95:5 + 50μL acetic acid). The solvent was evaporated and the residue was taken up with AcOEt (25 mL) and washed with a saturated aqueous solution of $NaHCO_3$; the organic layer was dried over anhydrous Na_2SO_4 and the solvent removed under vacuum. The residue was chromatographed on silica gel (eluant: petroleum ether/AcOEt 7:3) to give 2.80 g (overall yield: 92%) of **11** as a colorless oil. R_f: 0.49 (petroleum ether/AcOEt 8:2). ^{1}H NMR ($CDCl_3$): 1.42 (s, 9H), 2.50 (m, 2H), 3.72 (s, 3H), 4.36 (dd, J = 6.6, 13.9 Hz, 1H), 5.02 (bd, J = 7.5 Hz, 1H), 5.14 (m, 2H), 5.68 (m, 1H). Anal. ($C_{11}H_{19}NO_4$): C, H, N.

Ethyl (5*R,2′*S**)-5-(2-*tert*-Butoxycarbonylamino-2-methoxycarbonylethyl)-4,5-dihydroisoxazole-3-carboxylate (12) and Ethyl (5*R**,2′*R**)-5-(2-*tert*-Butoxycarbonylamino-2-methoxycarbonylethyl)-4,5-dihydroisoxazole-3-carboxylate (13).** To a solution of **11** (2.8 g, 12.30 mmol) in AcOEt (40 mL) was added ethyl 2-chloro-2-(hydroxyimino)acetate[15] (2.79 g, 18.45 mmol) and $NaHCO_3$ (5 g). The mixture was vigorously stirred for 3 days at room temperature; the progress of the reaction was monitored by TLC (petroleum ether/AcOEt 7:3). Water was added to the reaction mixture and the organic layer was separated and dried over anhydrous Na_2SO_4. The crude material, obtained after evaporation of the solvent, was chromatographed on silica gel (petroleum ether/AcOEt 4:1) to give 1.58 g of **12** and 2.52 g of **13**. The overall yield was 97%.

Compound 12. Colorless oil. R_f: 0.23 (petroleum ether/AcOEt 7:3). ^{1}H NMR ($CDCl_3$): 1.36 (t, J = 7.2 Hz, 3H), 1.43 (s, 9H), 2.14 (m, 2H), 2.91 (dd, J = 10.5, 17.8 Hz, 1H), 3.37 (dd, J = 7.2, 17.8 Hz, 1H), 3.78 (s, 3H), 4.32 (q, J = 7.2 Hz, 2H), 4.34 (m, 1H), 4.92 (m, 1H), 5.34 (bd, J = 6.7 Hz, 1H). Anal. ($C_{15}H_{24}N_2O_7$): C, H, N.

Compound 13. Colorless needles from diisopropyl ether. Mp: 94.5 °C. R_f: 0.21 (petroleum ether/AcOEt 7:3). ^{1}H NMR ($CDCl_3$): 1.36 (t, J = 7.0 Hz, 3H), 1.43 (s, 9H), 2.00 (m, 1H), 2.22 (ddd, J = 7.0, 8.0, 12.3 Hz, 1H), 2.88 (dd, J = 7.0, 16.1 Hz, 1H), 3.33 (dd, J = 9.1, 16.1 Hz, 1H), 3.75 (s, 3H), 4.34 (q, J = 7.0 Hz, 2H), 4.46 (m, 1H), 4.91 (m, 1H), 5.3 (bd, J = 7.5 Hz, 1H). Anal. ($C_{15}H_{24}N_2O_7$): C, H, N.

(5*R,2′*S**)-5-(2-Amino-2-carboxyethyl)-4,5-dihydroisoxazole-3-carboxylic Acid (8A). Step A.** Derivative **12** (800 mg, 2.32 mmol) was dissolved in MeOH (10 mL) and treated with 1 N NaOH (10 mL) at room temperature overnight. The disappearance of the starting material was monitored by TLC ($CHCl_3$/MeOH 95:5 + 50 μL acetic acid). The aqueous layer was washed with CH_2Cl_2, made acidic with 2 N HCl, and extracted with EtOAc. The organic phase was dried over anhydrous Na_2SO_4 and after evaporation of the solvent a white powder was obtained (420 mg, 1.39 mmol, yield 60%).

Step B. The crude material, obtained from the previous transformation (420 mg, 1.39 mmol), was treated with a 30% CH_2Cl_2 solution of trifluoroacetic acid (1.07 mL, 13.9 mmol) at 0 °C. The solution was stirred at room temperature for 3 h until the disappearance of the starting material (TLC: *n*-butanol/water/acetic acid 4:2:1). The volatiles were removed under vacuum, and the residue was washed with MeOH and Et_2O to yield 37% (104 mg).

Amino Acid 8A. White prisms. R_f: 0.37 (*n*-butanol/water/acetic acid 4:2:1). Mp: 161−169 °C (dec). pK_a: < 2.0 (α-COOH), 2.6 (ω-COOH), 9.1 (NH_3^+). ^{1}H NMR (D_2O): 2.02 (ddd, J = 8.1, 10.3, 15.1 Hz, 1H), 2.14 (ddd, J = 3.3, 5.3, 15.1 Hz, 1H), 2.83 (dd, J = 6.6, 18.1 Hz, 1H), 3.29 (dd, J = 10.6, 18.1 Hz, 1H), 3.86 (dd, J = 5.3, 8.1 Hz, 1H), 4.94 (dddd, J = 3.3, 6.6, 10.3, 10.6 Hz, 1H). ^{13}C NMR (D_2O): 35.66, 40.16, 52.25, 80.98, 155.98, 164.83, 172.55. Anal. ($C_7H_{10}N_2O_5$): C, H, N.

(5*R,2′*R**)-5-(2-Amino-2-carboxyethyl)-4,5-dihydroisoxazole-3-carboxylic Acid (8B). Step A.** Derivative **13** (800 mg, 2.32 mmol) was dissolved in MeOH (10 mL) and treated with 1 N NaOH (10 mL) at room temperature overnight. The disappearance of the starting material was monitored by TLC ($CHCl_3$/MeOH 95:5 + 50 μL AcOH). The aqueous layer was washed with CH_2Cl_2, made acidic with 2 N HCl, and extracted with AcOEt. The organic phase was dried over anhydrous Na_2SO_4, and after evaporation of the solvent, a white powder was obtained (408 mg, 1.35 mmol, yield 58%).

Step B. The crude material, obtained from the previous transformation (408 mg, 1.35 mmol), was treated with a 30% CH_2Cl_2 solution of trifluoroacetic acid (1.00 mL,13.5 mmol) at 0 °C. The solution was stirred at room temperature for 3 h until the disappearance of the starting material (TLC: *n*-butanol/water/acetic acid 4:2:1). The volatiles were removed under vacuum, and the residue was washed with MeOH and Et_2O to yield 41% (112 mg).

Amino Acid 8B. White prisms. R_f: 0.27 (*n*-butanol/water/acetic acid 4:2:1). Mp: 175−185 °C (dec). pK_a: < 2.0 (α-COOH), 2.5 (ω-COOH), 9.0 (NH_3^+). ^{1}H NMR (D_2O): 2.08 (ddd, J = 3.9, 6.5, 13.7 Hz, 1H), 2.15 (ddd, J = 4.4, 9.3, 13.7 Hz, 1H), 2.84 (dd, J = 7.3, 18.0 Hz, 1H), 3.30 (dd, J = 10.7, 18.0 Hz, 1H), 3.92 (dd, J = 4.4, 6.5 Hz, 1H), 4.84 (dddd, J = 3.9, 7.3, 9.3, 10.7 Hz, 1H). ^{13}C NMR (D_2O): 34.90, 49.91, 51.82, 80.04, 155.92, 164.71, 172.45. Anal. ($C_7H_{10}N_2O_5$): C, H, N.

Ethyl 2-*tert*-Butoxycarbonylamino-5-methylhex-4-enoate (14). Step A. Under a nitrogen atmosphere at −78 °C, DMPU (3.25 mL, 26.84 mmol) and *N*-(diphenylmethylene)-glycine ethyl ester (7.17 g, 26.84 mmol) were added to a solution of 2.5 M BuLi in hexane (10.74 mL, 26.84 mmol) and diisopropylamine (3.76 mL, 26.84 mmol) in THF (10 mL). The mixture was stirred for 10 min and then 1-bromo-3-methyl-2-butene (3.09 mL, 26.84 mmol) was added. The progress of the reaction was monitored by TLC (petroleum ether/AcOEt 9:1). Stirring was continued for 2 h more at −78 °C and then a saturated solution of ammonium chloride was added and the mixture was allowed to reach room temperature. THF was evaporated and the aqueous layer extracted with Et_2O. The organic phase was dried over anhydrous Na_2SO_4 and the solvent removed under vacuum. Hydrolysis of the alkylated imine with 1 N aqueous HCl/THF afforded the crude amine (3.67 g) in 80% yield.

Step B. At 0 °C a solution of di-*tert*-butyl dicarbonate (5.71 g, 26.16 mmol) in CH_2Cl_2 (10 mL) was added to a solution of the crude amine (3.67 g, 21.47 mmol) and triethylamine (3.95 mL, 28.36 mmol) in CH_2Cl_2 (20 mL). The mixture was stirred at room temperature overnight and the progress of the reaction was monitored by TLC (AcOEt). The mixture was washed with 2 N HCl, the organic phase was dried over anhydrous Na_2SO_4, and the crude material, obtained after the evaporation of the solvent, was chromatographed on silica gel (petroleum ether/AcOEt 95:5) to give 5.24 g (yield 90%) of **14** as a colorless oil. R_f: 0.52 (petroleum ether/AcOEt 9:1). ^{1}H NMR ($CDCl_3$): 1.22 (t, J = 7.3 Hz, 3H), 1.42 (s, 9H), 1.60 (s, 3H), 1.64 (s, 3H), 2.50 (m, 2H), 4.08 (q, J = 7.3 Hz, 2H), 4.30 (m, 1H), 5.02 (m, 1H), 5.02 (m, 1H). Anal. ($C_{14}H_{25}NO_4$): C, H, N.

Ethyl (4*R,2′*R**)-4-(2-*tert*-Butoxycarbonylamino-2-ethoxycarbonylethyl)-5,5-dimethyl-4,5-dihydroisoxazole-3-carboxylate (15) and Ethyl (4*R**,2′*S**)-4-(2-*tert*-Butoxycarbonylamino-2-ethoxycarbonylethyl)-5,5-dimethyl-4,5-dihydroisoxazole-3-carboxylate (16).** To a solution of **14** (5.0 g, 18.45 mmol) in AcOEt (40 mL) was added ethyl 2-chloro-2-(hydroxyimino)acetate (3.35 g, 22.14 mmol) and $NaHCO_3$ (5

g). The mixture was irradiated in a microwave oven at 50 W for 20 min in a sealed vessel. The same amount of ethyl 2-chloro-2-(hydroxyimino)acetate was then added and the reaction was heated again for 20 min. The progress of the reaction was monitored by TLC (petroleum ether/AcOEt 9:1). Water was added to the reaction mixture and the organic layer was separated and dried over anhydrous Na_2SO_4. The crude material, obtained after evaporation of the solvent, was chromatographed on silica gel (petroleum ether/AcOEt 9:1) to give 1.85 g of **15** and 1.80 g of **16**. The overall yield was 52%.

Cycloadduct 15. Yellow oil. R_f: 0.43 (petroleum ether/AcOEt 9:1). ^{1}H NMR ($CDCl_3$): 1.27 (t, J = 7.3 Hz, 3H), 1.37 (t, J = 7.3 Hz, 3H), 1.38 (s, 3H), 1.45 (s, 9H), 1.57 (s, 3H), 1.84 (m, 1H), 2.09 (m, 1H), 3.15 (dd, J = 2.2, 10.6 Hz, 1H), 4.18 (q, J = 7.3 Hz, 2H), 4.32 (q, J = 7.3 Hz, 2H), 4.33 (m, 1H), 5.15 (bd, J = 9.2 Hz, 1H). Anal. ($C_{18}H_{30}N_2O_7$): C, H, N.

Cycloadduct 16. Yellow oil. R_f: 0.33 (petroleum ether/AcOEt 9:1); ^{1}H NMR ($CDCl_3$): 1.30 (t, J = 7.3 Hz, 3H), 1.37 (t, J = 7.3 Hz, 3H), 1.39 (s, 3H), 1.44 (s, 9H), 1.47 (s, 3H), 2.05 (m, 2H), 3.22 (m, 1H), 4.23 (q, J = 7.3 Hz, 2H), 4.34 (q, J = 7.3 Hz, 2H), 4.35 (m, 1H), 5.22 (bd, J = 7.7 Hz, 1H). Anal. ($C_{18}H_{30}N_2O_7$): C, H, N.

(4*R,2′*R**)-4-(2-Amino-2-carboxyethyl)-5,5-dimethyl-4,5-dihydroisoxazole-3-carboxylic Acid (10A). Step A.** Derivative **15** (500 mg, 1.29 mmol) was dissolved in EtOH (10 mL) and treated with 1 N NaOH (4 mL) at room temperature overnight. The disappearance of the starting material was monitored by TLC ($CHCl_3$/MeOH 8:2 + 100 μL acetic acid). The aqueous layer was washed with CH_2Cl_2, made acidic with 2 N HCl, and extracted with AcOEt. The organic phase was dried over anhydrous Na_2SO_4 and, after evaporation of the solvent, a white powder was obtained (383 mg, 1.16 mmol, yield 90%).

Step B. The crude material, obtained from the previous transformation (383 mg, 1.16 mmol), was treated with a 30% CH_2Cl_2 solution of trifluoroacetic acid (894 μL, 11.6 mmol) at 0 °C. The solution was stirred at room temperature for 3 h until the disappearance of the starting material (TLC: *n*-butanol/water/acetic acid 4:2:1). The volatiles were removed under vacuum, and the residue was crystallized from EtOH and Et_2O to yield 38% (101 mg).

Amino Acid 10A. White prisms. R_f: 0.31 (*n*-butanol/water/acetic acid 4/2/1). Mp: 160−170 °C (dec). pK_a: < 2.0 (α-COOH), 2.5 (ω-COOH), 8.8 (NH_3^+). ^{1}H NMR (D_2O): 1.29 (s, 3H), 1.33 (s, 3H), 1.93 (m, 1H), 2.20 (m, 1H), 3.27 (dd, J = 6.4, 6.4 Hz, 1H), 3.98 (dd, J = 6.9, 6.9 Hz, 1H). ^{13}C NMR (D_2O): 20.21, 26.72, 28.62, 50.39, 52.49, 90.96, 158.79, 165.16, 172.47. Anal. ($C_9H_{14}N_2O_5$): C, H, N.

(4*R,2′*S**)-4-(2-Amino-2-carboxyethyl)-5,5-dimethyl-4,5-dihydroisoxazole-3-carboxylic Acid (10B). Step A.** Derivative **16** (500 mg, 1.29 mmol) was dissolved in EtOH (10 mL) and treated with 1 N NaOH (4 mL) at room temperature overnight. The disappearance of the starting material was monitored by TLC ($CHCl_3$/MeOH 8:2 + 100 μL acetic acid). The aqueous layer was washed with CH_2Cl_2, made acidic with 2 N HCl, and extracted with AcOEt. The organic phase was dried over anhydrous Na_2SO_4 and, after evaporation of the solvent, a white powder was obtained (362 mg, 1.09 mmol, yield 85%).

Step B. The crude material obtained from the previous transformation (362 mg, 1.09 mmol) was treated with a 30% CH_2Cl_2 solution of trifluoroacetic acid (839 μL, 10.9 mmol) at 0 °C. The solution was stirred at room temperature for 3 h until the disappearance of the starting material (TLC: *n*-butanol/water/acetic acid 4:2:1). The volatiles were removed under vacuum, and the residue was crystallized from EtOH and Et_2O to yield 36% (90 mg).

Amino Acid 10B. White prisms. R_f: 0.30 (*n*-butanol/water/acetic acid 4:2:1). Mp: 120−130 °C (dec). pK_a: < 2.0 (α-COOH), 2.15 (ω-COOH), 8.95 (NH_3^+). ^{1}H NMR (D_2O): 1.22 (s, 3H), 1.29 (s, 3H), 2.05 (m, 1H), 2.15 (m, 1H), 3.17 (m, 1H), 3.93 (m, 1H). ^{13}C NMR (D_2O): 20.04, 26.72, 28.49, 49.13, 51.72, 91.36, 158.06, 165.06, 172.17. Anal. ($C_9H_{14}N_2O_5$): C, H, N.

Ethyl (3a*R,5*R**)-3,3-Dimethyl-7-oxo-3,3a,4,5,6,7-hexahydroisoxazolo[3,4-*c*]pyridine-5-carboxylate (17) and Ethyl (3a*R**,5*S**)-3,3-Dimethyl-7-oxo-3,3a,4,5,6,7-hexahydroisoxazolo[3,4-*c*]pyridine-5-carboxylate (18).** Derivative **15** (100 mg, 0.258 mmol) was treated with a 30% CH_2Cl_2 solution of trifluoroacetic acid (168 μL, 2.58 mmol) at 0 °C. The solution was stirred at room temperature for 3 h until the disappearance of the starting material (TLC: AcOEt). The volatiles were removed under vacuum, and the residue was dissolved in a saturated solution of $NaHCO_3$. The aqueous layer was extracted with AcOEt, the organic phase was dried over anhydrous Na_2SO_4, and the solvent evaporated to give lactam **17** in 90% yield (56 mg, 0.232 mmol).

The same procedure applied to derivative **16** gave lactam **18** in comparable yield.

Lactam 17. Colorless oil. R_f: 0.50 (AcOEt). ^{1}H NMR ($CDCl_3$): 1.22 (s, 3H), 1.30 (t, J = 7.3 Hz, 3H), 1.57 (s, 3H), 1.83 (m, 1H), 2.45 (ddd, J = 12.8, 4.4, 3.7 Hz, 1H), 3.22 (dd, J = 12.8, 4.4 Hz, 1H), 4.23 (dd, J = 12.8, 3.7 Hz, 1H), 4.28 (q, J = 6.9 Hz, 2H), 6.54 (bs, 1H). Anal. ($C_{11}H_{16}N_2O_4$): C, H, N.

Lactam 18: Colorless oil. R_f: 0.43 (AcOEt). ^{1}H NMR ($CDCl_3$): 1.22 (s, 3H), 1.30 (t, J = 7.3 Hz, 3H), 1.57 (s, 3H), 2.08 (ddd J = 13.5, 13.5, 5.1 Hz, 1H), 2.38 (ddd, J = 13.5, 5.1, 1.1 Hz, 1H), 3.12 (dd, J = 13.5, 5.1 Hz, 1H), 4.24 (q, J = 7.0 Hz, 2H), 4.23 (m, 1H), 7.40 (bs, 1H). Anal. ($C_{11}H_{16}N_2O_4$): C, H, N.

X-ray Analysis of Ethyl (5R*,2′R*)-5-(2-*tert*-Butoxycarbonylamino-2-methoxycarbonylethyl)-4,5-dihydroisoxazole-3-carboxylate (13). Crystal Data. Single crystals suitable for X-ray diffraction studies were grown from di-*n*-propyl ether. $C_{15}H_{24}N_2O_7$, M_r 344.4, orthorhombic, space group *Pbca* (No 61), a = 11.721(2) Å, b = 9.682(2) Å, c = 31.642(5) Å, V = 3590.8(11) Å^3, Z = 8, D_c = 1.274 Mg/m^3, F(000) = 1472, μ(Mo Kα) = 0.101 mm^{-1}, crystal size: 0.06 × 0.26 × 0.3 mm.

Data Collection and Reduction. A single crystal was mounted and immersed in a stream of nitrogen gas [T = 122-(1) K]. Data were collected, using the graphite-monochromated Mo Kα radiation source (λ = 0.71070 Å) on a KappaCCD diffractometer. Data collection and cell refinement were performed using COLLECT[30] and DIRAX.[31] Data reduction was performed using EvalCCD.[32] Correction for absorption was performed using SADABS.[33]

Structure Solution and Refinement. Positions of all non-hydrogen atoms were found by direct methods (SHELXS97).[34,35] Full-matrix least squares refinements (SHELXL97),[36] were performed on F^2, minimizing $\Sigma w(F_o^2 - kF_c^2)^2$, with anisotropic displacement parameters of the non-hydrogen atoms. The position of most of the hydrogen atoms were located in subsequent difference electron density maps, and except for the hydrogen atoms of methyl groups, all hydrogen atoms were refined with fixed isotropic displacement parameters (U_{iso} = 1.2U_{eq} for NH, CH, and CH_2). The positions of the hydrogen atoms of methyl groups were included in idealized positions and refined with a riding model with fixed isotropic displacement parameter (U_{iso} = 1.5U_{eq}) of the parent atom. Extinction correction was applied;[36] extinction coefficient = 0.0019(2). Refinement (250 parameters, 3149 unique reflections) converged at R_F = 0.048, wR_F^2 = 0.085 [2581 reflections with F_o > 4$\sigma(F_o)$; $w^{-1} = (\sigma^2(F_o^2)+(0.0251P)^2 + 2.4415P)$, where $P = (F_o^2 + 2F_c^2)/3$; S = 1.167]. The residual electron density varied between −0.19 and 0.20 e Å^{-3}. Complex scattering factors for neutral atoms were taken from the *International Tables for Crystallography* as incorporated in SHELXL97.[36]

Further details of crystal structure of compound **13** can be obtained, free of charge, on application to CCDC (CCDC 241975), 12 Union Road, Cambridge CB2 lEZ UK.

Pharmacology. Receptor Binding and Electrophysiology Assays at iGluRs. The membrane preparations used in receptor-binding experiments were prepared according to Ransom and Stec[37] with slight modifications, as previously described.[38]

Affinity for AMPA,[25] KA,[26] and NMDA[24] receptor sites was determined using 5 nM [^{3}H]AMPA, 5 nM [^{3}H]kainic acid in the absence of $CaCl_2$, and 2 nM [^{3}H]CGP 39653 ([^{3}H]-(±)-(*E*)-

2-amino-4-propyl-5-phosphono-3-pentenoate), respectively. The tritiated ligands were all purchased from New England Nuclear (NEN).

The amount of bound radioactivity was determined using a Packard TOP−COUNT microplate scintillation counter.

The rat cortical wedge preparation, in a modified version, was used for the determination of the depolarizing effects of the compounds under study.[27] Tests for agonist activities of **8A/8B** and **10A/10B** were performed by their application for 90 s. Tests for antagonist activities of **8A/8B** and **10A/10B** were performed by their preapplication for 90 s followed by coapplication of the compound under study with a standard agonist, AMPA (5 μM), NMDA (10 μM), or KA (5 μM), for further 90 s.

Data Analysis. Binding data were analyzed by the nonlinear curve fitting program GRAFIT 3.0.[39] Data were fitted to the following equation: $B = 100 - (100 \times [\text{inhibition}]^n)/(IC_{50}^n + [\text{inhibitor}]^n)$, where B is the binding, expressed as a percentage of total specific binding, and n is the Hill coefficient.

Metabotropic Testing. The mGluR subtypes mGluR1α, mGluR2, mGluR4a and mGluR5a were expressed in Chinese hamster ovary (CHO) cell lines. Cell were maintained and tested as previously described.[23]

In Vivo Pharmacology. Male DBA/2 mice (6−12 g; 22−26 days old) were used. The animals were housed in groups of 10 in PVC cages (260 mm × 440 mm × 120 mm) at a temperature of 21−23 °C and a relative humidity of 57 ± 2%; a 12 h light/dark cycle was applied (light on in the interval 7:00 a.m. to 7:00 p.m.). Food and water were available ad libitum.

Procedure. DBA/2 mice were exposed to a loud sound stimulus which produced a whole-body clonus.[40] Drug (or vehicle, water) was administered intraperitoneally (ip) in a volume of 100 μL/10 g body weight. Prior to the test for sound-induced seizures, mice were observed for drug related abnormal motor behavior effects. Anticonvulsant tests were carried out on individual mice 45 min after drug (or vehicle) administration under a Perspex dome (58 cm in diameter) fitted with a doorbell generating a sound of 110 dB for a period of 60s or until the onset of a clonic seizure. The sound stimulus produced a sequential seizure response, consisting of a wild running phase (score 1), clonic seizures (score 2), tonic extension (score 3), and occasionally respiratory arrest (score 4) as previously reported.[40] The compounds were dissolved in NaOH (0.1 mM) and the pH adjusted to 7.3−7.7 with a phosphate buffer solution for ip injection.

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Supporting Information Available: Tables containing the X-ray crystallographic crystal data of derivative **13**. This material is available free of charge via the Internet at http://pubs.acs.org.

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